THE INTERACTION OF A MODEL STEROID WITH PHOSPHOLIPID STRUCTURES

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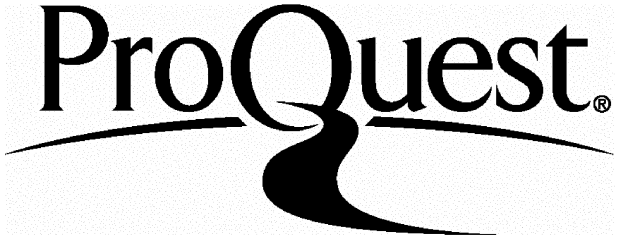

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Abstract

This thesis describes investigations into the entrapment of beclomethasone dipropionate (BDP) into phospholipid structures, in order to understand the behaviour of this drug within monolayers and bilayers, and to help optimise future steroid entrapment in liposomes for drug delivery.

BDP monohydrate forms a solvate when recrystallised from chloroform, and its size and shape may affect liposomal entrapment. BDP crystals are present in liposomes at concentrations of 1.5-2 mole % steroid, and form on the hydration of dry phospholipid films. Unentrapped material was efficiently removed from liposomal suspensions by centrifugation in deuterated water, with crystals forming a pellet. An HPLC assay for the determination of BDP was developed, and the maximum entrapment of this steroid in dipalmitoylphosphatidylcholine (DPPC) liposomes was found to be 2.5 mole % BDP.

Fluorescence studies of monolayers indicated that BDP increases the surface pressure at which DPPC solid domains form. BDP is expelled out from compressed DPPC, distearoylphosphatidylcholine, dipalmitoylphosphatidic acid and dipalmitoyl-phosphatidylglycerol monolayers, into the hydrophobic super-phase region. BDP increased the surface pressures of condensed dilaurylphosphatidylcholine monolayers, as the hydrophobic regions of this phospholipid are near to the aqueous sub-phase surface.

DSC investigations into the effects of BDP on phospholipid bilayers found that the optimal interaction between steroid and phospholipid molecules, monitored by transition peak width values, occurs with C_{18} phospholipid chains (distearoyl) and large head-groups (phosphatidylglycerol). BDP molecules seem to be located at the terminal chain regions, probably due to their size and shape. Domain formation by BDP affects thermal profiles only when domains increase and modify the mid-chain region.

The relative instability of BDP molecules in the disordered region of bilayers may explain why BDP, although hydrophobic, does not incorporate into liposomes to any great extent. These results have important practical implications for achieving higher BDP entrapment efficiencies in liposomes.

List of Abbreviations

Å	angstrom
A_o	molecular area
AUFS	Arbitrary units for sensitivity
BDP	beclomethasone dipropionate
21-BMP & 17-BMP	21- & 17-beclomethasone monopropionate
°C	° centigrade
CP	cross-polarisation
DIC	differential interference contrast
DLPC	dilaurylphosphatidylcholine
DMPC	dimyristoylphosphatidylcholine
DPPA	dipalmitoylphosphatidic acid
DPPC	dipalmitoylphosphatidylcholine
DPPE	dipalmitoylphosphatidylethanolamine
DPPG	dipalmitoylphosphatidylglycerol
DRV	dehydrated-rehydrated vesicle
DSC	differential scanning calorimetry
DSPC	distearoylphosphatidylcholine
DTA	differential thermal analysis
DTPA	diethylenetriamine pentacetic acid
ESR	electron spin resonance
HHW	half-height width
HPLC	high performance liquid chromatography
HSM	hot-stage microscopy
J/g	joules/gram
LUV	large unilamellar vesicle
MLV	multi-lamellar vesicle
MW	molecular weight
NMR	nuclear magnetic resonance
ODS	octyldecylsilane
PC	phosphatidylcholine
PE	phospatidylethanolamine
PG	phosphatidylglycerol
PTFE	polytetrafluoroethylene
REV	reverse phase evaporation vesicle
SCG	sodium cromoglycate
SOD	superoxide dismutase
SP-C	pulmonary surfactant protein
SUV	small unilamellar vesicle
$T_{½}$	half height width
T_m	maximum transition temperature
T_o	transition onset temperature
UHQ-PS	ultra-high quality purification system
UV	ultraviolet

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For Mum and Dad.

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1. INTRODUCTION

1.1. Structure and phase behaviour of liposomes

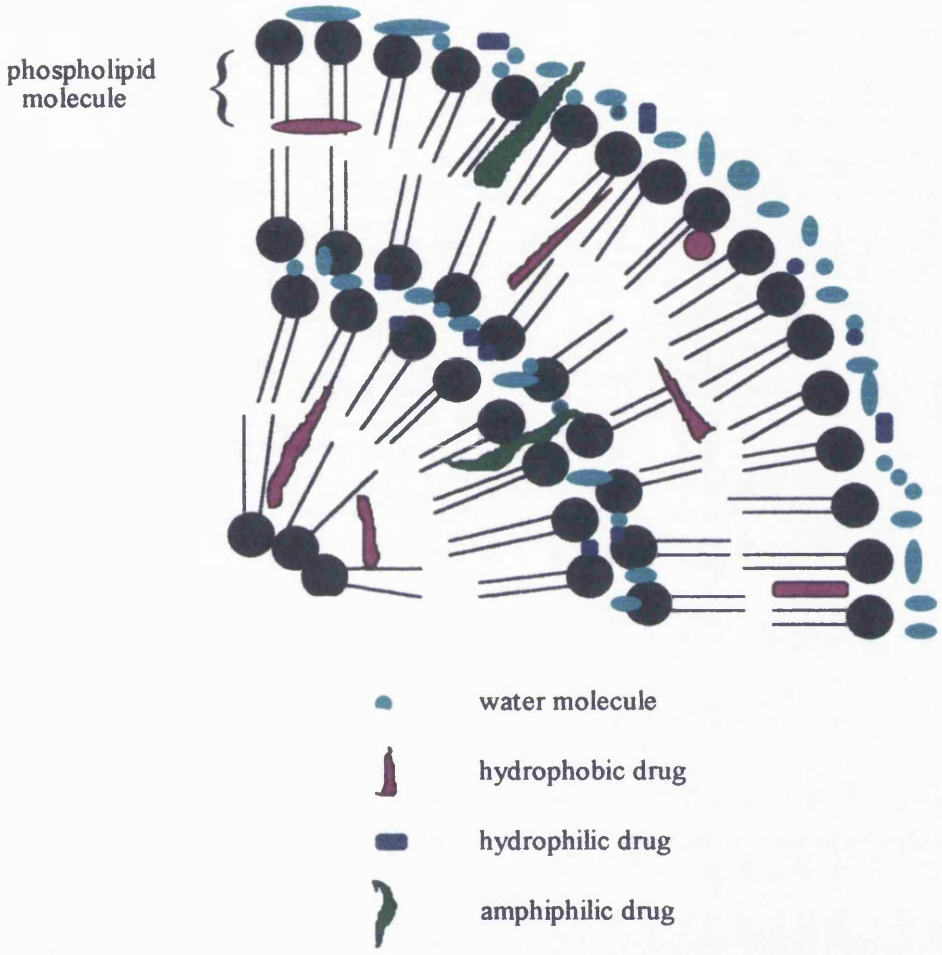
Liposomes are vesicles in which an aqueous core is enclosed by one or more bilayers composed of phospholipid molecules. They form spontaneously when these lipids are dispersed in aqueous media, and can be constructed from natural components so that the bilayer structure is essentially identical to the lipid portion of natural cell membranes (Lewis and Hadgraft, 1990; Reig et al, 1992) and are therefore generally non-toxic *in vivo*. The similarity between liposomes and cell membranes may be exploited in areas such as drug penetration studies of cell membranes. Liposomes may also be used as drug delivery systems since they provide safe and efficacious vehicles for medical applications.

The great value of liposomes as drug carriers is the variety of materials which they are able to encapsulate and release (Figure 1.1.). Because the membrane is a relatively fluid medium composed of molecules associated by non-covalent interactions, it will readily accept and retain a wide range of hydrophobic compounds within the hydrocarbon region of bilayers, without the need for any chemical structural specificity. Amphiphilic compounds can be located at the boundary between the aqueous phase and the phospholipid membrane, and water-soluble molecules can be entrapped in the enclosed aqueous compartments. Because the means of incorporation is physical, no restrictions are placed on the chemical nature of these agents.

1.1.1. Types of liposomes

Multilamellar vesicles (MLVs) consist of up to ten to twelve bilayers with diameters ranging from 100 nm to 10 or 20 μm. They are traditionally prepared by producing a thin film of phospholipid and any lipid-soluble components from an organic solution, via evaporation of the solvent. This film is then hydrated with aqueous phase containing any water-soluble components, at a temperature exceeding the main phase transition temperature of the phospholipid (see Section 2.3.2.2.), followed by agitation (Bangham et al, 1965).

Figure 1.1. Schematic representation of a section of a multi-lamellar liposome, incorporating a variety of drug molecules.



Size reduction of MLVs by probe sonication produces small unilamellar vesicles (SUVs). The energy input disrupts the liposome membrane to produce smaller liposomes. Alternatively, MLV suspensions may be extruded through polycarbonate filters, so that part of the liposome is squeezed off and subsequently reseals to form smaller liposomes (Olson et al, 1979). SUVs range in size from 25 to 100 nm, according to the ionic strength of the aqueous medium and the phospholipid composition of the liposomes.

Several approaches to increasing the trapping efficiency of liposomes have been developed. Thus, large unilamellar vesicles (LUVs) have been prepared by injection of an ethereal solution of phospholipid into a warmed aqueous phase (Deamer and Bangham, 1976), resulting in a dilute dispersion. LUVs are approximately 1000 nm in

size, and are three to four times more efficient at solute entrapment than MLVs of comparable size. For unilamellar vesicles, the phospholipid content is related to the surface area of the vesicles, which is proportional to the square of the radius, while the entrapped volume varies as the cube of the internal radius. Also, because of the finite thickness of the membrane, as vesicle size decreases, their aqueous volume also decreases since the phospholipids occupy more of the internal space. Therefore for a given quantity of lipid, large liposomes entrap a greater volume than do small liposomes.

High encapsulation levels have been achieved by preparing reverse-phase evaporation vesicles (REVs) (Szoka and Papahadjopoulos, 1978). A "stable" water-in-oil emulsion is produced, consisting of phospholipid, aqueous phase and organic solvents. The organic phase is then removed by evaporation under reduced pressure to produce a viscous gel which collapses to produce the REVs, which are unilamellar and/or oligolamellar in nature and of the order of approximately 0.5 to 1 μm.

Kirby and Gregoriadis (1984) have used dehydration-rehydration technology to produce liposome with a high encapsulation ratio known as dehydration-rehydration vesicles (DRVs). They are prepared by mixing a solution containing drug with a suspension of blank (water-containing) SUV liposomes, and freeze-drying the mixture. DRVs are formed upon controlled rehydration and contain on average 40% more of the original drug in entrapped form, than an equivalent MLV preparation. DRVs are usually unilamellar and 1 μm or less in diameter.

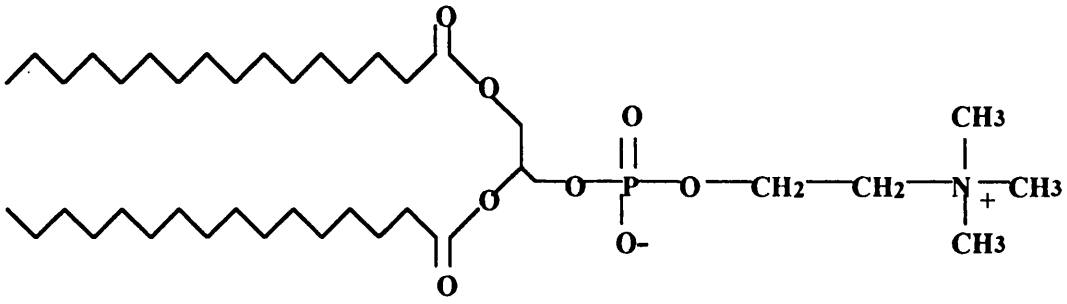
A freezing and thawing process can be used to rupture and re-fuse SUVs. The resultant, primarily unilamellar liposomes are larger in size, and therefore have a greater entrapment volume than the original SUVs. However this method requires the presence of a charged species in the bilayer (for the formation of ice crystals to aid in the rupture-fusion process), and high phospholipid concentrations (greater than 40 mg/ml) may reduce the trapping efficiency (New, 1990a).

1.1.2. Phosphatidylcholines

Phospholipids are the major structural components of biological membranes, and the most common phospholipids are the amphiphilic phosphatidylcholine (PC)

molecules, in which a glycerol bridge links the two hydrophobic long chain fatty acids with a hydrophilic phosphoryl moiety. In aqueous media PC molecules arrange themselves into planar bilayer sheets in order to minimise the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. These interactions are eliminated when bilayers form closed sealed vehicles. Micellar structures are not formed preferentially, because the PC molecule has a tubular shape more suitable for aggregation in planar sheets. The structure of a dipalmitoylphosphatidylcholine (DPPC) molecule may be seen in Figure 1.2.

Figure 1.2. Structure of a dipalmitoylphosphatidylcholine (DPPC) molecule.



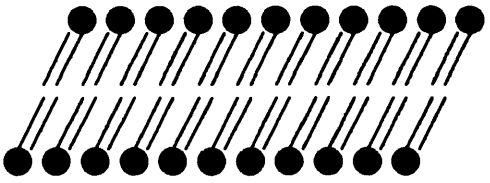
At the membrane boundary where the phospholipid head-groups come into contact with the bulk aqueous phase, their limited motion results in the phospholipid molecules aligning into a two-dimensional arrangement, with the molecules all adopting a set distance and orientation with respect to each other. In the liquid-crystalline phase (described in Section 1.1.3.) the glycerol backbone of the phospholipid molecule is the most severely restricted in its motion. Towards the end of the chain, the motion becomes progressively less restricted. The alignment of the glycerol bridge perpendicular to the plane of the bilayer would be expected to reduce the distance between the positive and negative charges within the molecule. The bulky head-group occupies an area of approximately 0.42 nm^2, whereas the two straight chains occupy 0.39 nm^2 (Street, 1993). Therefore the chains tilt to fill the space created by the head-groups, and van der Waals and other non-covalent interactions are maximised.

1.1.3. Main phase transition of phosphatidylcholines

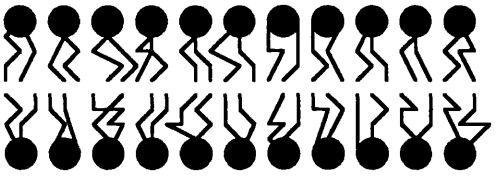
Depending on the phospholipid and environmental temperature, PC bilayers can exist in different phases, the most consistently observed of which are the highly ordered "gel" phase (or $P_{b'}$ phase), and the more fluid "liquid-crystalline" phase (or L_a state) (nomenclature following that of Tardieu et al, 1973). Diagrammatic representations of these phases are shown in Figure 1.3.

Figure 1.3. Different phases of phosphatidylcholine bilayers.

(a) gel phase



(b) liquid-crystalline phase



The transition from the gel (Figure 1.3(a)) to the liquid-crystalline phase (Figure 1. 3(b)) occurs because an increase in temperature alters the rotation of the carbon-carbon single bonds of the hydrocarbon chains.

In the membrane, packing abnormalities such as point defects, line defects and grain boundaries occur because of the presence of impurities or because molecules adopt an altered configuration. These abnormalities result in an increased permeability of small molecules through these regions of the membrane. The main gel to liquid-crystalline transition is associated with these packing abnormalities such that, as the temperature increases and the main transition becomes favourable, so the duration and number of defects increases. Since both phases (liquid-crystalline and gel) are found to

co-exist at the phase transition temperature (Jain et al, 1975; Weis, 1991), the incidence of grain boundary defects increases, particularly at the interfaces of the two phases. Consequently, membrane permeability at the phase transition temperature is greatly increased and loss of entrapped material is temperature dependent, being greatest around the transition temperature (T_m) of the phospholipid (Papahadjopoulos et al, 1973).

1.1.4. Other phase transitions of phosphatidylcholines

In reality the transition from gel to liquid-crystalline phase of liposomes is not a single melting process as, depending on the amount of water present, phospholipids exist in one or more intermediate forms. Section 4.1.2. describes the events that occur at each transition.

There is some evidence that other phospholipid-mediated thermal events, not directly related to the melting of the hydrocarbon chains, can also occur. A variety of techniques have suggested the possible presence of pre-melting and pre-freezing phenomena, liquid-liquid phase separations and grain boundary defects in the solid and glassy state in some model membranes (McElhaney, 1982).

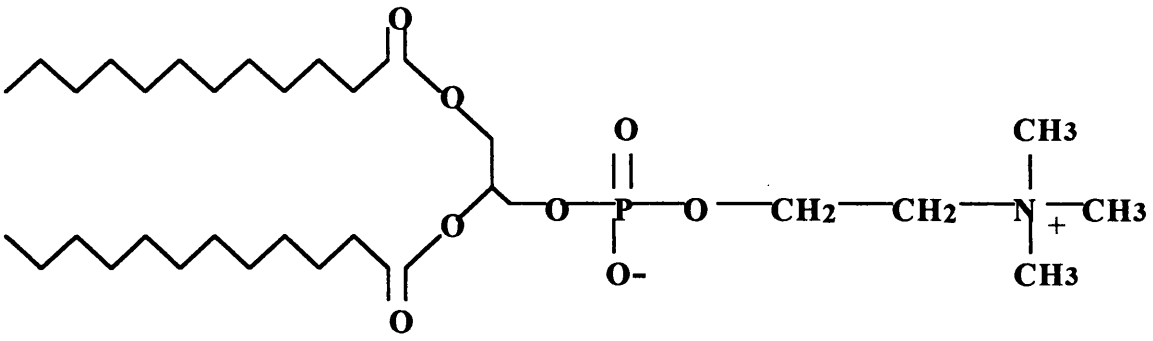
1.1.5. Other phospholipids

Thermal profiles of liposomes vary according to the alkyl chain and head-group of the constituent phospholipid(s). Section 4.1.4. describes the effect of altering one or both of these features, such as chain length and/or head-group charge, on phospholipid phase transitions.

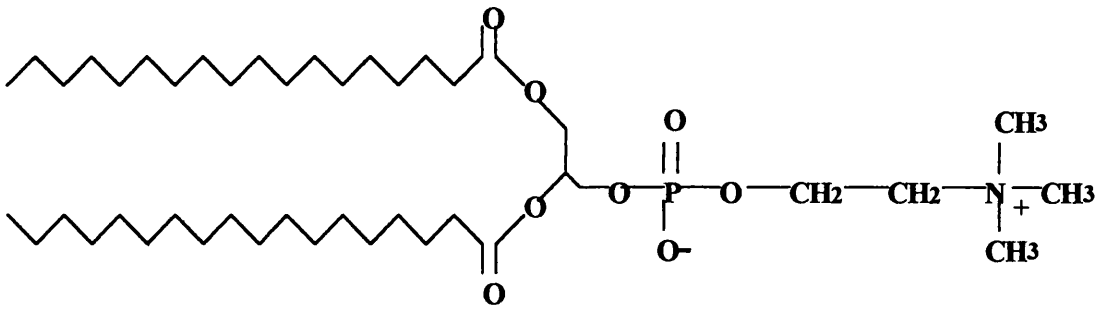
The structures of several phospholipids, differing in chain length and/or head-group may be seen in Figure 1.4. Distearoylphosphatidylcholine (DSPC) has the same head-group as DPPC but a longer chain (C_{18}), whereas dilaurylphosphatidylcholine (DLPC) has a shorter chain length (C_{12}).

Figure 1.4. Structures of several phospholipid molecules.

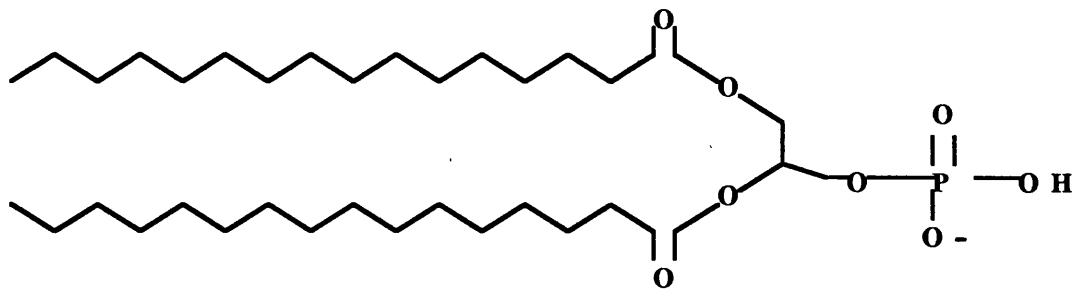
(a) Dilaurylphosphatidylcholine (DLPC)



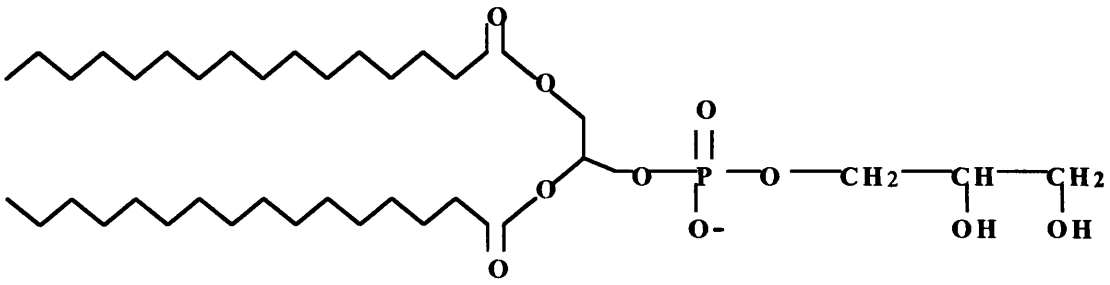
(b) Distearylphosphatidylcholine (DSPC)



(c) Dipalmitoylphosphatidic acid (DPPA)



(d) Dipalmitoylphophatidylglycerol (DPPG)



1.1.6. Phase behaviour of liposomes other than MLVs

SUVs show a thermal profile different to that of MLVs or LUVs (Biltonen and Lichtenberg, 1993). For example, high-sensitivity DSC studies have shown that freshly sonicated vesicles of disaturated phosphatidylcholines have no pre-transition and a decreased enthalpy of the main transition (Suurkuusk et al, 1976). A more detailed description of the effect of liposome size on phospholipid phase transitions can be found in Section 4.1.3.

1.1.7. Measurement of phase transitions

DPPC has a main phase transition temperature (T_m) of approximately 41°C and a pre-transition temperature of approximately 34-35°C, as determined by differential scanning calorimetry (DSC) (Fildes and Oliver, 1978; Fuldner, 1981). Since there is an increase in fluidity of the bilayer at the T_m, other techniques employed to measure the T_m utilise probes which measure molecular motion, such as nuclear magnetic resonance (NMR) (Lewis et al, 1984) and electron spin resonance (ESR) spectroscopy (Cater et al, 1974).

Historically DSC has been used primarily to study the thermally induced transitions of phospholipid bilayers and biological membranes (Ladbrooke and Chapman, 1969). However, whilst DSC can provide useful information regarding the changes associated with the phospholipid transformation, it does not provide direct information about the structural state of the phospholipid under a given set of conditions.

The principles of power-compensated DSC involve simultaneously heating a sample and an inert reference (that is one that does not undergo any transition within the temperature range of interest) at the same rate. The temperatures of both pans initially increase linearly with time and the temperature difference between them is maintained at zero. If the sample undergoes a thermally-induced event, the control system senses the resulting temperature differential between the two pans, and supplies more or less heat to the sample to hold its temperature equal to that of the reference. The recorded DSC parameter is excess specific heat or differential heat as a function of temperature. Thermal events manifest as endotherms or exotherms and the magnitude of deflection depends on the magnitude of the differential heating rate.

The main transition is the temperature at which excess specific heat reaches a maximum. T_m represents the temperature at which the transition is half complete. However for asymmetric traces (characteristic of certain phospholipids), the T_m does not represent the midpoint of the transition and instead $T_{½}$ may be reported. $T_{½}$ is the width of the peak at half its height, and is also known as the HHW (half height width). More details of the principles of DSC and the interpretation of thermal data, are given in Sections 4.1.5. and 4.1.6., respectively.

1.1.8. Thermal analysis of liposomes

Liposomes have been proven to be a key tool in the study of the physical characteristics of membrane lipids, since they are made up of phospholipids which have characteristic phase transition profiles. Therefore when compounds such as drug molecules interact with bilayers, the effect on these profiles can be measured using a number of techniques such as DSC (Fildes and Oliver, 1978) and solution calorimetry (Taylor et al, 1990a). The information gained, such as the maximum amount of drug which may be entrapped (Fildes and Oliver, 1978), and the location of the entrapped material in the bilayer (Jain and Wu, 1977), can help in the design of liposomal formulations for drug delivery.

The information derived from heat capacity curves (those constructed using heat capacity and temperature as the y and x axes, respectively) has been limited to the determination of standard enthalpy changes, and the onset and peak transition

temperatures. The integral under the curve is equal to the enthalpy of the transition but the shape of the curve can be used to quantitate the interaction of compounds with phospholipid bilayers (as described in Section 4.1.6.), by measuring HHW values (Jain and Wu, 1977).

The phase behaviour of liposomal systems containing drug, determines properties such as the permeability, fusion and aggregation tendencies of these liposomes, which in turn affects the stability of dosage forms with respect to drug release, since the permeability of bilayers is related to bilayer fluidity.

1.2. Entrapment of drugs into liposomes

1.2.1. Introduction

Studies have demonstrated the ability of liposomes to encapsulate a diverse assortment of drugs. As discussed in Section 1.1, the nature of the entrapped material determines its location within a liposome. Hence hydrophobic materials are associated with the membrane's hydrocarbon region, whilst hydrophilic materials are found nearer the polar head-group region. Amphiphiles may be located at the interface between the polar and the non-polar regions. Materials with poor solubility in aqueous and organic solvents, together with materials which are highly soluble in both media, do not incorporate particularly well into liposomes because of the relative ease by which they are able to permeate through the bilayer (New, 1990b). Consequently the method by which drugs are entrapped must be appropriate for the type of drug.

Entrapment of drugs into liposomes may be achieved either passively or actively. In the first instance passive entrapment of hydrophilic materials may be achieved by adding the water-soluble drug to the aqueous phase during the hydration stage of liposome manufacture, and such drugs would be expected to be located mainly in the aqueous core, and also in the aqueous channels between bilayers. Therefore the volume of water contained in the aqueous compartments plays an important role because it varies considerably with different types and sizes of liposomes (Mayer et al, 1992). Conversely, hydrophobic drugs which are by definition poorly soluble in water, may be homogeneously mixed with phospholipids in an organic solution during the initial stage of liposome manufacture. In this way a hydrophobic drug may be

passively incorporated into the phospholipid bilayers. Consequently any interaction between the two components may dictate the amount of hydrophobic drug entrapped, which is highly dependent on phospholipid properties, such as chain length (Shaw et al, 1976), degree of chain saturation (Stamp and Juliano, 1979), the affinity of the drug for the bilayer (its hydrophobicity and partition coefficient) (Perkins et al, 1993), the total phospholipid concentration (Fildes and Oliver, 1978) and the physical state of the bilayer (Stamp and Juliano, 1979; Ma et al, 1991). In the second instance, active entrapment involves establishing ion gradients to entrap mostly amphiphilic agents (see Section 1.2.4.).

Generally the term drug entrapment has been used to discuss how much drug is entrapped within a liposome, but drugs may be incorporated, loaded, associated, bound or otherwise attached to liposomes or their bilayers. The entrapment efficiency is usually expressed as a percentage of the starting amount of the drug. Thus the optimum entrapment would be 100 %. Alternatively entrapment efficiency may be expressed as the amount of drug entrapped per unit weight of phospholipid, or the number of moles of drug entrapped per mole of phospholipid.

It is important to determine how much drug has been entrapped into liposomes, as other physical characteristics, such as size and lamellarity, can then be related to the drug concentration within a liposome. Once a sample of drug-containing liposomes is administered to an animal or patient, the amount of drug entrapped (and hence the dose administered) can also be related to any pharmacological effects observed. Thus entire clinical and physico-chemical profiles of a liposomal system may be compiled, and with this information, future entrapment may be optimised.

The selection of the entrapment technique must be appropriate for the type of material to be entrapped, and the factors which may affect aqueous and hydrophobic entrapment, together with examples, have been highlighted below.

1.2.2. Entrapment of hydrophilic materials

Passive aqueous entrapment relies on the ability of liposomes to capture a certain aqueous volume during liposome manufacture. This aqueous volume is known as the captured volume. Once incorporated into liposomes, water-soluble drugs may interact with the polar head-groups of lipid bilayers. Hence, the extent of entrapment

of a water-soluble drug is mainly dependent on the number of polar head-groups present - in other words, the volume of water enclosed within the aqueous compartments of a liposome. The captured volume differs for liposomes of varying size and type, and it can be altered by various experimental parameters such as the length of time the phospholipid film has been left to hydrate, the method by which the lipid is dispersed, the thickness of the phospholipid film and the concentration and composition of the phospholipid phase (Olson et al, 1979). For example the presence of charged moieties in the bilayer can result in bilayer repulsion, increasing the aqueous volume and hence aqueous entrapment (Bangham et al, 1967). Drug properties such as aqueous solubility may also be a limiting factor in aqueous entrapment.

Captured volume can be measured by incorporating an impermeable aqueous marker into the hydrating solution. After the formation of liposomes, unencapsulated marker is separated from the encapsulated marker, and the ratio of solute:lipid is measured to give an indication of the distribution of the solute within the sample (Perkins et al, 1993). However this method cannot be used if the marker is unevenly distributed throughout all aqueous compartments, which has been shown to be the case for MLVs formed by the simple hydration of dry phospholipid films (Gruner et al, 1985).

If the external medium of a liposomal system is replaced with a spectroscopically inert fluid and the water signal is measured, for example by NMR, then the quantity of water captured internally by the liposomes may be measured (Pidgeon et al, 1986). If the aqueous solubility of the drug is assumed to be the same as its solubility in the aqueous compartments within a liposome, then it is possible to estimate how much drug is entrapped.

There are various ways of improving the entrapment of aqueous materials into liposomes. The presence of a drug in the aqueous compartment is less likely to be affected by the nature of the phospholipid used (Stamp and Juliano, 1979) so the phospholipid composition may be of minor importance. As mentioned earlier, passive entrapment is highly dependent on the type and size of the vesicle employed. SUVs have a low captured volume per mole of lipid when compared to LUVs and MLVs. LUVs have a high aqueous:phospholipid compartment ratio making them particularly suitable for the entrapment of aqueous materials (New, 1990c). Within each liposome type there are further differences depending on the preparation method used and the

total phospholipid concentration. Therefore there are factors which may be altered in order to increase entrapment.

For example, if a sample of SUVs that do not contain any drug is frozen and lyophilised together with the material to be entrapped, there is intimate contact between the phospholipid and the solute in the dry state. Consequently when the suspension is rehydrated, vesicles with a high capture efficiency are formed, known as DRVs (dehydration-rehydration vesicles also described in Section 1.1.1.) (Kirby and Gregoriadis, 1984). For truly hydrophilic substances, the concentration of entrapped drug increases linearly with captured volume (Perkins et al, 1993). Therefore if vesicles with a large internal aqueous volume are produced, so the entrapment of hydrophilic drugs may be increased. This was demonstrated by Szoka and Papahadjopoulos (1978) with the formation of REVs (also described in Section 1.1.1.) which produce liposomes with large aqueous volumes and high drug/lipid ratios. For example, Taylor et al (1990b) incorporated sodium cromoglycate (SCG), a highly polar drug, into MLVs. Entrapment into REVs was found to be twice that of entrapment into MLVs of comparable size.

Another way in which to increase captured volume is through the use of charged phospholipids. MLVs composed of neutral phospholipids have very tightly packed adjacent bilayers with very little aqueous space between them. The presence of charged phospholipids in the membrane results in electrostatic repulsion of the bilayers (Johnson, 1973), thus increasing the liposome size. This may be used as a method by which drug entrapment into aqueous regions may be increased (Alpar et al, 1981). For example, stearylamine is an agent that can be used to confer a positive charge to the bilayer (Gregoriadis, 1973) in order to promote bilayer repulsion. This may also be achieved by repeatedly freezing and thawing liposomes composed of neutral phospholipids (Mayer et al, 1986).

Generally the entrapment efficiency of hydrophobic drugs is higher than that of polar drugs, and therefore there have been attempts in the past to increase aqueous entrapment of compounds through the synthesis of their hydrophobic pro-drugs (Ma et al, 1991). These structures are designed to bind to the bilayer during delivery, but readily degrade to the parent compound in vivo.

Examples of the many hydrophilic materials that have been incorporated into liposomes include penicillin (Gregoriadis, 1973), cytarabine (Stamp and Juliano, 1979;

Juliano and McCullough, 1980), hydroxycobalamin (Alpar et al, 1981), salbutamol (Farr et al, 1989) and lignocaine (Sharma et al, 1994).

1.2.3. Entrapment of hydrophobic materials

Hydrophobic drugs are poorly soluble in water, and may be homogeneously mixed with phospholipids in an organic solution during liposome manufacture. As mentioned in Section 1.2.1., the amount of hydrophobic drug entrapped is dependent on the nature and concentration of phospholipid, the affinity of the drug for the bilayer and the physical state of the bilayer. For example, encapsulation into membranes that are in the liquid-crystalline state has been shown to be favoured, in comparison to encapsulation into membranes in the gel state (Stamp and Juliano, 1979). This is due to the fact that the alkyl chains in the liquid-crystalline state are less rigid and are able to accommodate drugs into their structure with more ease.

MLVs are more suited for entrapping hydrophobic drugs than unilamellar vesicles, because the interior of MLVs is occupied by more bilayers, as oppose to a large aqueous compartment (New, 1990d). The entrapment efficiency of MLVs can be increased by increasing their size and phospholipid concentration (resulting in more bilayers and therefore a greater hydrophobic area). 100 % entrapment (that is 100 % of the total starting amount of drug) may be achieved provided that this quantity of material is accommodated within the structure of the MLV with minimal disruption to bilayer packing. The lamellae within a MLV provide a stable environment for a hydrophobic drug, and the extent of entrapment may be approximated by the drug's oil/water partition coefficient (Perkins et al, 1993).

Examples of hydrophobic drugs that have been incorporated into liposomes include actinomycin D (Gregoriadis, 1973), hydrocortisone palmitate (Shaw et al, 1976; Fildes and Oliver, 1978), hydrocortisone 21-octanoate (Arrowsmith et al, 1983a and b; Farr et al, 1989) and atropine (Meisner et al, 1989).

1.2.4. Entrapment of amphiphilic materials

Amphiphilic materials are the most difficult to retain within a liposome structure because of the rapid rate at which they are able to permeate the liposome bilayer. However, since a large number of drugs are amphiphilic, ways to improve their entrapment have been investigated. For example, the environment within the interior of the liposome can be prepared so that the drug - usually a lipophilic amine - is always in the ionised form when in the liposome, but in its uncharged form when not in the liposome (Mayer et al, 1992). In this way a drug may enter a liposome by diffusion, and may be unable to leave the liposome because its lipophilicity is reduced by its conversion to a charged species. Therefore drugs are able to redistribute across phospholipid bilayers in response to a change in membrane potentials or transmembrane proton gradients. Since drug uptake in response to a change in pH does not require the use of exogenous ionophores, this method is of a more practical use (Mayer et al, 1986). Doxorubicin and vinblastine are antineoplastic agents that have been entrapped successfully into liposomes in response to either membrane potentials or proton gradients (Mayer et al, 1986). These methods may be used to entrap drugs once the liposomal carriers have been formed. This is known as remote loading and is particularly useful when delivery systems require immediate administration after preparation (for drugs that are labile, for example).

1.2.5. Separation and calculation of the amount of entrapped drug within liposomes

In order to measure the quantity of material entrapped within a liposome, un-incorporated (free) material must usually first be removed. There are several ways in which separation of free and entrapped drug may be achieved, namely dialysis, centrifugation, ultrafiltration or gel filtration chromatography.

Sephadex G-50 is the material most widely used for column chromatographic separation, where the removal of encapsulated drug is dependent on the size differences between liposomes and free drug, the latter remaining on the column whilst liposomes containing drug are eluted into the void volume. Medium or coarse grades

of Sephadex (with a particle size of 50 - 150 μm) are suitable for MLVs, whilst finer grades are preferable for SUVs. However leakage of entrapped material may occur if any phospholipid within the sample interacts with the surface of the beads of the column, which is noticeable with low phospholipid concentrations. This phenomenon may be prevented by initially saturating the column with empty liposomes. Whilst this technique is useful for separating SUVs and MLVs within the same sample, problems may be encountered when separating unentrapped material that is similar in size to the liposomes within the sample, leading to inefficient separation (New, 1990e). Stamp and Juliano (1979) achieved separation and determined encapsulation efficiencies from the size of the void volume (liposomes) and retarded volume (free drug) peaks. Separation by dialysis is also dependent on size differences, and may be a time-consuming process. The membranes used are constructed from polycarbonate, and an area of the membrane is covered by pores. Dialysis usually requires several days to efficiently separate unentrapped material since the diffusion of large molecules may be slow and difficult. Dialysis has been found to be inadvisable for some materials such as steroids, which have been reported to adsorb onto the polymeric membranes used in dialysis (Arrowsmith et al, 1983a) thus hindering the separation of unincorporated steroid. It has been reported that ultrafiltration is not an efficient method for separating entrapped material (Ryman and Tyrrell, 1979), although prolonged ultrafiltration has been used to separate free from liposome-entrapped ^{99m}Tc-DTPA (Barker et al, 1994). The basis behind separation of free material by centrifugation is that liposomes containing entrapped material will be more dense than water or saline. Thus when suspended in either of these media and subjected to a high gravitational field, these liposomes would be expected to sediment, whilst any free material should remain in the supernatant. This has been the method of choice for many authors (Taylor et al, 1990b; Meisner et al, 1989; Ma et al, 1991), but a possible drawback of this method is the inability to efficiently sediment smaller vesicles (Tyrell et al, 1976). Alternatively liposomes may be suspended in a medium of higher density, resulting in the liposomally entrapped material floating on top (Fraley et al, 1980). This may be useful if the entrapped material has a high molecular weight, or is entrapped at high concentrations, making sedimentation difficult.

After separation of the free drug, the measurement of aqueous entrapment may be determined even if the concentration within the liposomes is not directly measured. For example, Taylor et al (1990b) incorporated a polar drug, sodium cromoglycate (SCG) into REVs. Centrifugation and UV assay was used to determine the concentration of free drug, and from a knowledge of the initial total amount of drug, the amount of entrapped drug was calculated by subtraction of the unentrapped fraction. To confirm that the total amount of drug in the preparations does actually correspond to the concentration of the entrapped and unentrapped drug, Triton-X-100 may be added (final concentration of 1 % v/v, for example). This agent serves to rupture liposomal membranes and therefore release any entrapped hydrophilic drug, which may be measured directly from UV absorbance readings.

Ethanol is another agent which may be used to rupture liposomal membranes. For example, Ma et al (1991) investigated the partitioning of an homologous series of alkyl-p-aminobenzoates into MLVs. The free drug was separated by ultracentrifugation, and the pellet and the supernatant were both analysed for drug content by UV spectroscopy. Both these fractions were then dissolved in a known amount of ethanol and the UV absorbances of each were measured at the wavelength of maximum absorbance of the drug. The concentration of the solute in both phases was determined from a Beer-Lambert plot.

Aqueous entrapment in liposomes may be directly measured by determining the amount of drug released on their lysis. For example, Sharma et al (1994) centrifuged a liposome suspension containing lignocaine (a hydrophilic drug) to separate the free drug, and the liposome pellet was resuspended in fresh buffer to remove the drug completely. Once it was assumed only liposomally entrapped drug was present, a solution of Triton X-100 was added to lyse the liposomes, and the concentration of the entrapped drug was determined by UV spectroscopy.

The amount of hydrophobic drug within a bilayer cannot be directly measured without altering the original liposome system, and so it can be more difficult to measure hydrophobic entrapment than aqueous entrapment. For instance the use of agents such as Triton X-100 or ethanol may be futile, since the rupture of liposomal membranes will not lead to the release of any hydrophobic contents since these drugs may remain associated with the resultant mixed micelle whilst hydrophilic drugs leak out easily (Stamp and Juliano, 1979). The presence of hydrophobic drugs within a

bilayer (above certain concentrations), will itself alter bilayer properties such as fluidity (Jain and Wu, 1977). This characteristic may be exploited when determining hydrophobic entrapment since bilayer properties may be measured by a variety of techniques such as fluorescence polarisation spectroscopy (Lentz, 1993) and DSC (McElhaney, 1982).

1.3. Entrapment of steroids into liposomes

Successful management of asthmatic patients usually depends on achieving adequate delivery of inhaled drugs to the lungs. Many problems have been encountered with the delivery of steroids used for their anti-inflammatory effects. Whilst there are clear advantages to the local administration of steroids via inhalation (such as a rapid onset of action with fewer systemic adverse effects experienced), there are also disadvantages and possible problems associated with nocturnal asthma (the short duration of activity requires frequent dosing which may be unpleasant for the patient). Therefore there is justification in investigating alternative delivery systems which are able to efficiently carry and deliver steroid to the alveolar region of the lungs. One of these systems is liposomes.

Many authors have incorporated a range of steroids into liposomal systems and the entrapment has been determined. Some examples have been highlighted. Devoiselle et al (1992) analysed SUVs containing flumethasone and dexamethasone. The non-entrapped fraction of steroid was removed by ultracentrifugation. Steroid entrapment was calculated by HPLC with a UV/visible detector, and calibration curves were constructed on the basis of peak area measurements for each steroid. Arrowsmith et al (1983a) radiolabelled the polypolar steroids, cortisone and hydrocortisone, before incorporating them into liposomes. Hydrocortisone-21-octanoate (a model hydrophobic drug) was shown to partition into phospholipid bilayers. During the preparation of the liposomes, drug and phospholipid were combined in an organic solution before the solvent was evaporated off and the phospholipid film hydrated. The ratio of supernatant to suspension radioactivity was then used to determine the percentage of steroid released with time.

DSC has been used to quantify the maximum incorporation of drugs into the bilayers of liposomes (see Section 1.1.8) by the measurement of the maximum width of

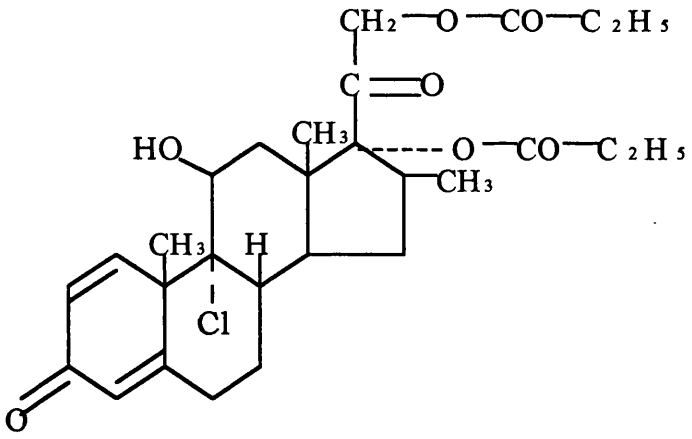
the main transition peak at half its height (HHW) (Fildes and Oliver, 1978). Low entrapment values for hydrocortisone have been reported (Fildes and Oliver, 1978) using this measurement, and an initial phase of rapid drug loss has also been reported for other hydrophobic drugs (Shaw et al, 1976; Arrowsmith et al, 1983a). This may reflect a loss from the liposome structure or loss of surface-associated material. The use of the palmitate ester of hydrocortisone to "anchor" the steroid to the bilayer (Shaw et al, 1976) was investigated. Whilst previous work has implied hydrocortisone interacts with the polar head-groups (Cleary and Zatz, 1973), the ester was found to have a limited affinity for the phospholipid, the excess forming a discrete phase or acting as insoluble particles stabilised by adsorbed phospholipid. Steroid esters have been reported to have limited solubilities in phospholipid systems (Smith et al, 1980). The low aqueous solubilities of steroids may result in material in excess of this limit being present in a crystalline form. This may be external or internal to the liposome, and therefore release rates may be partially attributable to the presence of this excess rather than efflux of drug from liposomes. The initial rapid release of synthetic steroids was also suggested to be due to an incompatible fit of the steroid in the liposome bilayer (Shaw et al, 1976; Radhakrishnan, 1991). Shaw et al (1976) incorporated hydrocortisone palmitate into liposomes composed of either DMPC, DPPC or DSPC, and noted that whilst the maximum amount of steroid entrapped was independent of the nature of the phospholipid, the rate of drug release was related to the length of the alkyl chain. DPPC liposomes were found to exhibit enhanced retention, reflecting an optimum compatibility between the ester and the hydrophobic chains. Similarly Arrowsmith et al (1983a) studied the release of cortisone esters (hexadecanoate and octadecanoate) from liposomes composed of either DMPC, DPPC or DSPC, and found that efflux from DMPC liposomes was the fastest, whilst release rates from DSPC and DPPC liposomes were similar. Further, the longer chained ester was released more slowly than the shorter chained ester from DSPC liposomes. This indicates that release rates are independent of chain length for DMPC and DPPC liposomes, but suggests that for DSPC liposomes, the length of the ester chain is similar to that of the phospholipid alkyl chain length.

1.4. Entrapment of BDP into liposomes

1.4.1. BDP structure and properties

Beclomethasone dipropionate (BDP) is a halogenated synthetic analogue of hydrocortisone, and it has a full name of 9 alpha chloro-11 beta, 17 alpha, 21-thrihydroxy-16 beta-methylpregna-1,4-diene-3, 20-dione 17, 21-dipropionate (Figure 1.5.). It has a poor aqueous solubility of only 54 μg/ml (Radhakrishnan, 1991). However, it is freely soluble in acetone and 96 % ethanol, and very soluble in chloroform (British Pharmacopoeia, 1993). The partition coefficient for BDP between octanol and phosphate buffer saline at a pH of 7.4 is such that approximately 95 % is associated with the octanol (Radhakrishnan, 1991).

Figure 1.5. Structure of beclomethasone dipropionate (BDP) molecule.



1.4.2. BDP clinical uses and action

BDP is currently used for oral inhalation and as a nasal spray, for the treatment of bronchial asthma and perennial and seasonal rhinitis. The typical inhalation dose of BDP for the treatment of asthma, from a metered dose inhaler, is 200 μg twice daily, or 100 μg in 3-4 divided doses. In addition BDP is available as a dry powder for inhalation (BNF, 1997). Beconase® nasal spray is formulated as an aqueous suspension containing 50 μg\metered spray. Becotide® suspension for nebulisation

contained 50 μg/ml of the active ingredient as an aqueous suspension, and is no longer marketed.

Whilst corticosteroids have been used in the treatment of asthma for many years, their mode of action is not yet fully understood. But they probably reduce bronchial mucosal inflammation, and therefore reduce mucus secretions and oedema (Rang and Dale, 1991). They also suppress the bronchial hyper-reactivity associated with asthma (Davies, 1993).

The pharmacological action of steroids is thought to result in part from a direct interaction with phospholipid in membranes. Steroids may act like cholesterol in membranes, and associate with the acyl chains of the lipid molecules.

The inhaled BDP that reaches the lung, acts partly as a pro-drug, as it is converted by hydrolytic cleavage to 21 beclomethasone monopropionate (21-BMP) and 17 beclomethasone monopropionate (21-BMP) (Davies, 1993). Whilst it was previously thought that BDP was more active than its BMP metabolites, infact 17-BMP has a 30-fold greater affinity for glucocorticosteroid receptors and is more active than BDP (Pavord and Knox, 1993). Also 17-BMP has a higher aqueous solubility and can therefore dissolve in bronchial secretions, unlike BDP. Since BDP is so poorly water-soluble it has been suggested that BDP may act as a reservoir of 17-BMP in the lung (Davies, 1993). In addition both BDP and 17-BMP have short half lives (approximately 30 minutes) (Pavord and Knox, 1993), favouring topical effects as oppose to systemic ones.

1.4.3. BDP formulations

BDP for inhalation is formulated as a microcrystalline suspension in chlorofluorocarbon propellants, whilst Becotide® suspension for nebulisation contains only 50μg /ml of BDP in an aqueous medium (water preserved with benzalkonium chloride) and produced very poor, if any, alveolar deposition, and therefore is no longer marketed. The use of crystalline suspensions and organic solvents may cause tissue irritation, and be painful or impossible to administer by certain routes (Radhakrishnan, 1991). Liposomes may be suitable vehicles for the delivery of drugs to the respiratory system since they may be prepared from materials endogenous to the

lung as components of lung surfactant. DPPC is the major component of lung surfactant (van Golde, 1976). The use of liposomes as solubilising agents for steroids in aqueous, inhaled suspensions may eliminate the use of potentially toxic halogenated hydrocarbon propellants and other solvents, and ensures that the drug stays in a stable suspension. Liposome formulation may also prevents the lung irritation caused by drug sedimentation and crystallisation, often encountered with conventional steroidal suspension preparations (Radhakrishnan, 1991).

The entrapment of BDP into liposomes is not, however, an easy or efficient process, with the phenomenon of BDP crystal formation a limiting factor. Hydrophobic materials in an aqueous environment cause structuring of the surrounding water. This is turn leads to a negative entropy change and energetically favours self-association and partitioning into a hydrophobic phase. This means that whilst partitioning into the aqueous phase is restricted, interactions between steroids and bilayers may be promoted (Arrowsmith et al, 1983a). More importantly, self-association also results in the steroid molecules forming large crystals which may be harmful if administered. For example, the administration of a liposomal sample containing drug crystals might lead to lung irritation as experienced on the administration of non-entrapped drug. Therefore there is a need to remove any crystals from the liposomal sample prior to its characterisation and administration.

BDP is a highly hydrophobic steroidal drug and when incorporated into phospholipid films or bilayers, would be expected to be associated with the hydrocarbon chain region of the lipid molecules. Taylor et al (1990a) found that the incorporation of BDP into DMPC films at 5 mole % resulted in a decrease in the total enthalpy of the liposome formation process. However, a further increase in the proportion of drug had a much less pronounced effect. It was suggested that BDP is affecting liposome formation most probably by partitioning into the phospholipid and interfering with the van der Waals interactions between the phospholipid hydrocarbon chains, which play an important role in determining the enthalpy of the transition (Nagle, 1980). The smaller responses produced on addition of larger proportions of drug indicates that perhaps ideal mixing is not occurring, and that a solid drug phase is co-existing with a fluid liquid-crystalline phospholipid phase. Therefore additional BDP has little opportunity to interfere with either the hydration or the phase transition

of the phospholipids. Therefore it has been identified that the formation of a separate BDP phase occurs only when a certain concentration has been exceeded.

Waldrep et al (1994) incorporated BDP into MLVs by dissolving the drug and lipid in an organic solution prior to formation of the dry film. Liposome preparations were checked for the presence of drug crystals and lipid aggregates, but how they were removed was not documented. A Centrifree Micropartition System was used to remove any free drug, and the encapsulation efficiency (determined using HPLC) was found to be 97 - 99 % of the original starting amount of BDP. The reason for such a high entrapment yield may be because the limit at which BDP forms crystals was not exceeded.

Radhakrishnan (1991) proposed the theory that BDP tends to crystallise due to an incompatible steric fit between the steroid and the bilayer. When a conventional liposome sample, prepared from phospholipid components, was prepared, a large amount of crystalline steroid was detected after extrusion and on storage. Therefore an alternative non-conventional liposome system (composed of non-phospholipid components such as cholesterol and its derivatives) was produced, which accommodated BDP molecules and thus resulted in an increased entrapment efficiency.

One of the most extensively researched approaches in drug delivery is the entrapment of hydrophobic drugs into liposomes, and the optimisation of this process. However, the behaviour of these drugs within the liposome remains unknown. For a drug such as BDP, its poor aqueous solubility is an advantage since interactions with the hydrophobic bilayer may be promoted. However the compatibility of BDP and liposomes is also dependent on the size and spatial structure of the BDP molecule, together with any chemical functions that may help stabilise BDP within the bilayer (Sampedro et al, 1993). Therefore, hydrophobicity alone does not ensure compatibility of drug and liposome, and the requirements of such a compatibility should be fully investigated.

1.5. Aims of the PhD

The general aims of this PhD were therefore to investigate the interaction between BDP and phospholipid structures, namely monolayers and bilayers, not only to help optimise future steroid entrapment for drug delivery purposes, but also to gain a further understanding of the behaviour of this particular drug within these structures. Specifically, the aims of this PhD include characterising the nature of the steroid molecules within phospholipid films and bilayers, in order to determine the solvate properties of BDP, and to accurately identify the concentration at which drug crystals form in liposomal suspensions. In addition, it was hoped that techniques to efficiently separate unentrapped crystalline material would be improved, and a drug assay developed to determine the concentration of drug entrapped within liposomes. There is a strong justification in investigating the occurrence of BDP crystals, as the knowledge of the limiting concentration at which they form, could result in the optimisation of liposome preparation methods, such that the formation of crystals may be avoided, and therefore samples would not require any crystal removal.

Whilst it is generally accepted that liposomes have applications as vehicles for drug delivery, they may also act as model systems for studying the interactions of phospholipid membranes with drug molecules. Membranes may be made up of one or more bilayers of phospholipid, as seen in a liposome structure, but monolayers of the same phospholipid molecules can also be constructed on an aqueous surface. These monomolecular films might serve as a physical model for cellular membranes, since they are coherent, interfacial and organised, and therefore useful in obtaining information on the orientation and arrangement of components within that monolayer (Zatz and Cleary, 1975). Therefore other aims of this PhD include the use of monolayers constructed from various phospholipids, to investigate the incorporation of BDP into these structures, with a view to developing a predictive test for the behaviour of BDP in equivalent bilayer systems. In addition, thermal analysis of liposomes containing varying amounts of drug will be performed to determine the effects of BDP on the bilayers of liposomes composed of different phospholipids.

The behaviour of BDP in phospholipid monolayers under high compression, will be of prime importance in predicting behaviour in liposomes, since it is this region that is most indicative of the bilayer region of liposomes. In addition, thermal analysis

may be useful to determine the effects of BDP on phospholipid bilayers, particularly the hydrophobic chain region. Therefore it is hoped that a profile may be built up as to how a model steroid, such as BDP, behaves in phospholipid monolayers and bilayers, and this may be of use in suggesting ways in which BDP entrapment into liposomes may be enhanced, for the purposes of drug delivery.

2. ENTRAPMENT OF BDP IN PHOSPHOLIPID FILMS AND LIPOSOMAL SUSPENSIONS

2.1. Aims of studies

Successful liposomal entrapment of a hydrophobic drug is thought to be the result of combining a high concentration of the drug within phospholipid bilayers, and a poor aqueous drug solubility (and thus low association with hydrophilic regions) (Stamp and Juliano, 1979). BDP is a hydrophobic drug with a low aqueous solubility, and thus interactions with the hydrocarbon regions of liposomal bilayers would be expected to be promoted. However the tendency to form crystals above certain concentrations may severely limit the extent of BDP entrapment. The behaviour of BDP within liposome bilayers is still poorly understood, as is the form in which the drug exists within such an environment. Therefore, an aim of these investigations was directed towards further understanding the solvate nature of BDP when dissolved in a variety of solvents, with a view to investigating crystal formation in liposomal suspensions. Other aims focused mainly on developing techniques to identify the presence of BDP crystals and the concentration at which excess BDP crystallises into separate domains, with a view to preventing their formation in future work.

Whilst various techniques to investigate the effects of drugs on liposomal bilayers have been developed, such as DSC (described fully in Chapter 4), there is still a need to quantify the amount of steroid entrapped, as the drug concentration within a liposomal sample can then be accurately related to other characteristics of such a sample, such as liposome size, lamellarity and bilayer fluidity. Clinical and physico-chemical profiles of a liposomal system containing drug may be compiled and optimised to achieve maximum drug entrapment (which may be expressed as the amount of drug entrapped per unit weight of phospholipid, or the number of moles of drug entrapped per mole of phospholipid).

Drug assays may be used to determine the concentration of entrapped drug in liposomal samples. Miyajima et al (1993) used a superoxide dismutase (SOD) assay when quantifying SOD entrapment, but recognised that the values obtained reflected not only entrapped SOD but also SOD associated with liposomal surfaces. This highlights a need for the development of techniques that determine the concentration

of entrapped drug alone. In addition, drug associated with liposomal surfaces and with non-liposomal phospholipid requires removal, since in a formulation this drug may become unassociated (and essentially released) prior to reaching the target site and will affect entrapment profiles, and thus bioavailability studies. Therefore these studies aimed to develop a separation method that would efficiently remove non-entrapped material. On completion of this step, it was hoped that a drug assay for BDP could be developed and optimised, so that future assays of liposomal samples would accurately reflect the liposomally-entrapped concentration of BDP.

2.2. Characterisation of BDP solvates and hydrates

2.2.1. Introduction

Some drug materials can crystallise in such a way that molecules of the solvent in which they were dissolved become incorporated into their crystal lattice. The solid is then referred to as a solvate, and stoichiometric or non-stoichiometric amounts of solvent may become entrapped within the crystal lattice. If the incorporated solvent molecules are water, then the solid that separates is known as a hydrate. Solvates may undergo desolvation under a variety of conditions. Some desolvate readily to a form that has the same crystal structure as the solvate. These forms are known as pseudoanhydrates, and are able to resolvate easily, under the appropriate conditions. However some crystals desolvate to produce forms with a different crystal structure to that of the original solvate, and these crystals require dissolution and recrystallisation in order to achieve resolvation (Haleblian, 1975).

Different solvates of a drug material have different physical properties. For example, one solvate may show 5 to 10 times the solubility and bioavailability of another solvate of the same drug (Haleblian, 1975). Also, the nature of the incorporated solvent may influence subsequent crystals shapes, which may be important when incorporating drugs into liposomes, since the bulkiness of the solvated molecule may dictate its entrapment profile. For example resorcinol crystallises from benzene into fine needles, but it crystallises from butyl acetate into squat prisms (Haleblian, 1975).

If a hydrate is formed, then the interaction between the drug and water that occurs in the crystal phase reduces the amount of energy liberated when the solid hydrate dissolves in water. Consequently, hydrated crystals tend to exhibit a lower aqueous solubility than their unhydrated forms. In contrast the solubilities of non-aqueous solvates are often greater than those of the unsolvated forms (Richards, 1988). BDP has an aqueous solubility of only 54.4 μg/ml, as reported by Radhakrishnan (1991), and 58 μg/ml (at 25°C) as reported by Kabasakalian et al (1966), and forms solvates with a variety of organic solvents including chloroform (Glaxo Wellcome in-house data). BDP is known to exist in three different crystal forms, namely the anhydrate, monohydrate and organic solvate (Glaxo Wellcome in-house data). Each form has a different arrangement of molecules within their lattices, whilst the solvates formed with trichloroacetic acid, isopropanol and ethyl acetate all have the same steroidal lattice structure. The solvate behaviour of BDP in phospholipid films and liposomal suspensions is as yet unknown, and therefore preliminary investigations into this area may provide some useful information on the entrapment of this drug into liposomes.

A variety of techniques may be used to characterise solvates, including DSC (Sekiguchi et al, 1968), thermogravimetric analysis (Hirtz et al, 1968), and X-ray diffraction (Haleblian et al, 1971). Thermal analysis is useful when investigating the removal of solvent molecules from solvates, since the temperature at which this loss occurs usually registers as an endotherm on DSC scans. The removal of solvent is inevitably accompanied by a loss in sample weight, and TGA is therefore useful to detect not only the temperature at which this loss occurs, but also to calculate the stoichiometric weight of the solvent. The use of DSC and TGA together, has been advocated in the past when studying polymorphism and pseudopolymorphism in organic compounds (Haleblian and McCrone 1969).

2.2.2. Materials and Methods

The solvents used in these investigations were all purchased from BDH Chemicals (Poole, Dorset), and were all HPLC Grade (HiPerSolv), unless otherwise stated. BDP monohydrate was supplied as a micronised powder by Glaxo Wellcome (Ware, Herts.).

Thermogravimetric analysis (TGA) is described in Section 2.2.2.1., whilst differential scanning calorimetry (DSC) is described fully in Chapter 4.

2.2.2.1. Thermogravimetric analysis

TGA principally involves monitoring sample weight as a function of temperature. The TGA apparatus uses an electronic null position balance, rather than a deflection type, which is ideal since it ensures that the sample maintains a constant position in the furnace. An aluminium pan containing the sample is placed on a hangdown wire attached to the balance. During heating of the sample in the TGA furnace, the system is able to sense if the balance moves from its null position and a restoring forces is applied, which is proportional to the change in the weight of the sample. The temperature within the furnace will vary due to its geometry, so by keeping the sample in the same position the temperature can be accurately controlled. A thermocouple in the furnace, situated approximately 2 mm above the sample pan, is used to monitor the environmental temperature. Heating rates and ranges are pre-programmable, and computer software allows for derivative recording, automatic temperature and mass calibration and zeroing. For pharmaceutical studies temperatures of 350°C are attainable for sample sizes that are typically up to 20 mg in weight. The furnace may be cooled between runs using a compressed air supply. In addition a water reservoir is connected to supply water continuously around the furnace during the experiments and while the furnace is cooling down. A purge gas is always used to prevent contamination of the furnace and balance assemblies, in this case, nitrogen. A large flow of gas (60 ml/minute) is sent horizontally across the furnace chamber, sweeping away any gaseous products of decomposition into the outlet tube. A smaller flow of gas (40 ml/minute) is sent down through the balance

chamber and then into the furnace, preventing decomposition products from entering this chamber and causing contamination.

2.2.2.2. Sample preparation

Approximately 15-20 mg of BDP monohydrate was dissolved in approximately 5 ml of either methanol, acetone or chloroform at ambient temperature. Each sample was placed in a watch-glass and the solvent was allowed to evaporate by placing the watch-glasses in an oven at 50°C overnight. The remaining powder was then stored in a glass jar in a desiccator at 19°C, since fresh samples may lose initial solvent content on atmospheric exposure at room temperatures. Each powder, together with ordinary BDP monohydrate, was analysed by TGA (HiRes TGA Model 2950, TA Instruments) in open aluminium pans. Sample sizes were typically 2-3 mg, and all runs were performed at heating rates of 10°C/minute, over a range of temperature from ambient to 230°C. In addition, samples were also placed into open aluminium pans (approximately 2-3 mg) and analysed by DSC (Model DSC7, Perkin Elmer) over a range of 0°C to 230°C, using a heating rate of 10°C/minute. All runs were carried out four times.

2.2.3. Results and Discussion

TGA data and DSC data were obtained for samples of BDP monohydrate that had been exposed to various organic solvents. Figure 2.1(a) is a typical TGA trace obtained for a sample of BDP monohydrate. Figures 2.1(b), (c) and (d) are typical TGA traces for BDP samples recrystallised from chloroform, methanol and acetone, respectively. The results are expressed as a plot of weight loss versus temperature, and a derivative curve is used to calculate the % weight loss per minute. Each run was repeated three times, and Table 2.1. presents the average weight losses for each sample.

Figure 2.1(a) TGA trace for sample of BDP monohydrate not exposed to solvent.

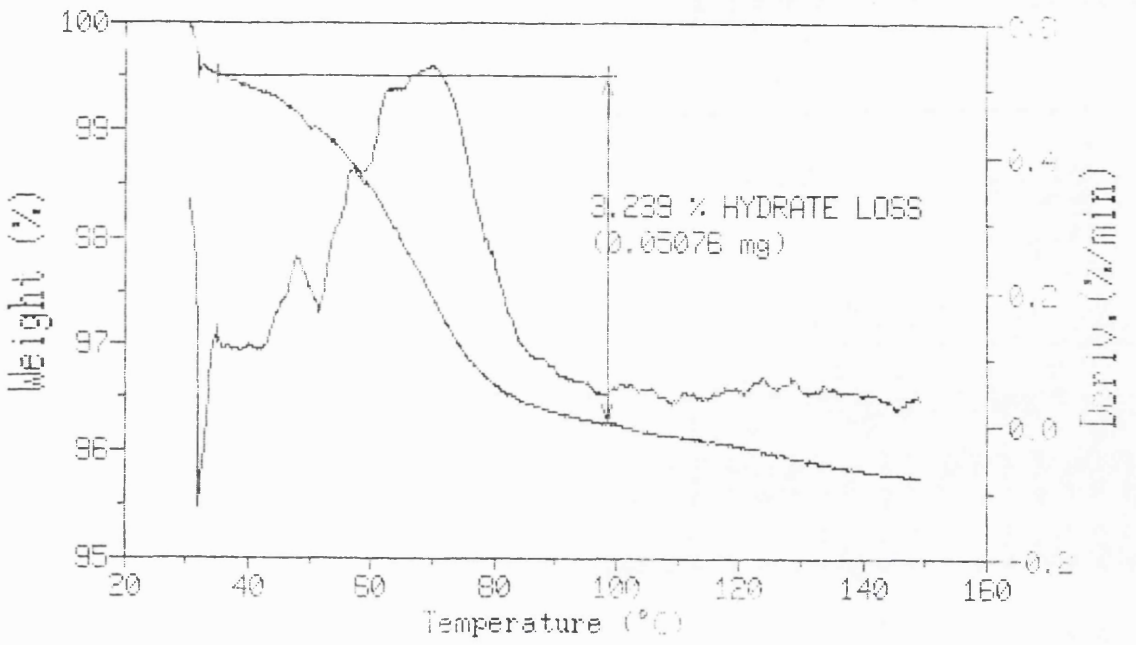


Figure 2.1(b) TGA trace for sample of BDP recrystallised from chloroform.

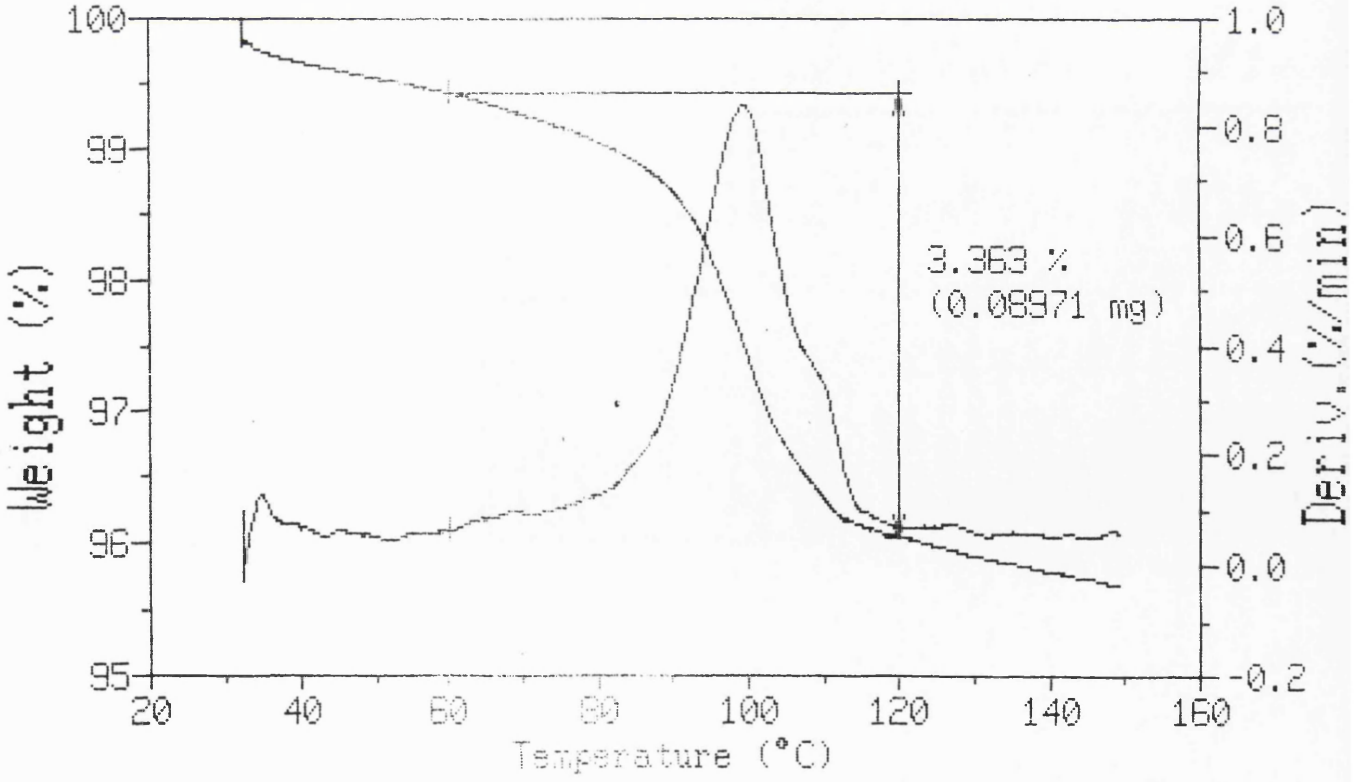


Figure 2.1(c) TGA trace for sample of BDP recrystallised from methanol.

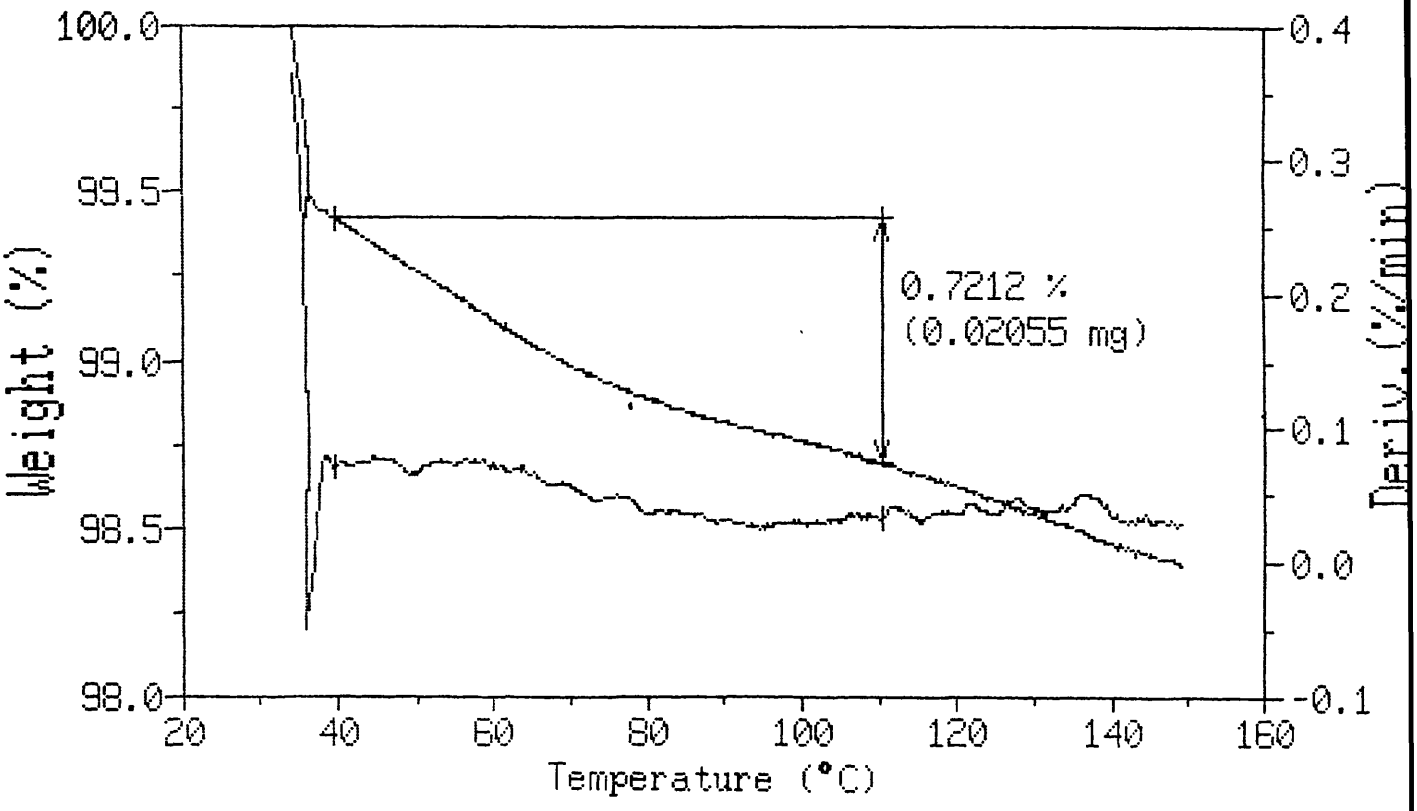


Figure 2.1(d) TGA trace for sample of BDP recrystallised from acetone.

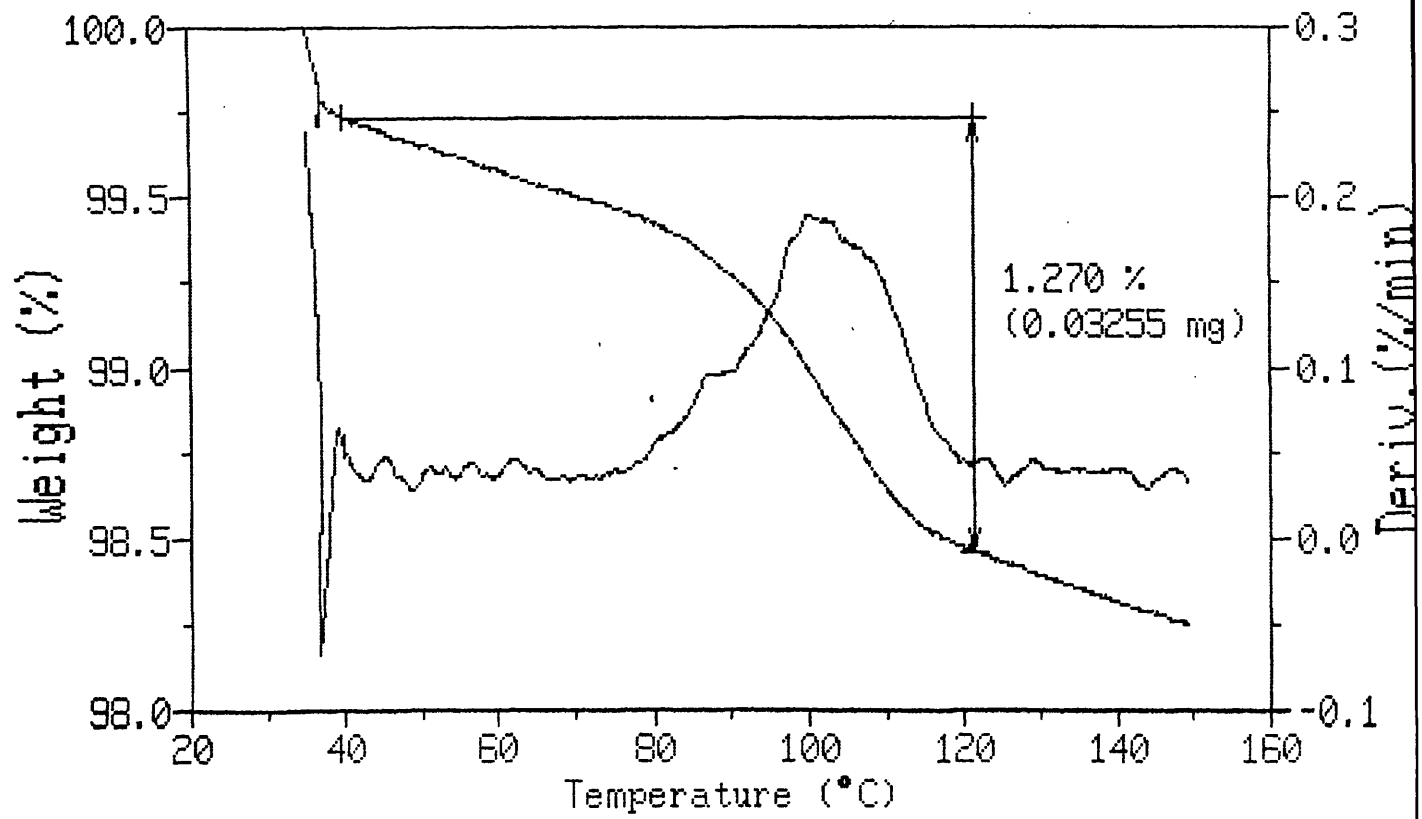


Table 2.1. TGA data for BDP monohydrate.

Solvent of exposure	Average weight loss (%) (± standard deviation)
None	3.277 ± 0.141
Methanol	0.837 ± 0.147
Chloroform	3.398 ± 0.042
Acetone	1.234 ± 0.179

Figure 2.2(a) is a typical DSC trace for a sample of BDP monohydrate, showing a broad endotherm, small exotherm and a melting endotherm (Glaxo Wellcome in-house data), whilst Figures 2.2(b), (c) and (d) are DSC traces for samples of BDP monohydrate exposed to chloroform, methanol and acetone, respectively. Runs were repeated three times and Tables 2.2(a), (b), (c) and (d) present the average peak temperatures obtained for each sample.

Table 2.2(a) DSC data for BDP monohydrate not exposed to solvent.

Transition	Average temperature (°C) (± standard deviation)	Average enthalpy (J/g) (± standard deviation)
Endotherm	84.653 ± 0.995	44.435 ± 14.424
Exotherm	121.123 ± 0.893	-10.000 ± 1.097
Endotherm	214.390 ± 0.457	78.660 ± 13.255

Table 2.2(b) DSC data for BDP recrystallised from chloroform.

Transition	Average temperature (°C) (± standard deviation)	Average enthalpy (J/g) (± standard deviation)
Exotherm	123.685 ± 14.044	-25.820 ± 10.571
Endotherm	214.030 ± 0.510	57.160 ± 8.745

Figure 2.2(a) DSC trace for sample of BDP monohydrate not exposed to solvent.

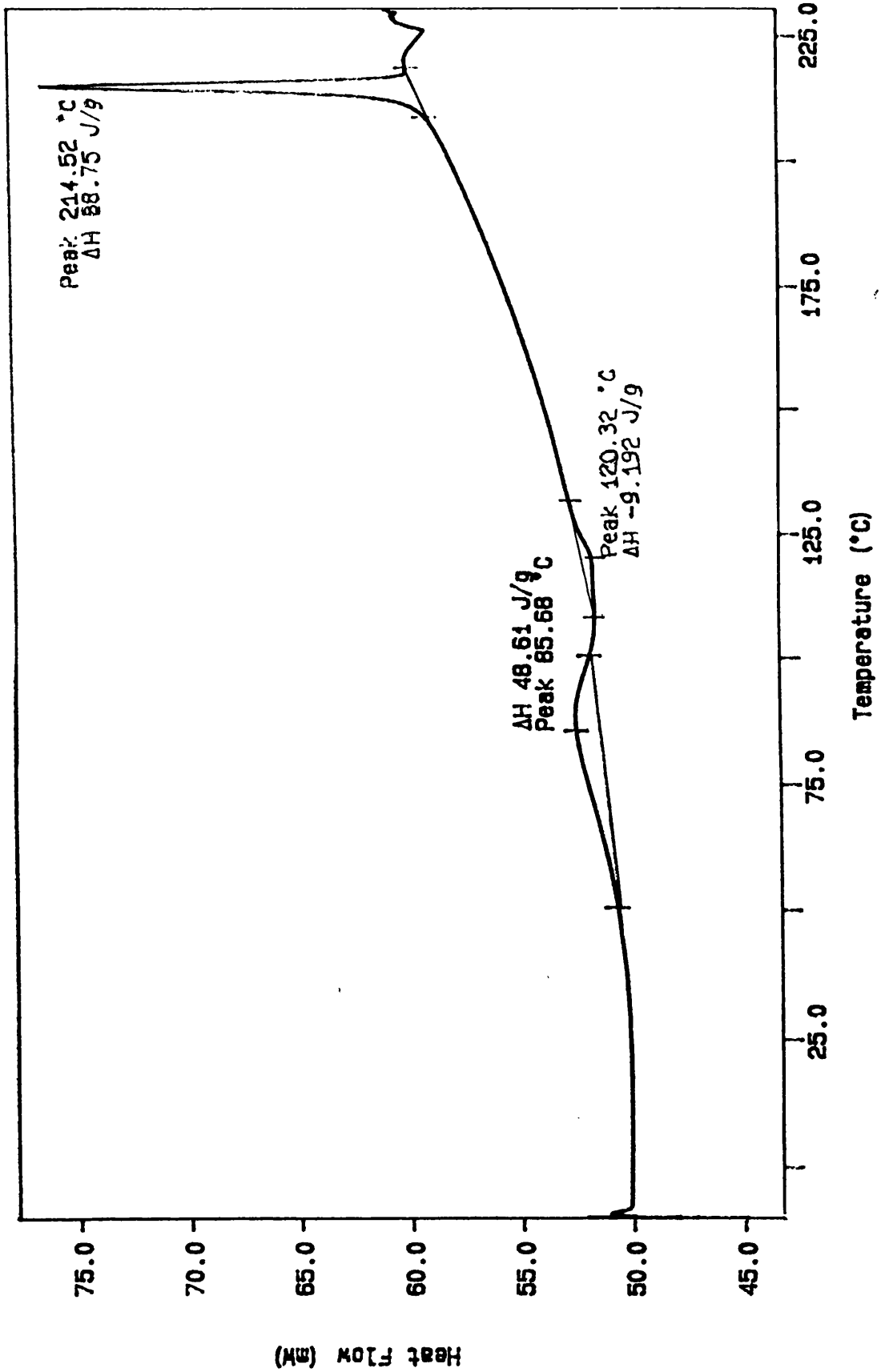


Figure 2.2(b) DSC trace for sample of BDP recrystallised from chloroform.

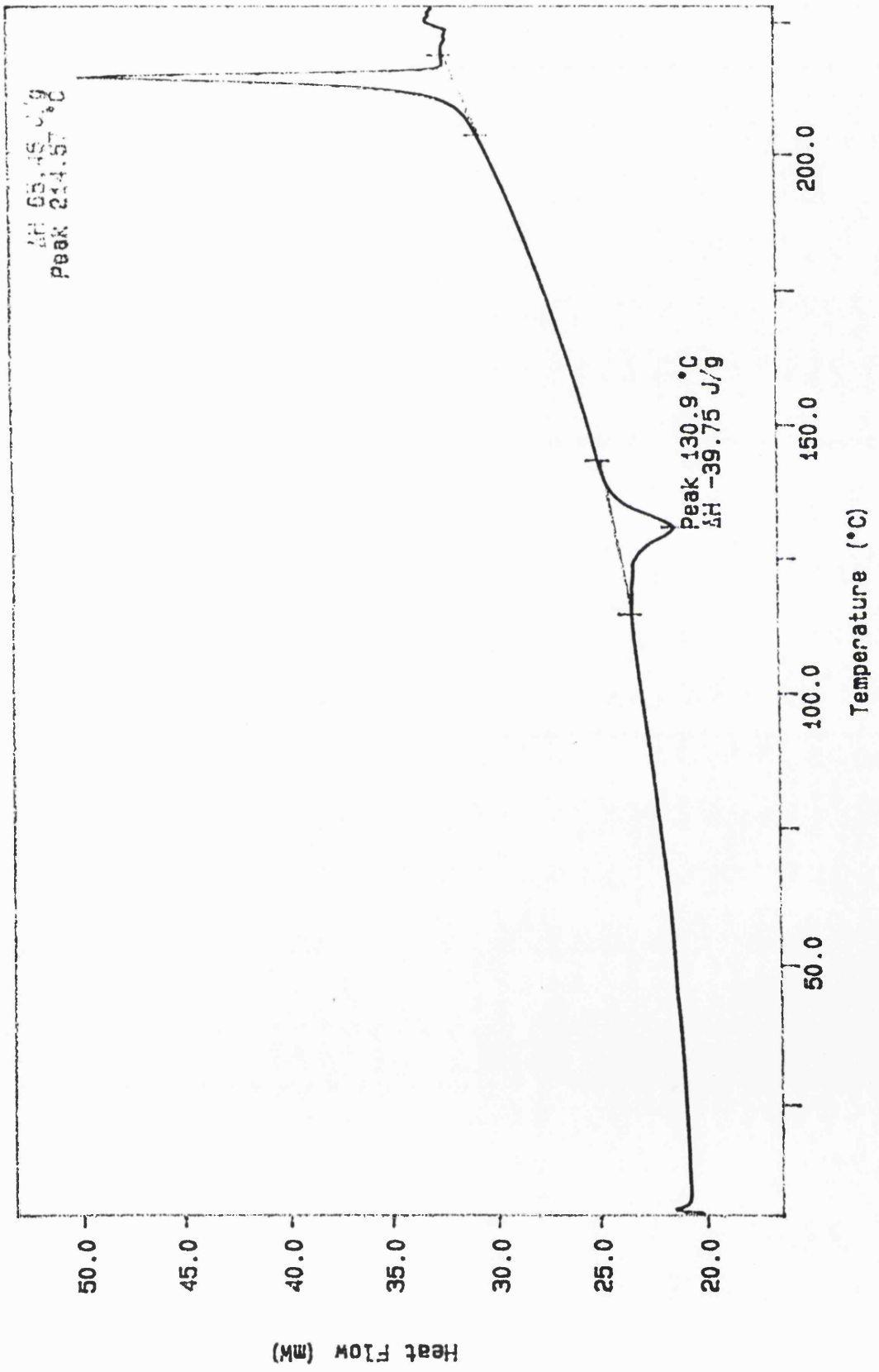


Figure 2.2(c) DSC trace for sample of BDP recrystallised from methanol.

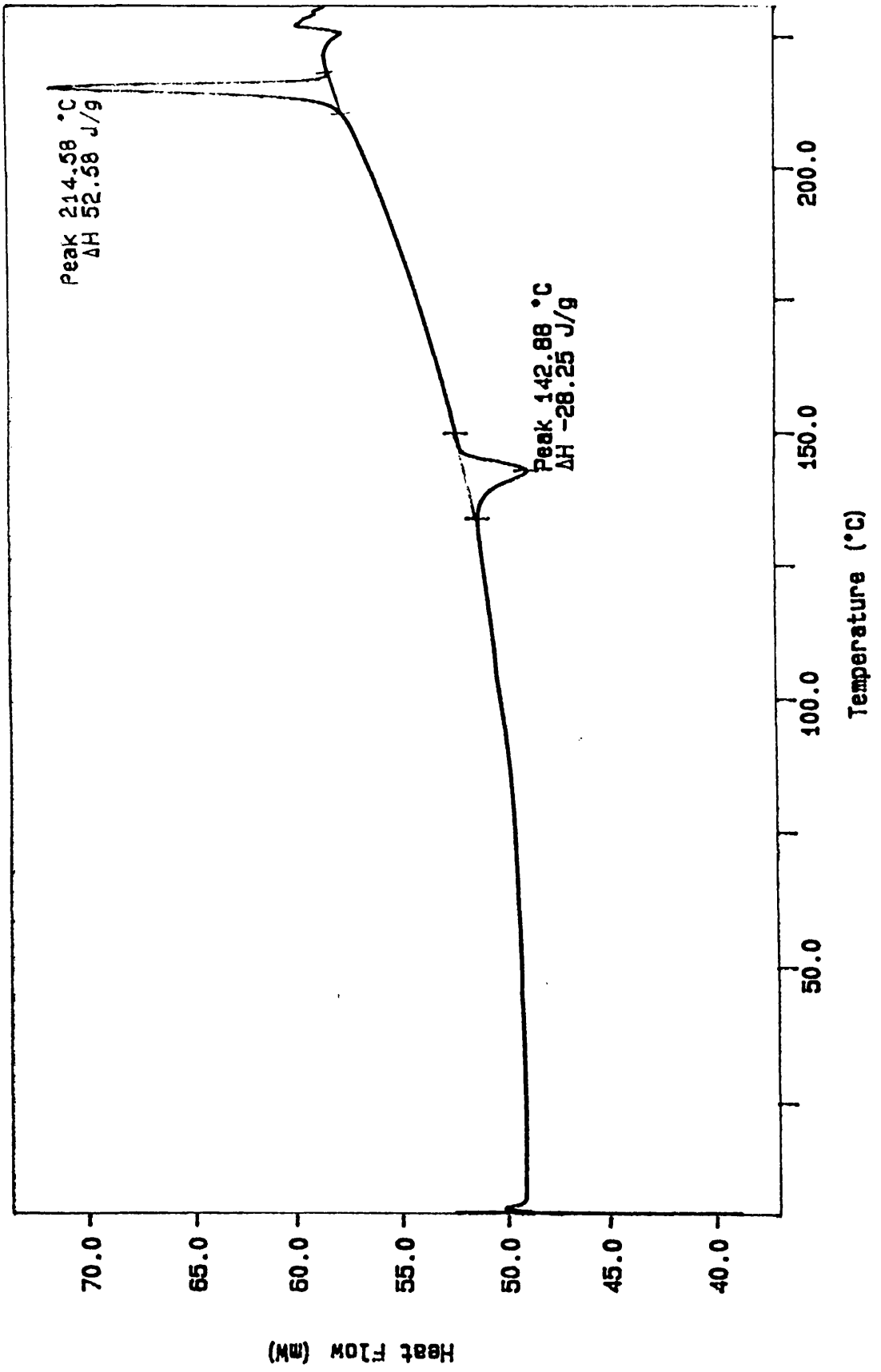


Figure 2.2(d) DSC trace for sample of BDP recrystallised from acetone.

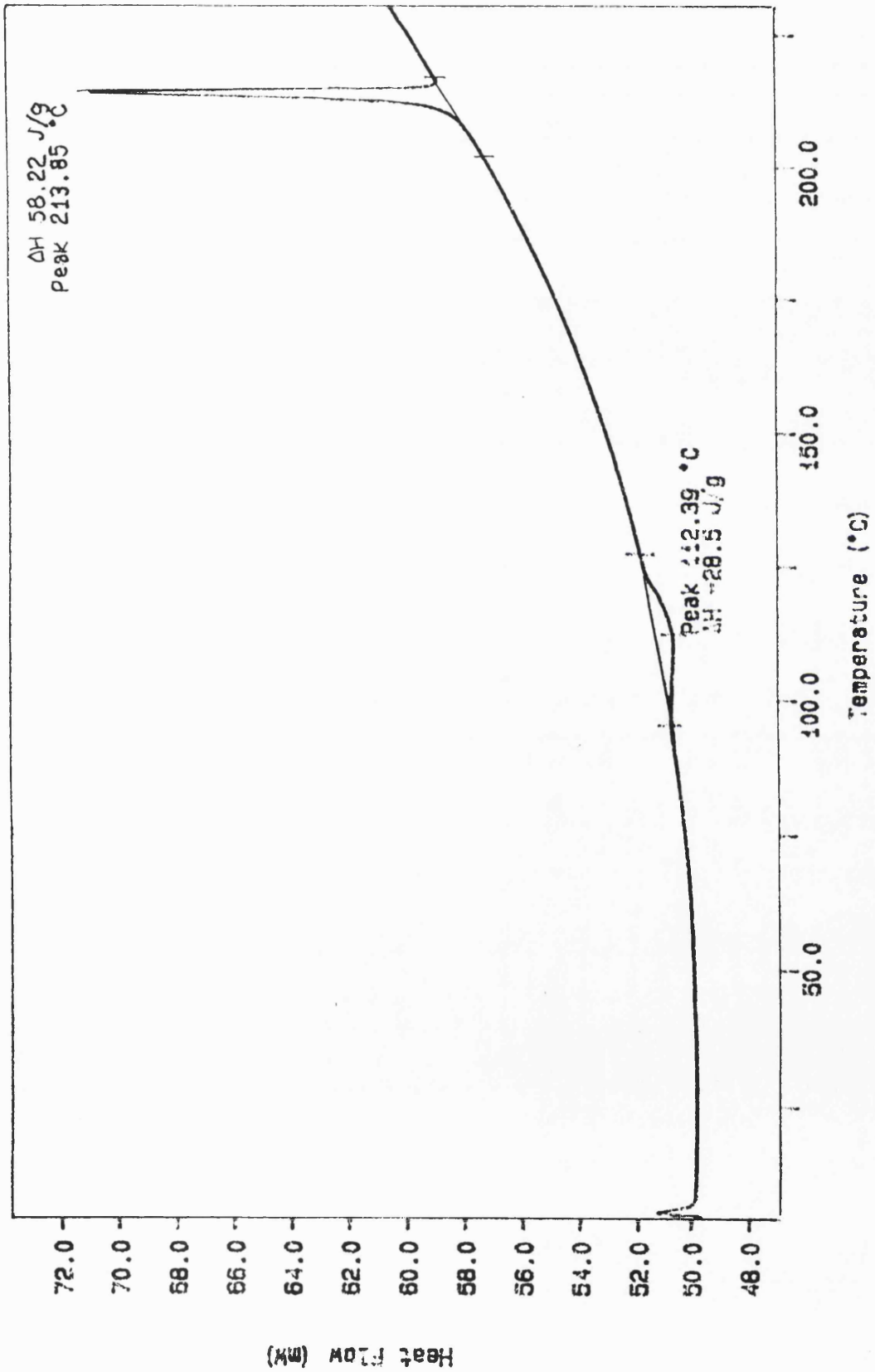


Table 2.2(c) DSC data for BDP recrystallised from methanol.

Transition	Average temperature (°C) (± standard deviation)	Average enthalpy (J/g) (± standard deviation)
Exotherm	142.470 ± 0.350	-34.915 ± 10.754
Endotherm	214.080 ± 0.383	62.278 ± 16.917

Table 2.2(d) DSC data for BDP recrystallised from acetone.

Transition	Average temperature (°C) (± standard deviation)	Average enthalpy (J/g) (± standard deviation)
Exotherm	114.837 ± 3.930	-27.530 ± 2.073
Endotherm	213.723 ± 0.242	61.285 ± 7.173

The weight losses associated with BDP monohydrate recrystallised from acetone and methanol are relatively small (approximately 1 %) compared to that of the monohydrate recrystallised from chloroform (approximately 3.4 %). Differences in the DSC profiles of conditioned (drug exposed to solvent) and unconditioned drug was noted to be especially prevalent in the region of the endotherm at approximately 85°C, with this endotherm notably absent for DSC traces of the conditioned drug (Figures 2.2(b), (c) and (d)).

Unconditioned drug (that is BDP monohydrate not exposed to solvent) showed a weight loss that reflected the loss of water from within the lattice. The monohydrate has a limiting stoichiometry originating in specific hydrogen bonding which serves to create a water molecule bridge within each individual steroid molecule. The 1:1 stoichiometry of the monohydrate ($C_{28}H_{37}ClO_7.H_2O$) requires that the water content is 3.34 %, by weight. Therefore the value in Table 2.1. reflects the complete removal of the 1 molecule of water of hydration per steroid molecule. As the temperature of the system is increased, the water of hydration is driven off (seen by a broad endotherm at approximately 85°C) (Table 2.2(a)). For micronised BDP monohydrate, the release of water molecules is achieved with initial retention of its lattice structure, and infra-red studies (Glaxo Wellcome in-house data) have shown that when the micronised

monohydrate is fully (or extensively) dehydrated, it is able to reconvert into the original monohydrate immediately on exposure to water vapour (at room temperature). Further heating causes the resultant vacant hydrate lattice (the pseudoanhydrate) to rearrange to the true anhydrate, characterised by the broad exotherm at 120-130°C. The subsequent thermal behaviour then corresponds to that of the normal anhydrate, with melting at 213°C and thermal decomposition (Table 2.2(a)), as suggested by previous work (Glaxo Wellcome in-house data). Dehydration of non-micronised BDP monohydrate results in collapse of this structure, and a concomitant rearrangement into the anhydrate (seen by a broad endotherm in the region of 85-95°C), which is metastable.

DSC of BDP recrystallised from solvents did not produce any endotherms at 85°C, confirming that none of the forms resembles that of the normal micronised monohydrate. In addition, exotherms for all samples were noted in the region of 110-140°C, which may correspond to the rearrangement of the empty crystal lattice into the anhydrate form. The constant value of the melting temperature (approximately 214°C) (Tables 2.2(b), (c) and (d)) suggests that all forms convert to the lattice structure of a normal anhydrate (either at the time of full solvent loss or after this). Solvates of BDP can be converted into the normal anhydrate by heating to 130-140°C (Glaxo Wellcome in-house data). This process is thought to be akin to the conversion of the monohydrate into the anhydrate, although different temperatures are required to bring about this conversion. In addition X-ray diffraction studies (Glaxo Wellcome in-house data) have demonstrated that whilst the solvate and hydrate crystal lattices both contain 'pockets' (spatial volume between two adjacent steroid molecules), the anhydrate does not. Therefore the solvate can easily undergo a conversion to the hydrate because water molecules in the gaseous phase can gain ready access to the empty pocket in the lattice of the solvate. However the solvate cannot easily convert to the anhydrate form, since the latter form takes up a smaller volume and this conversion requires high temperatures.

Table 2.1. shows that the weight losses associated with heating the monohydrate recrystallised from chloroform are approximately 3.4 %. This may indicate that the monohydrate exposed to chloroform remained unchanged in the presence of solvent and remained a monohydrate, since the weight loss correlates well

to the loss of water from within the lattice. However, the broad endotherm characteristic of the loss of water of crystallisation from micronised BDP monohydrate, was not apparent on DSC traces of monohydrate exposed to chloroform (Figure 2.2(b)). TGA traces (Figures 2.1(a) and (b)) show that the temperature range over which weight losses occured was 50-80°C for BDP monohydrate, but 90-120°C for BDP recrystallised from chloroform. This suggests that loss from the BDP recrystallised from chloroform was not due to the loss of water of crystallisation from the monohydrate, but the loss of solvent from an organic solvate. TGA data suggests that a solvate formed with chloroform has a solvent content of less than 1:1 steroid:solvent, the exact molar ratio being 1:0.15 BDP:chloroform. Studies on other BDP solvates have also indicated that solvates with organic solvents are non-stoichiometric and of variable composition, with a solvent content always less than 1:1 steroid:solvent (Glaxo Wellcome in-house data). BDP has been shown to form a solvate with chloroform (Glaxo Wellcome in-house data).

The weight losses associated with TGA of monohydrates exposed to methanol and acetone (approximately 0.8 and 1.2 %, respectively), suggest that these forms are not monohydrates, but may be solvates, with a very small solvent content. However, an alternative explanation may be put forward, since the weight losses are relatively low and this is expected for pseudoanhydrates. DSC results suggest that neither forms are monohydrates, and BDP has shown no solvate formation with either methanol or acetone (Glaxo Wellcome in-house data). Therefore, these findings are consistent with the theory that monohydrates exposed to methanol and acetone, recrystallise into pseudoanhydrates, whilst monohydrates exposed to chloroform recrystallise into solvates.

2.2.4. Conclusions

To conclude, unconditioned drug has confirmed the characteristics of micronised BDP monohydrate, both through TGA and DSC analysis. In addition BDP monohydrate forms a solvate when recrystallised from chloroform, but with a stoichiometry of less than 1:1 solvent: steroid. The results arising from monohydrates exposed to methanol and acetone, are a little more complicated. Glaxo Wellcome in-

house data has suggested that BDP does not form solvates with either of these solvents. No results were produced as to whether in such an environment, the monohydrate still loses its water of crystallisation (and essentially transforms itself into a pseudoanhydrate), or remains a monohydrate. The findings from these studies indicate that these samples form pseudoanhydrates.

These results have implications regarding the incorporation of this drug into phospholipid films and liposomes. The findings of these studies would seem to suggest that BDP is present as a chloroform solvate within dry phospholipid films. During liposome manufacture (Section 2.3.2.2.), BDP monohydrate is dissolved in chloroform, which is subsequently evaporated off under vacuum, at temperatures of 55°C, for example. On the addition of water to hydrate the film, this solvate may remain stable, or may revert to the monohydrate form. Further work is required in order to investigate the effect of vacuum and increased temperatures (mimicking the liposome formation process) on BDP solvates and monohydrates. From the weight losses obtained, the chloroform solvate was calculated to contain 0.15 moles of solvent (equivalent to approximately 18 g) to 1 mole of steroid (521.1 g BDP), and therefore has an identical molecular weight to 1 mole of BDP monohydrate (539.1 g). Therefore future work involving phospholipid films and liposomes, used the molecular weight of BDP monohydrate as representing 1 mole of steroid.

2.3. Incorporation of BDP into liposomes

2.3.1. Introduction

When hydrophobic drugs, such as steroids, are exposed to an aqueous environment, they cause structuring of the surrounding water which leads to a negative entropy change and energetically favours self-association and partitioning into a hydrophobic phase. This means that whilst partitioning into the aqueous phase is restricted, interactions between the drug and liposomal bilayer may be promoted (Arrowsmith et al, 1983a). However, self-association also results in the drug molecules forming large crystals which may be harmful if administered. For example, the pulmonary administration of a liposomal sample containing drug crystals might lead

to lung irritation, as experienced on administration of non-entrapped drug (Radhakrishnan, 1991). There is an obvious need to remove any crystals from liposomes prior to the characterisation and administration of such a sample. One possible method by which liposomal samples may be checked for the presence of drug crystals and unentrapped drug-phospholipid complexes is by microscopy (Radhakrishnan, 1991; Waldrep et al, 1994). By identifying the presence of crystals in such samples, liposome preparation methods may be amended in order to prevent crystal formation, or techniques developed to remove them. Therefore these studies aim to utilise microscopy as a method by which BDP crystals in liposomal preparations may be observed.

Whilst in the past a phase of rapid drug release has been reported for hydrophobic drugs from charged (Juliano and Stamp, 1979) and uncharged liposomes (Arrowsmith et al, 1983a), this may be due to a loss from within the liposome structure, or of surface-associated material. Shaw et al (1976) found that non-esterified steroids were released rapidly from liposomes after preparation, but their esterified forms were found to have a longer retention time, and were incorporated to a greater extent within liposomes. This was confirmed by further studies by Arrowsmith et al (1983b). However, the esters were found to have a limited affinity for the phospholipid, the excess forming a discrete phase or acting as insoluble particles stabilised by adsorbed phospholipid. Steroid esters have been reported in the past to have limited solubilities in phospholipid systems (Smith et al, 1980), and it is expected that the low aqueous solubilities of steroids will result in material, in excess of this limit, being present in a microcrystalline form. This may be external or internal to the liposome, and therefore release rates may be partially attributable to the dissolution of this excess rather than efflux of drug from liposomes.

As mentioned in Section 1.4.3., Taylor et al (1990a) suggested that after the addition of a certain concentration of BDP to DMPC liposomes, a solid drug phase begins to co-exist with a fluid liquid-crystalline phospholipid phase. Radhakrishnan (1991) suggested BDP forms a crystalline phase in liposomal suspensions because of an incompatible steric fit between both components. Unsaturated "conventional" phospholipid liposomes were found to incorporate 1-3 mole % BDP, whilst saturated liposomes (consisting for example, of fully hydrogenated soy phosphatidylcholine) did not even incorporate small amounts of steroid. Lyso phosphatidylcholine liposomes

were able to incorporate 2 mole % BDP but released the drug readily after preparation (detected as crystalline steroid). An alternative non-conventional liposome system composed of non-phospholipid components was found to have a greater percent BDP entrapment in liposomes which contained 30-70 mole % of a cholesterol ester salt (such as sodium cholesterol sulphate) and in combination with cholesterol (20-50 mole %). Therefore drug hydrophobicity and concentration do not ensure compatibility between drug and liposome, and the size and spatial fit of BDP into bilayers may be of prime importance in determining the stability of this steroid in liposome bilayers. In addition to observing drug crystals, it was hoped that microscopy would also help to identify the concentration at which excess BDP forms crystals.

2.3.2. Materials and Methods

The chloroform used in the manufacture of liposomes was purchased from BDH Chemicals (Poole, Dorset), and was HPLC Grade (HiPerSolv). BDP monohydrate was supplied as a micronised powder by Glaxo Wellcome (Ware, Herts.), and dipalmitoylphosphatidylcholine (DPPC)(approximately 99% pure) was purchased from Sigma Chemicals (Poole, Dorset). Deionised water was obtained from laboratory supplies (Model WP 700, Whatman).

2.3.2.1. Differential interference contrast and cross-polarisation microscopy

Differential interference contrast (DIC) is a microscopy technique used to introduce contrast into non-absorbent specimens. Plane-polarised light illuminating the sample passes through a Woolaston prism, which splits the beam into two. Whilst the "object beam" passes through a point in the sample, the other beam falls slightly to one side of this point (usually to the order of the resolving power). Another prism located above the objective acts to recombine the beams and the phase change introduced into the object beam by the sample is then converted into an amplitude of colour difference.

Cross-polarisation (CP) microscopy utilises plane-polarised light to capture images. Light normally consists of waves of different frequencies, vibrating in all planes. But for plane-polarised light, the vibrations are restricted to a single plane, and therefore when rays pass through crystalline material their behaviour is dependent on

their orientation with respect to the structure of the material. The velocity of plane-polarised light is different to that of light which would vibrate at right-angles to it, and therefore if two polarised waves were in phase prior to falling on a material, a phase difference between them would be generated as they leave that material. This is the basis behind obtaining contrast in the cross-polarisation microscope.

The microscope used in these studies (Olympus BX50) could be used in either the DIC or CP mode. Prints were taken using a Sony printer unit (Model SSC M370CE), and photographs were taken using a camera (Nikon F-601M).

2.3.2.2. Preparation of liposomes

Drug-containing MLVs were manufactured by weighing appropriate amounts of phospholipid and BDP into a round-bottomed flask, and adding chloroform to dissolve both components. The solvent was removed by rotary evaporation in vacuo, in a water bath at 55°C for approximately 15 minutes. The flask was then flushed with nitrogen to remove any traces of residual solvent, for 1-2 minutes. Distilled water was further purified by passing through an Elgastat Ultra High Quality Purification System, and an appropriate amount of this water was added to the dry film in the flask, via a Millipore filter with a pore size of 0.45 μm, in a 25 mm holder, to give a final phospholipid concentration of 5-50 mg/ml. Glass beads were added to aid mixing, and the flask was again flushed with nitrogen and gently rotated for 30 minutes in the water bath, until the film was no longer attached to the sides of the flask. The suspension was allowed to anneal for a further 2 hours in the water bath at 55°C, before finally being stored under nitrogen in a refrigerator at 2-8°C.

For microscopy studies, a small drop of the liposomal sample was deposited onto a clean glass slide by means of a pasteur pipette, and a cover-slip was gently placed on top. To confirm the presence/absence of drug crystals in liposome suspensions, and to determine the concentration at which they form, a series of liposomal samples containing different amounts of BDP (1, 1.5, 2, 3, 4, 5 and 10 mole %) were prepared and observed using differential interference contrast microscopy (DIC) and cross-polarisation microscopy (CP), described in Section 2.3.2.1.

2.3.3. Results and Discussion

Prints taken using DIC microscopy show the presence of drug crystals in liposomal preparations containing high concentrations of 5 and 10 mole % BDP (Figures 2.3. and 2.4., respectively). DIC and CP microscopy were also used to detect the presence of BDP crystals in liposomal samples containing 2 mole % BDP (Figures 2.5(a) and (b)), and 3 and 4 mole % BDP (photographs not shown). However, crystals were not detected in samples containing 1.5 mole % BDP (Figures 2.6(a) and (b)) suggesting that the limiting concentration for crystal formation in DPPC liposomes lies between 1.5 and 2 mole % BDP. Interestingly, both samples showed the presence of crystals after 3 days of storage in the refrigerator, under an inert atmosphere of nitrogen, suggesting the instability of BDP in DPPC liposomes and/or the temperature dependency of BDP solubility in DPPC bilayers.

2.3.4. Conclusions

The concentration at which excess BDP forms crystals in liposomal suspensions, was found to lie between 1.5 and 2 mole % BDP. However the liposomes were not particularly stable, with respect to BDP entrapment, and future work was carried out on the day of sample preparation. Microscopy was found to be an extremely useful technique with which to identify the presence of drug crystals, and is therefore suitable for use in future work.

2.4. Incorporation of BDP into phospholipid films

2.4.1. Introduction

The earlier problems encountered with the incorporation of BDP into liposomes were mainly concerned with the low entrapment efficiency of these vehicles with respect to this particular steroid. It is not known whether there is an "incompatibility" between the steroid and phospholipid molecules which is apparent during the formation of the dry film containing both components, or whether it arises as a consequence of film hydration.

Figure 2.3. DIC prints of liposomal suspension containing 5 mole % BDP (× 400).

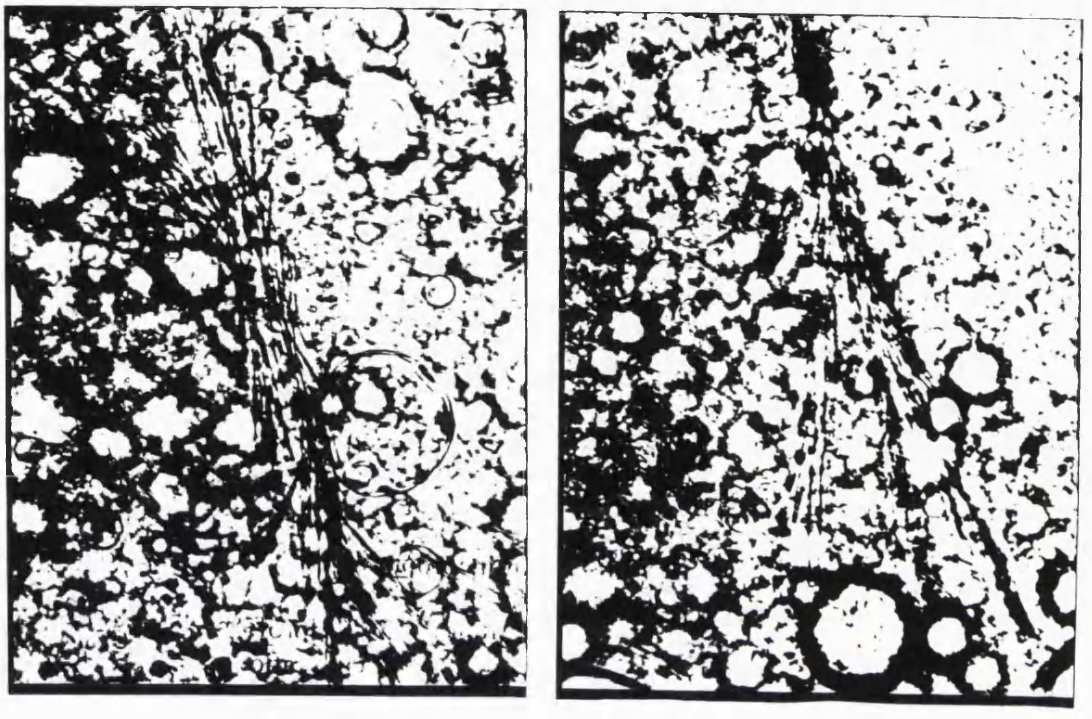


Figure 2.4. DIC prints of liposomal suspension containing 10 mole % BDP (× 400).

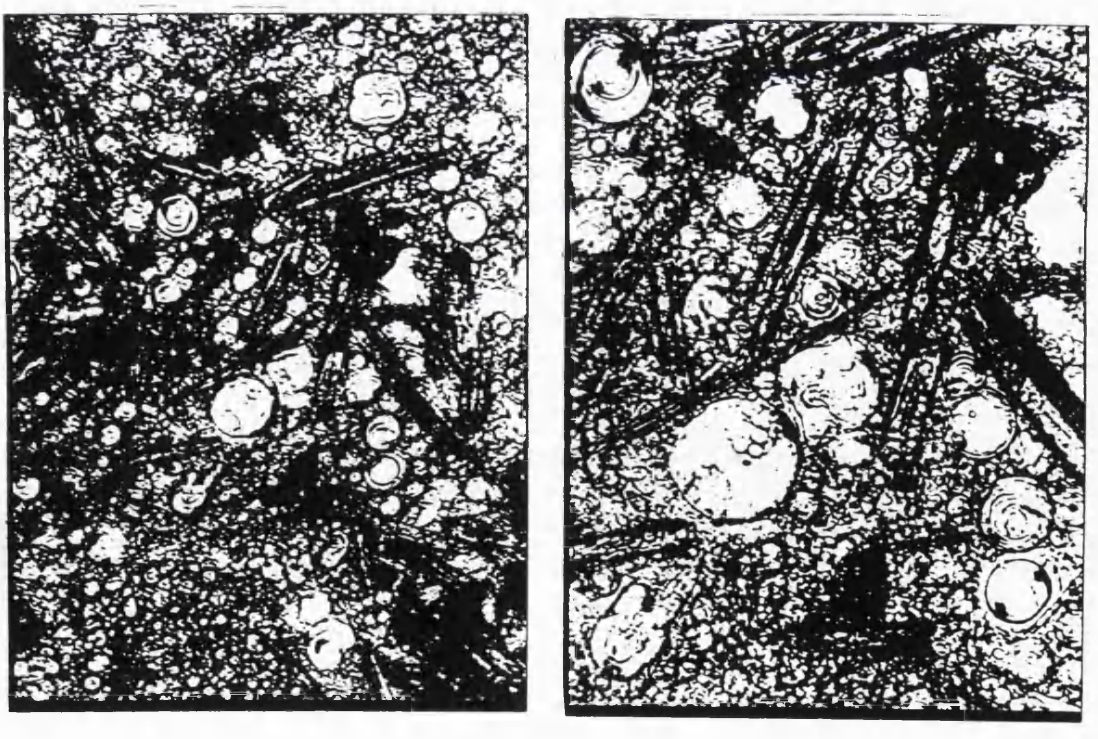


Figure 2.5(a) DIC photograph of liposomal suspension containing 2 mole % BDP (× 400).

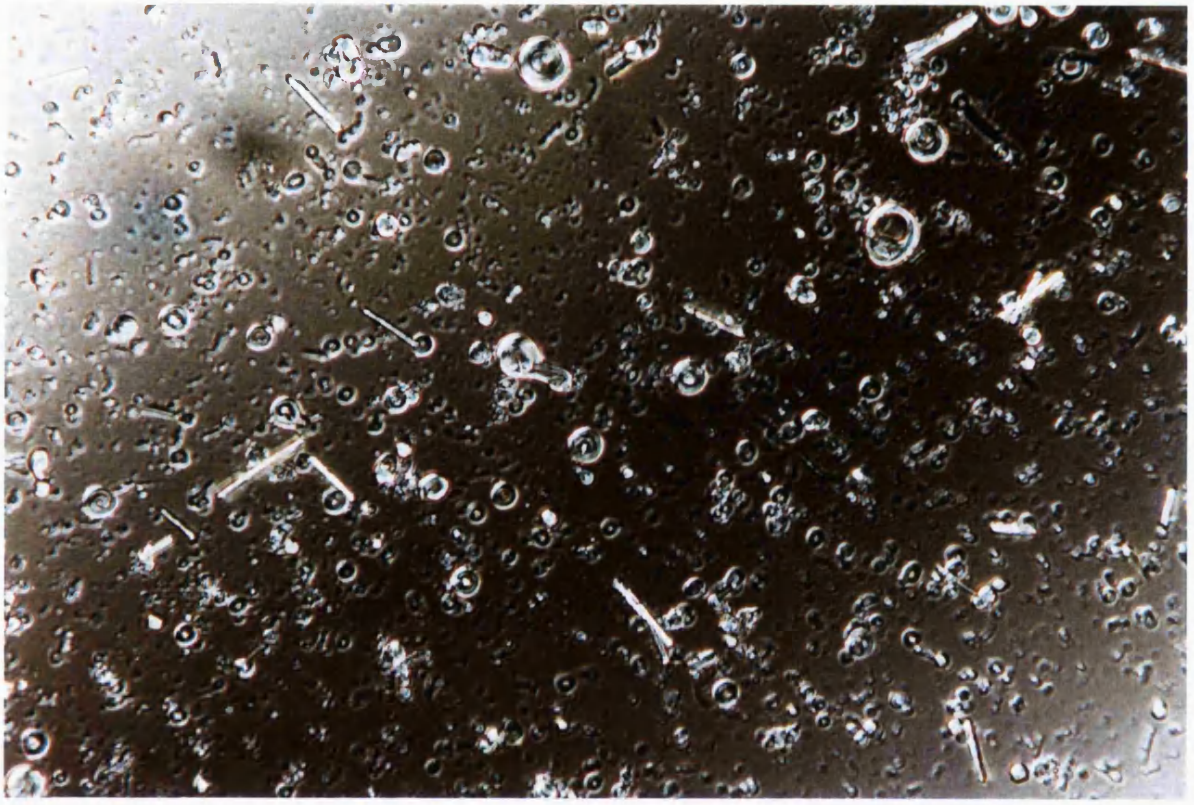


Figure 2.5(b) CP photograph of liposomal suspension containing 2 mole % BDP (× 400).

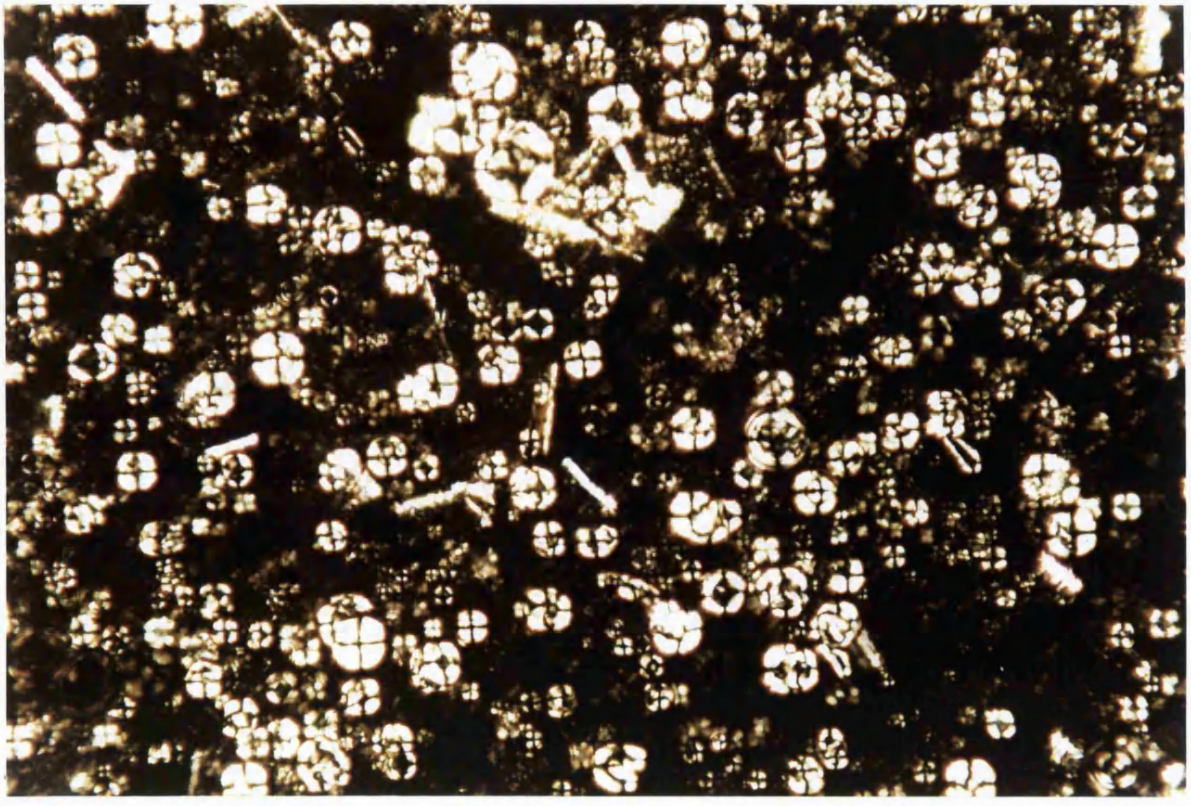
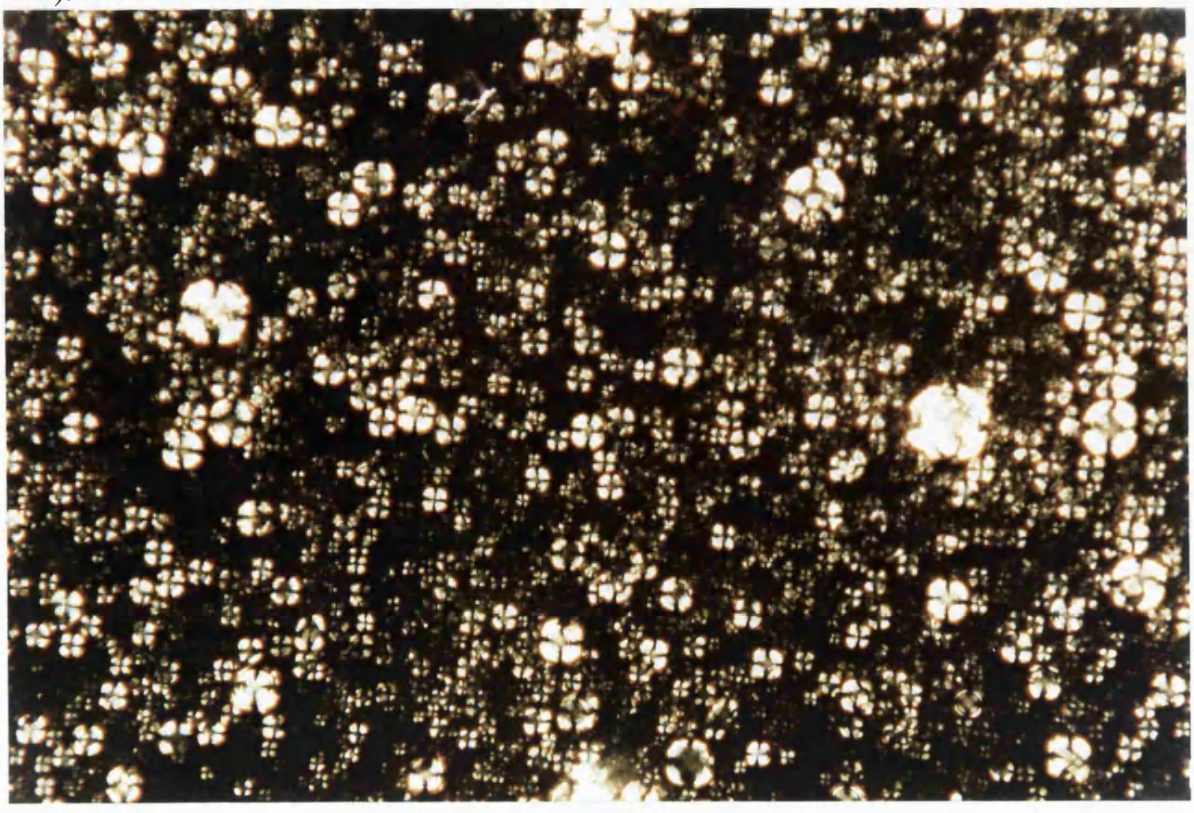


Figure 2.6(a) DIC photograph of liposomal suspension containing 1.5 mole % BDP (× 400).

Figure 2.6(b) CP photograph of liposomal suspension containing 1.5 mole % BDP (× 400).



Whilst the formation of crystals in liposomal suspensions (above 1.5-2 mole % BDP) may be due to the surrounding aqueous environment, BDP crystals may already be present in the dry phospholipid film. Therefore, the purpose of studies in this section was to prepare and microscopically examine slides of phospholipid films containing varying amounts of BDP. However, it is not always possible to use DIC microscopy since the films may be too thick to detect crystals. Therefore further studies involved the preparation of a series of slides of phospholipid films containing different concentrations of BDP, which were subsequently observed under a hot-stage microscope (HSM), described in Section 2.4.2.1.

2.4.2. Materials and Methods

Details of the materials used in these studies can be found in Section 2.3.2.

2.4.2.1. Hot-stage microscopy

Hot-stage microscopy is a useful technique since the sample slide can be heated, and the melting of various components may be observed. The apparatus (Model FP5, Mettler) consists of a hot-stage which may be placed onto the stage of any conventional microscope, and is connected to a power supply. The sample slide was held in place on the hot-stage by means of two metal clips, and a small hole in the hot-stage cover allowed a special objective lens (×10) to be lowered down for viewing purposes. The sample was heated up at a programmable heating rate, in this case 10°C/minute, up to temperatures of approximately 240°C. BDP has a melting point of approximately 213°C whilst DPPC has a melting point of approximately 234°C. Therefore samples containing both these components may be heated so that the presence (or absence) of any BDP crystals may be revealed.

2.4.2.2. Preparation of films

Dry DPPC films containing 100 mg/ml phospholipid and 5 or 10 mole % BDP were prepared by dissolving both components in chloroform. 50 µl of each solution was then deposited drop-wise onto clean glass slides using a microsyringe, and the solvent was left to evaporate by placing the slides in an oven (Townson and Mercer Ltd., Croydon) set at 200 mbar, 50°C for 1 hour. After this time slides were observed under HSM and/or DIC microscopy to determine if BDP crystals form prior to hydration of the phospholipid film. Phospholipid films containing 0, 5 and 10 mole % BDP were also prepared in welled slides, and the addition of approximately 50 µl of water to each slide, using a microsyringe (Hamilton, Nevada, USA), was observed microscopically.

For HSM studies, films containing approximately 3.5 mg/ml of BDP or DPPC were prepared by dissolving either component in chloroform, and then depositing 200 µl of each solution onto glass slides. Mixed films containing 2.5 mg/ml DPPC and 20 or 50 mole % BDP were also prepared and inspected under HSM.

2.4.3. Results and Discussion

BDP crystals were not observed when phospholipid films containing 5 (Figure 2.7.) and 10 mole % (Figure 2.8.) BDP were observed under DIC microscopy. This may be due to the fact that either the crystals were absent altogether, or the thickness of the film may have masked their presence. The films however, were seen to consist of "ridges and peaks". The addition of water to phospholipid films created on welled slides was also observed under the DIC microscope, but the presence of dark patches prevented adequate imaging to take place.

Hot-stage microscopy was used to heat films containing both phospholipid and drug. Whilst no crystals were detected on heating the film containing 20 mole % BDP (Figure 2.9.), a large crystal structure was seen to be present on heating the film containing 50 mole % (Figure 2.10.). However it was thought that this structure was the result of an artefact, and not a BDP crystal, since it rapidly disintegrated at only 89°C, and BDP films melted at much higher temperatures of approximately 200°C

Figure 2.7. DIC prints of phospholipid film containing 5 mole % BDP (× 100).

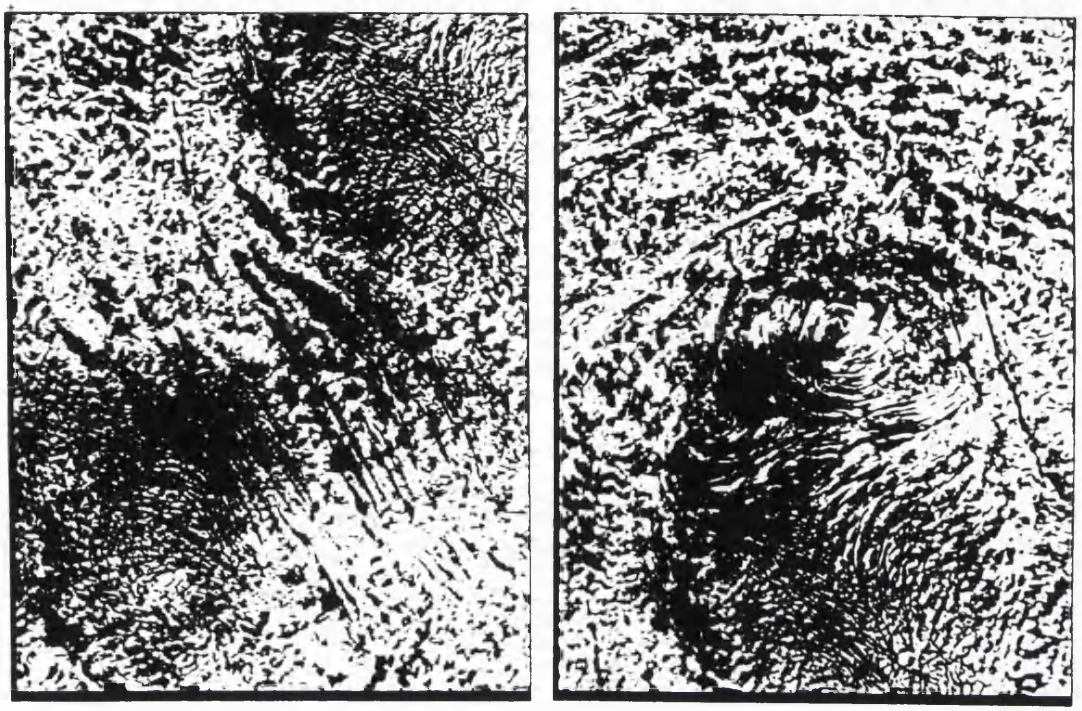


Figure 2.8. DIC prints of phospholipid film containing 10 mole % BDP (× 100).

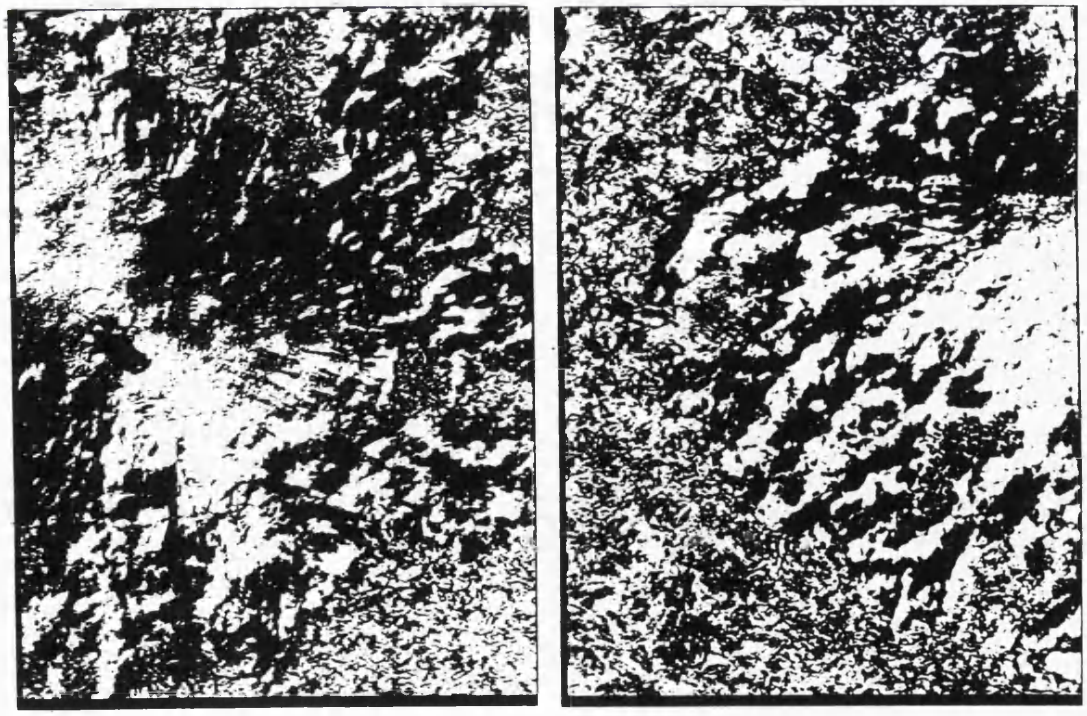
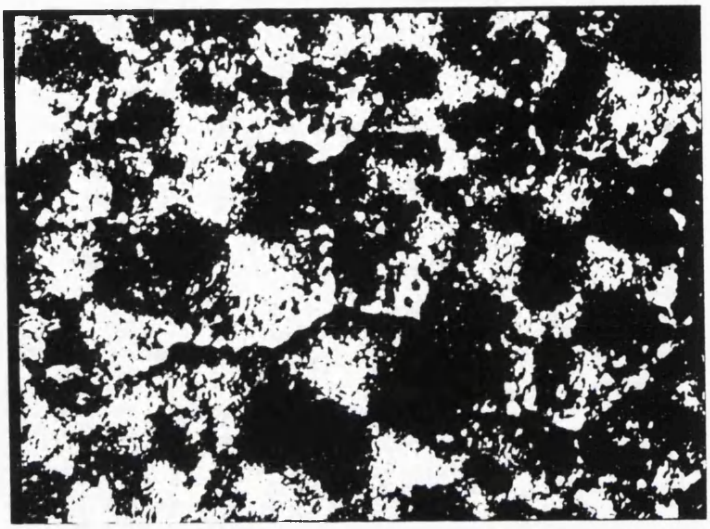
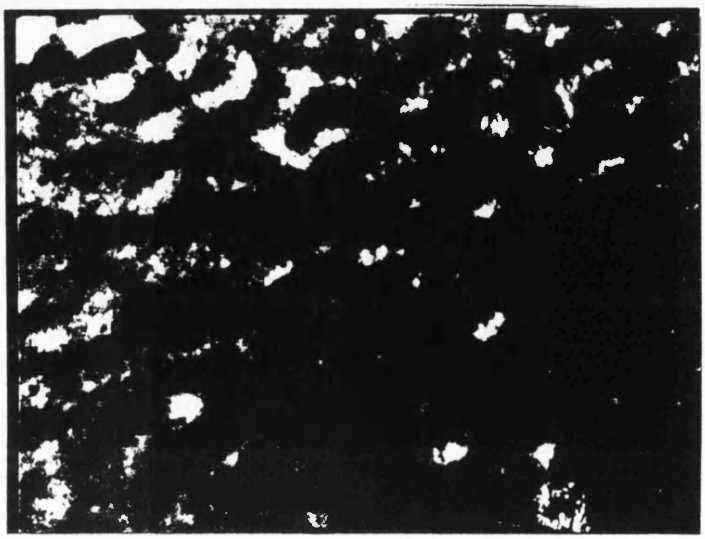


Figure 2.9. DIC prints of HSM of phospholipid film containing 20 mole % BDP (× 100).

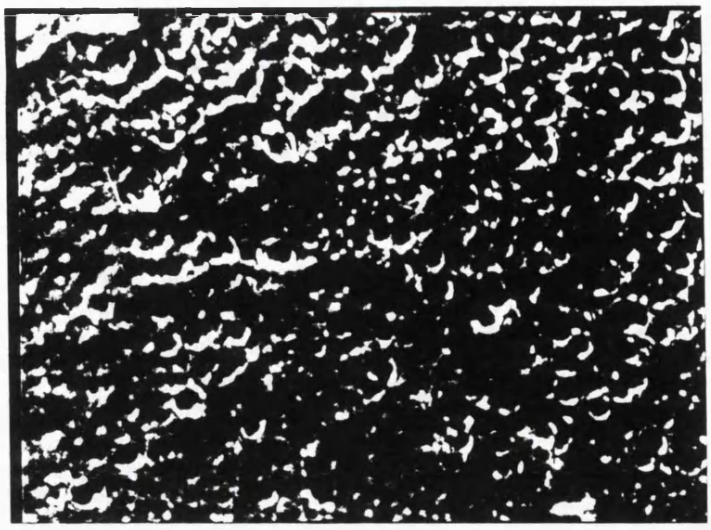


(a) 20°C

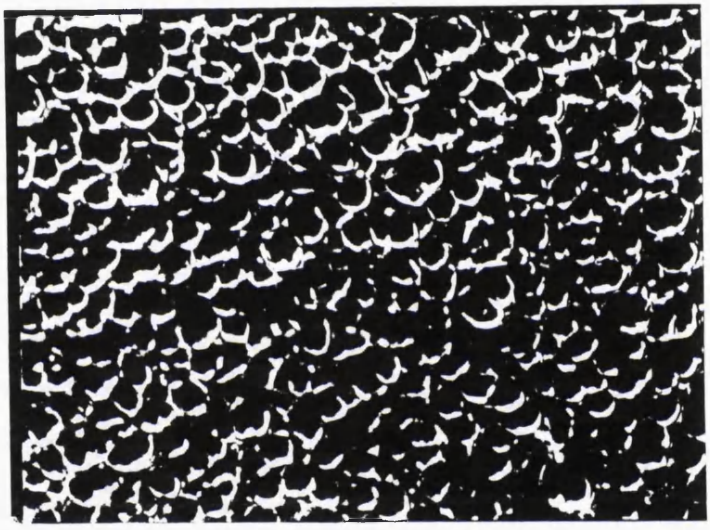
(b) 80°C



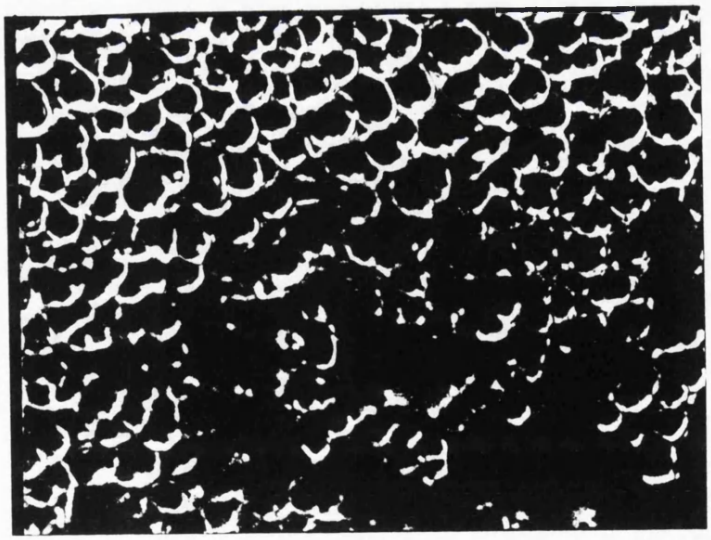
(c) 90°C



(d) 110°C

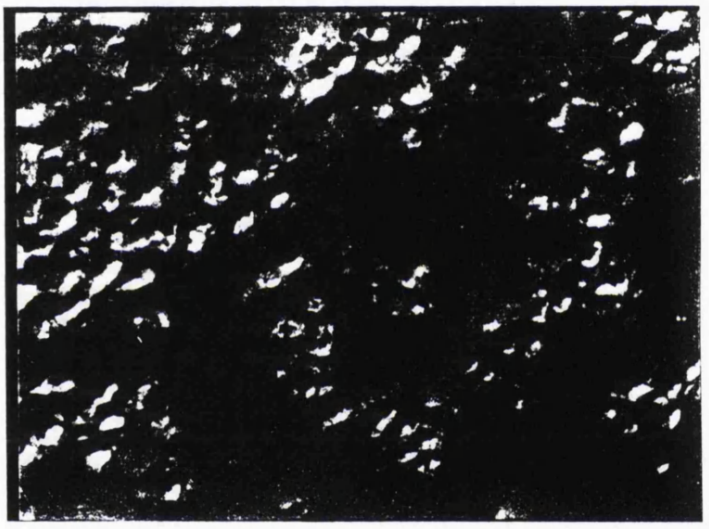


(e) 130°C



(f) 155°C

(g) 162°C

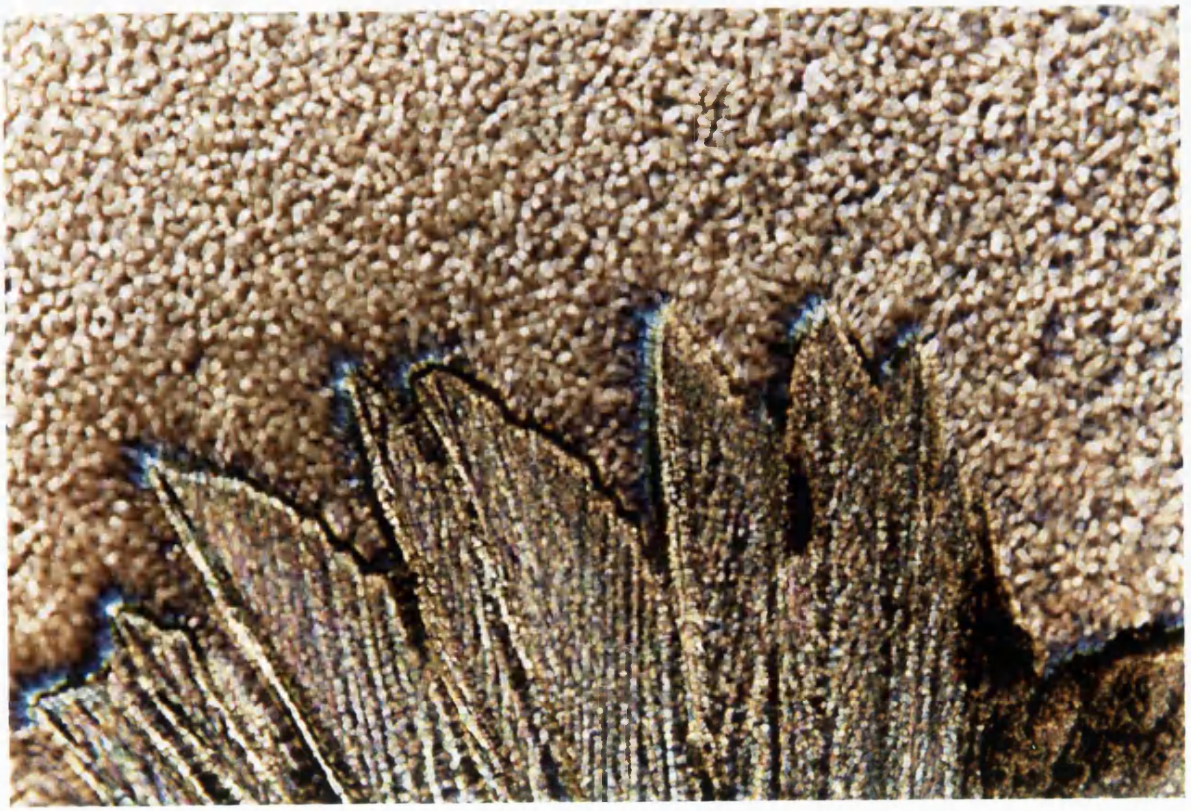


(h) 170°C

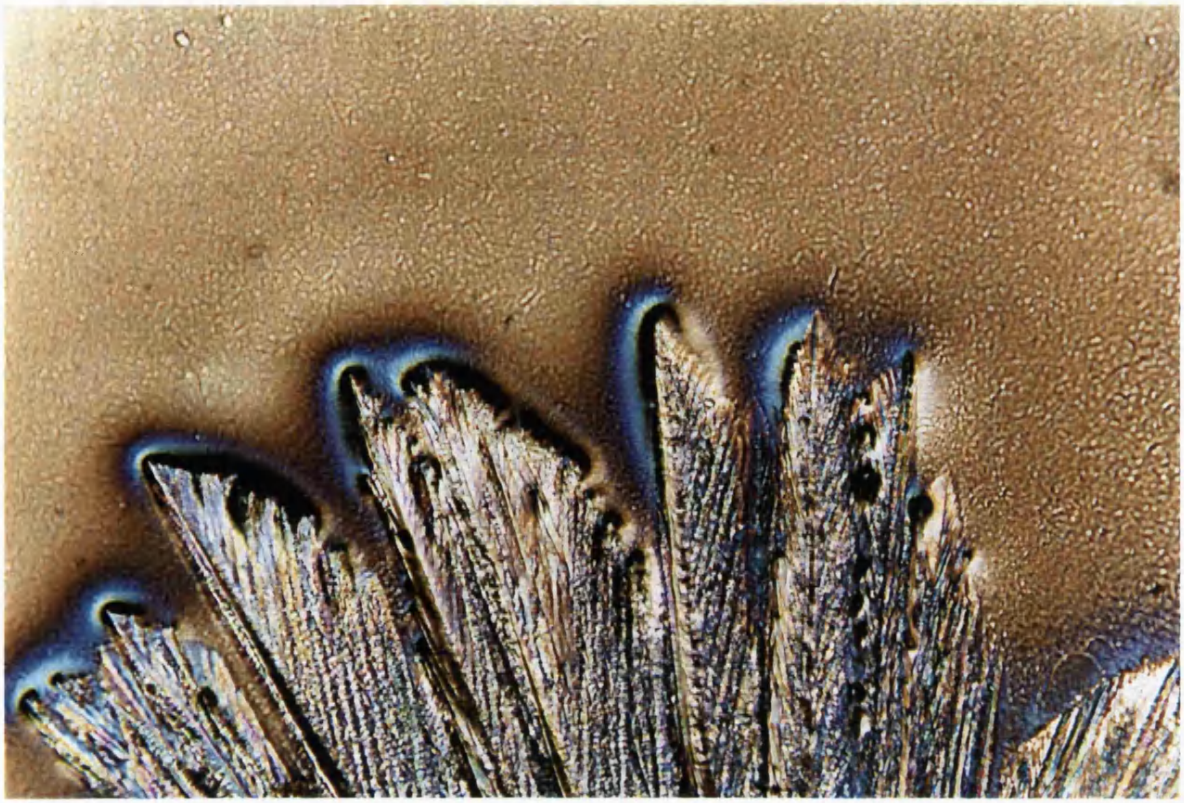
(j) 190°C

Figure 2.10. DIC photographs of HSM of phospholipid films containing 50 mole % BDP (× 100).

(a) 40°C

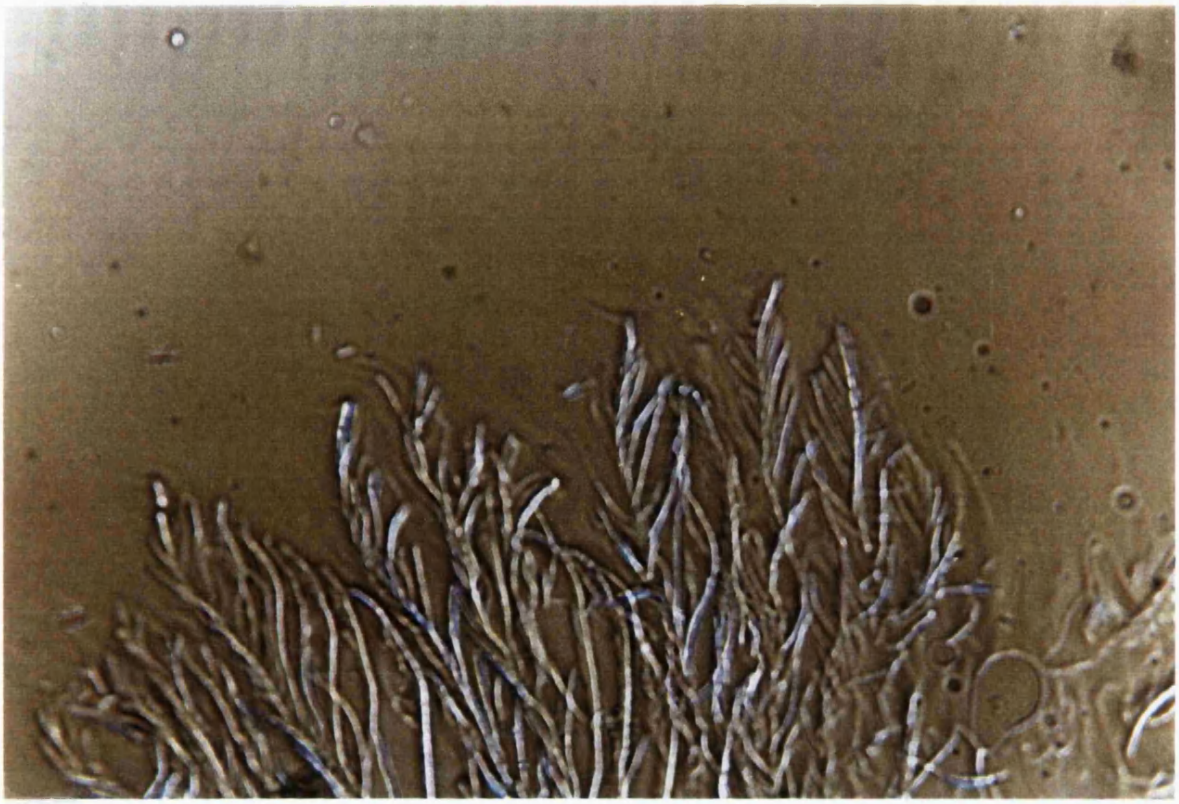


(b) 70°C

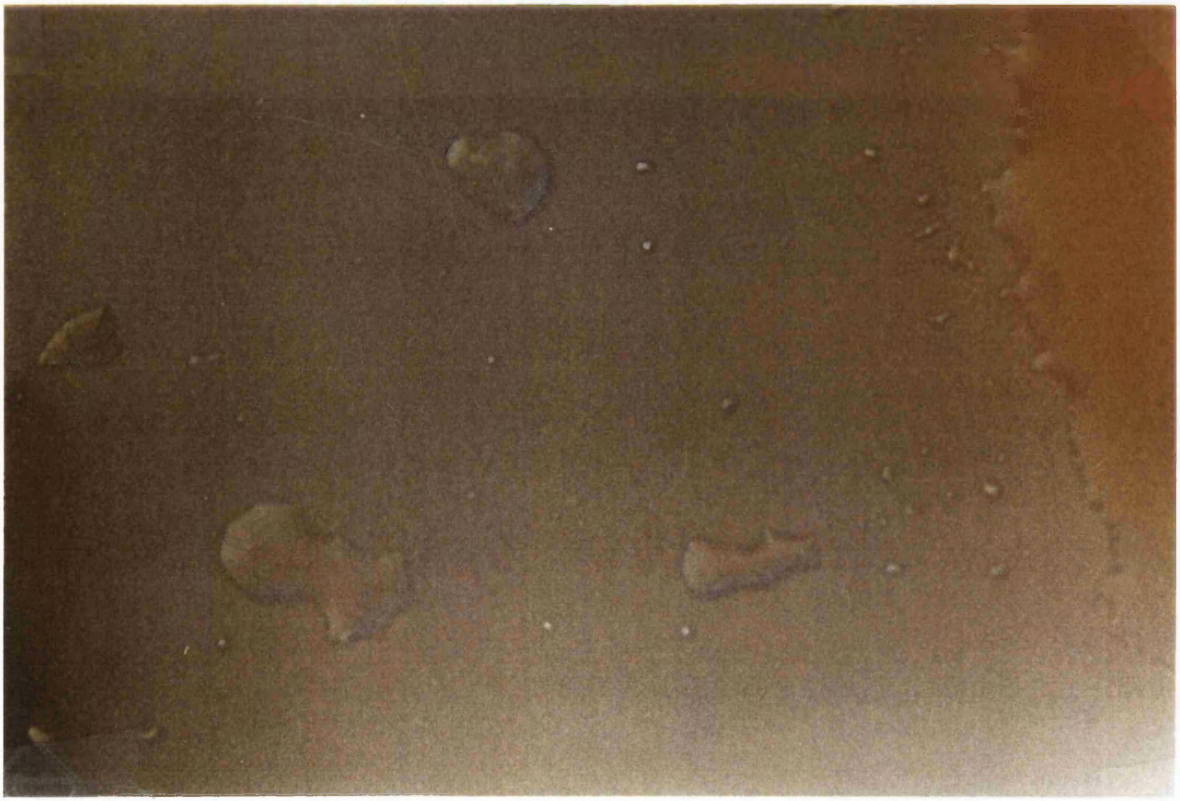


(c) 89°C

(d) 100°C



(e) 120°C



(f) 210°C

(Figure 2.11.). The presence of environmental impurities (such as dust and dirt from the oven) may have reduced the melting point of BDP. DPPC films melted at approximately 240°C (Figure 2.12.), but mixed films of DPPC and BDP (Figures 2.9. and 2.10.) were found to melt at approximately 200°C -210°C, indicating that the presence of BDP at these concentrations reduces the melting point of DPPC.

Therefore the absence of any crystalline material in DPPC films is probably due to the fact that BDP only exists as crystals when in an aqueous environment, such as a liposomal suspension. A hydrophobic drug such as BDP, with a poor aqueous solubility is expected to follow this behaviour, and therefore this result is not unexpected.

2.4.4. Conclusions

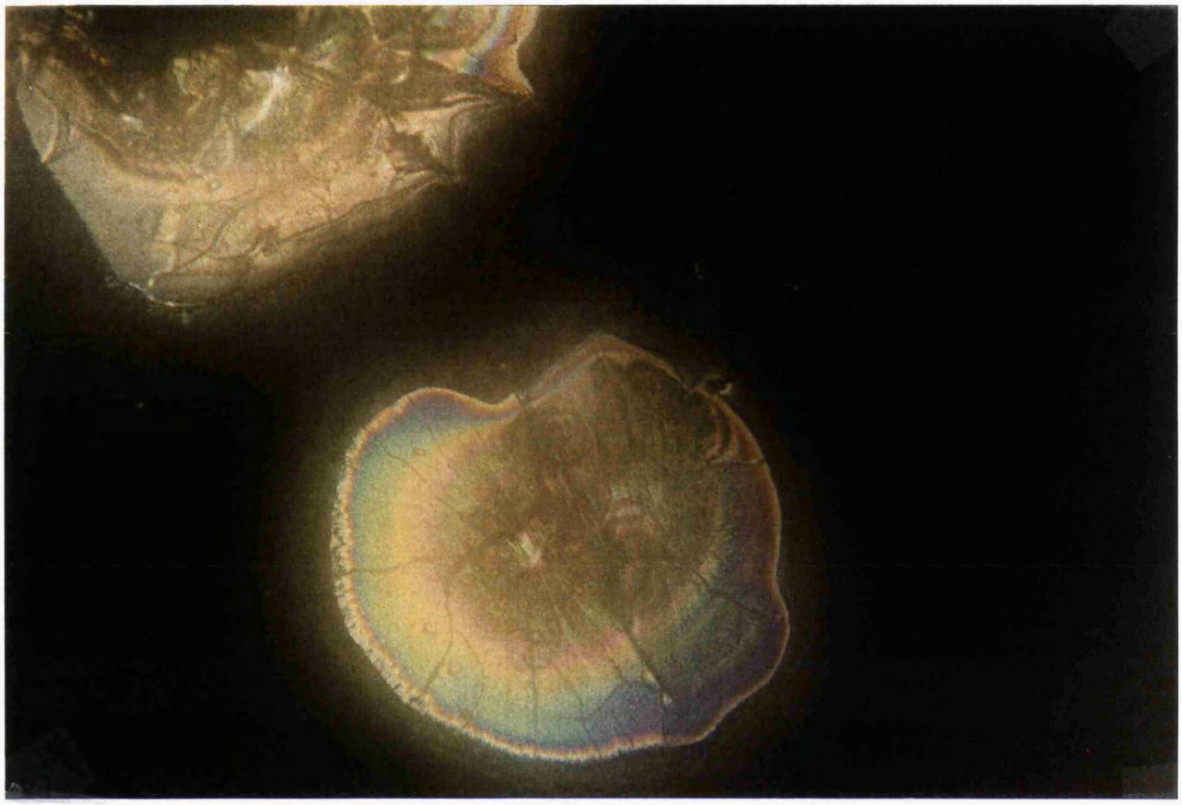
It was concluded that DIC was an unsuitable technique with which to inspect phospholipid films for the presence of BDP crystals. However, since HSM was also unable to detect the presence of any BDP crystals within a dry film of DPPC, we can assume that these crystals form only on the addition of water.

2.5. Determination of the extent of BDP entrapment in liposomes

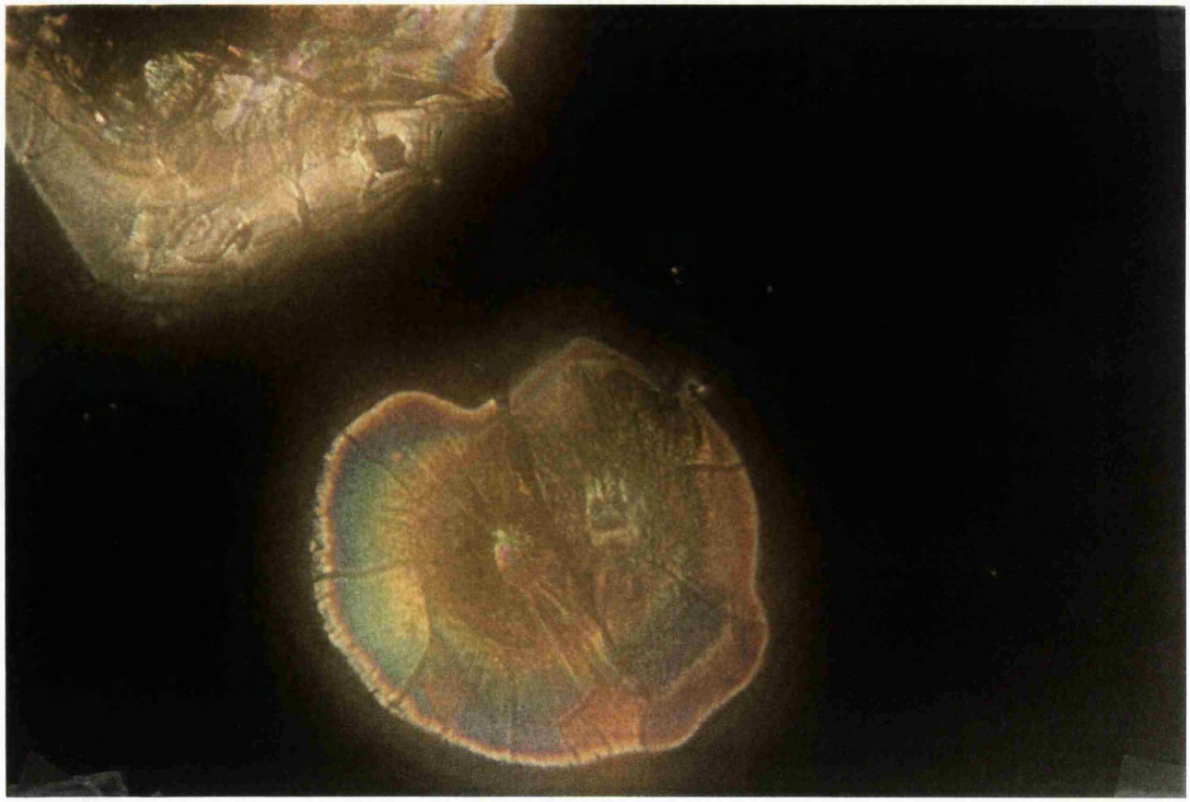
2.5.1. Introduction

Generally the term drug entrapment has been used to discuss how much drug is entrapped within a liposome, but drug may also be associated with liposomes or with non-liposomal phospholipid, as described in Section 2.1. Therefore the aims of these studies were to firstly, separate unentrapped material efficiently from liposome samples containing drug, and secondly, develop an assay to determine the quantity of BDP present as entrapped drug.

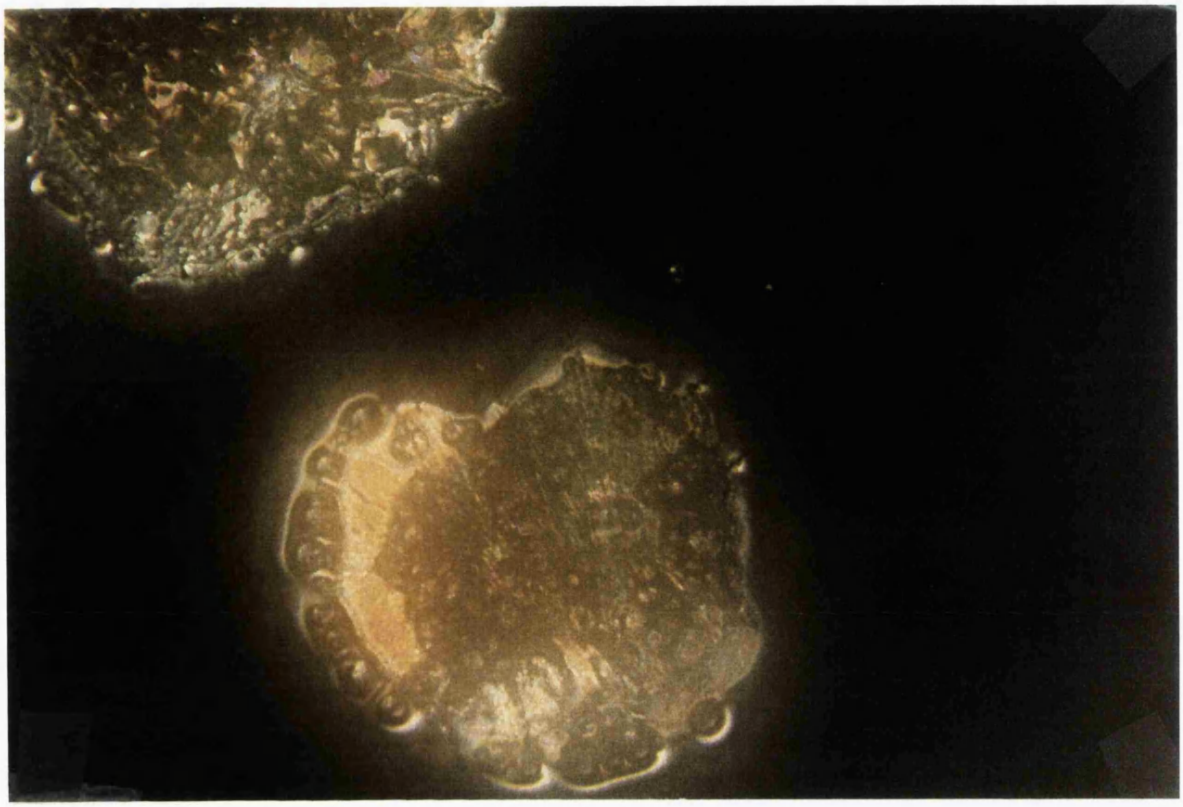
Figure 2.11. DIC photographs of HSM of BDP film (× 100).



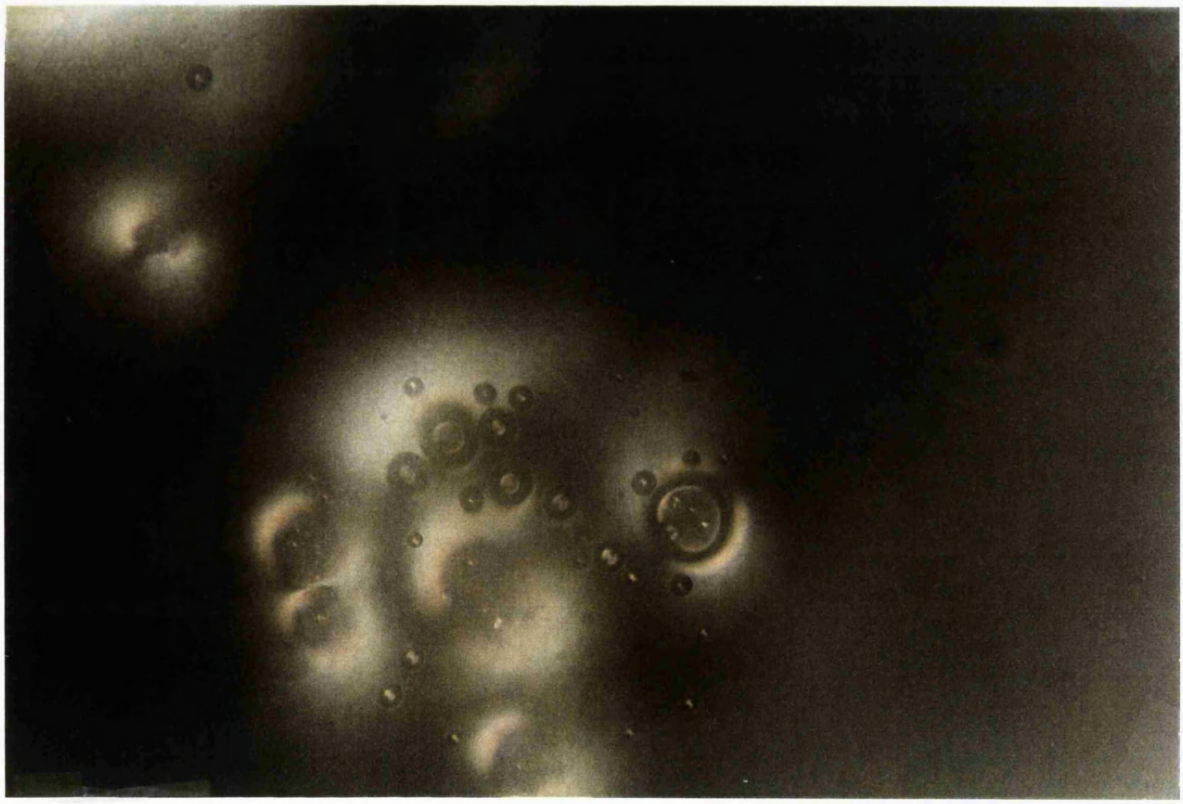
(a) 30°C



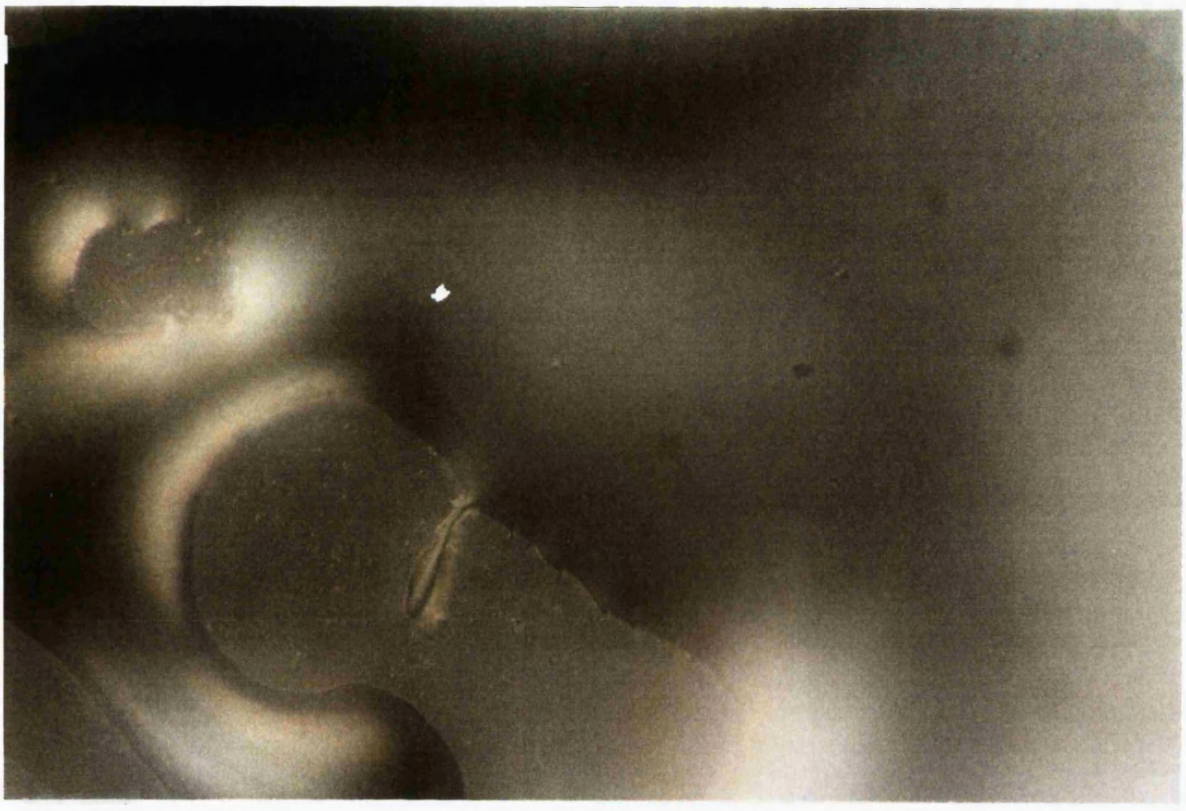
(b) 100°C



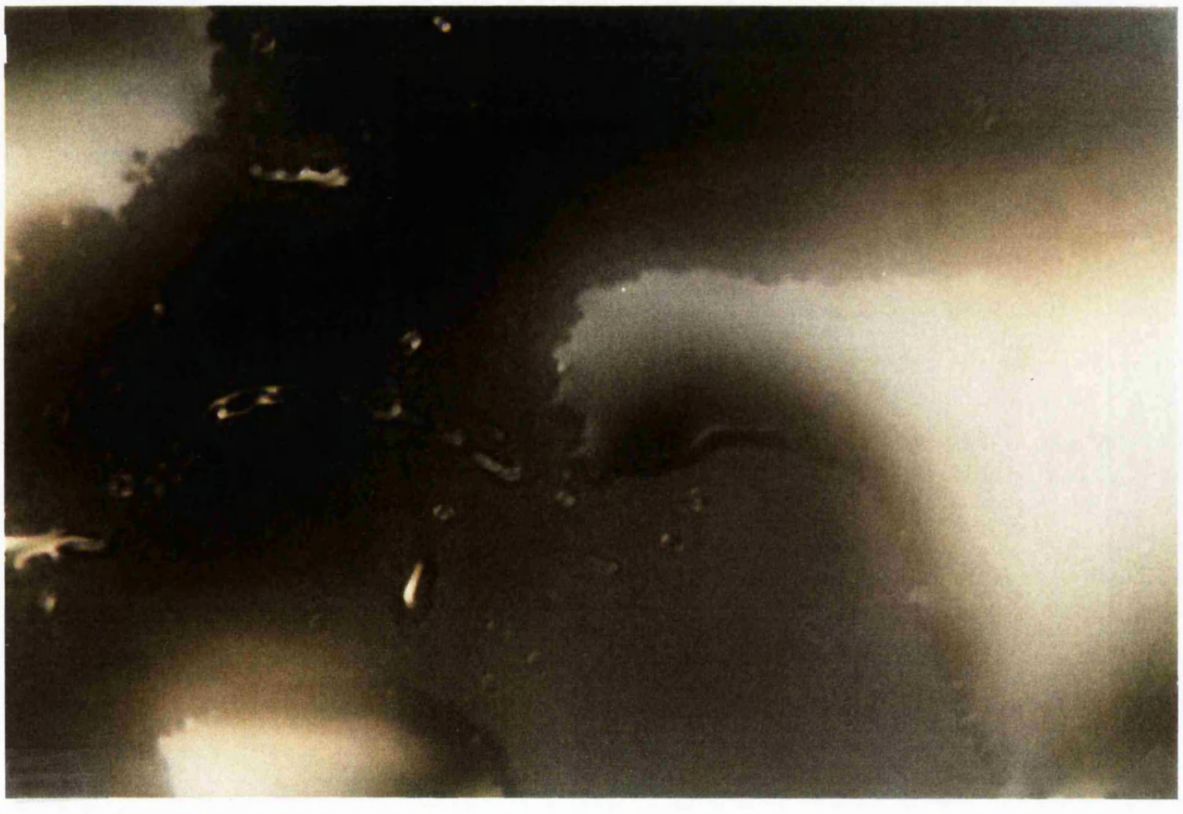
(c) 110°C



(d) 140°C



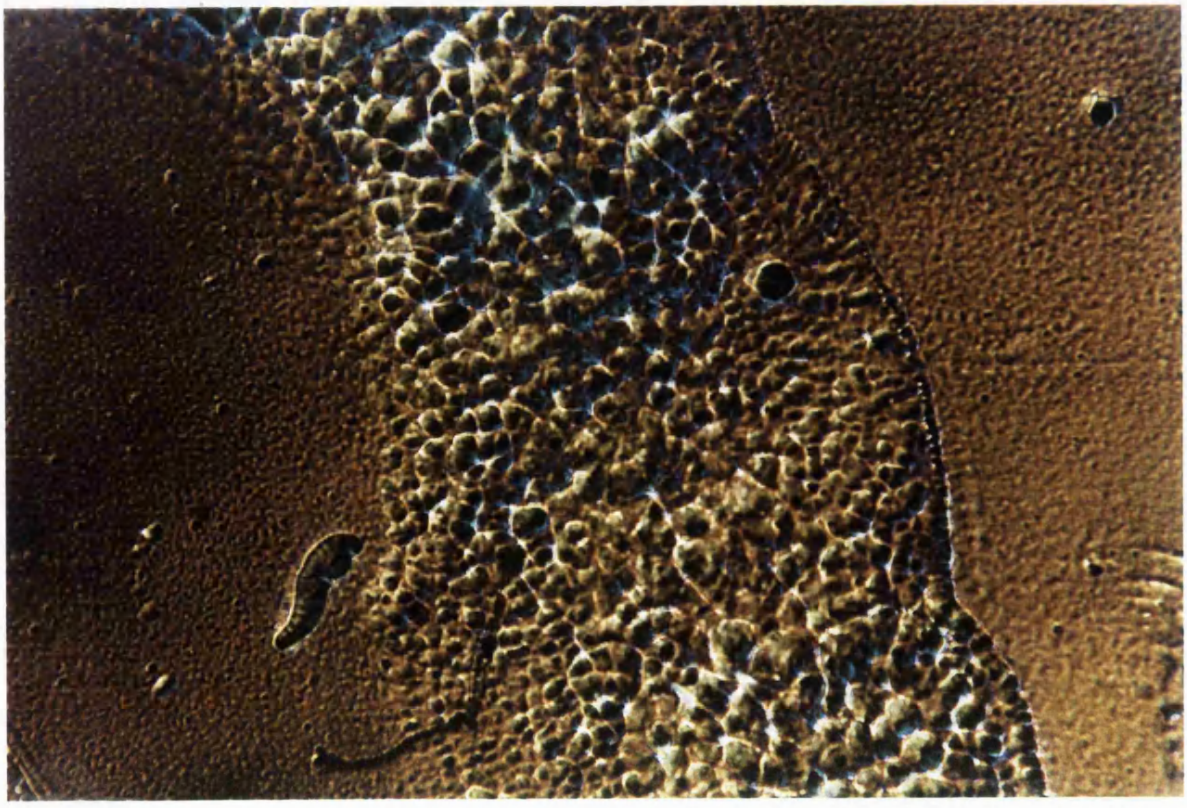
(e) 160°C



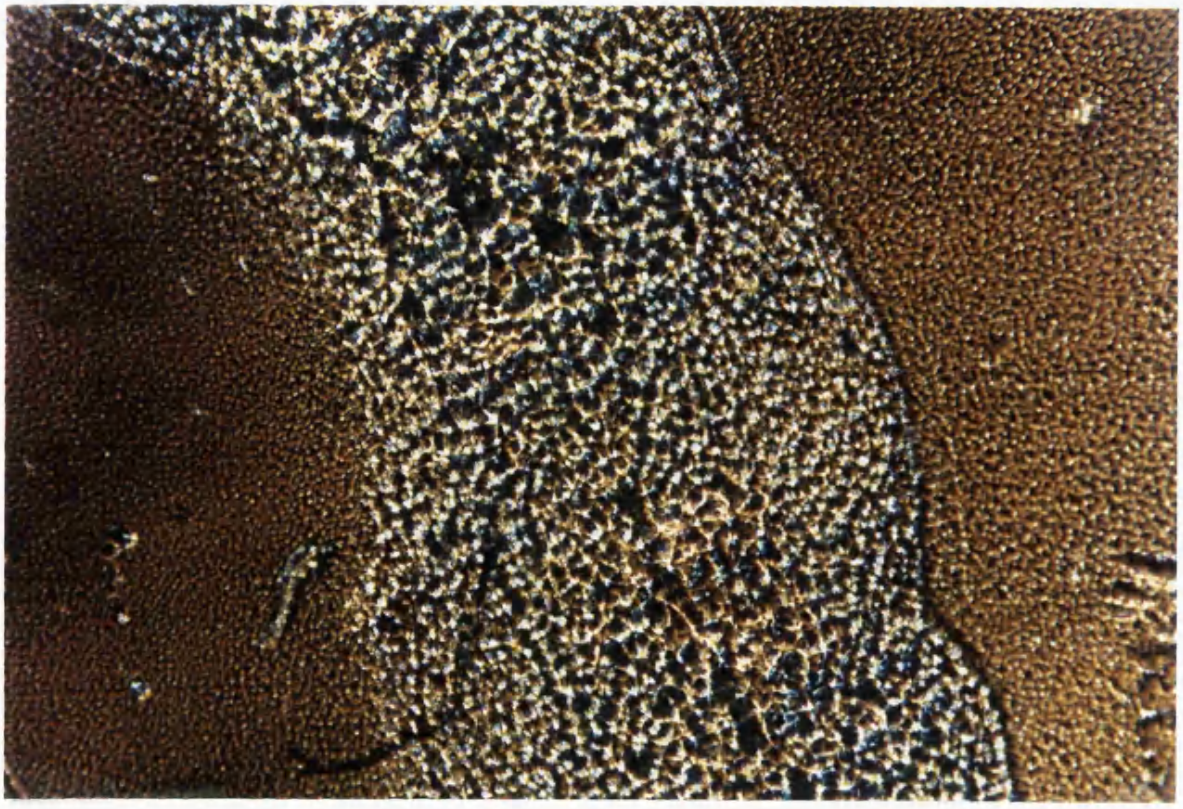
(f) 200°C

Figure 2.12. DIC photographs of HSM of DPPC film (× 100).

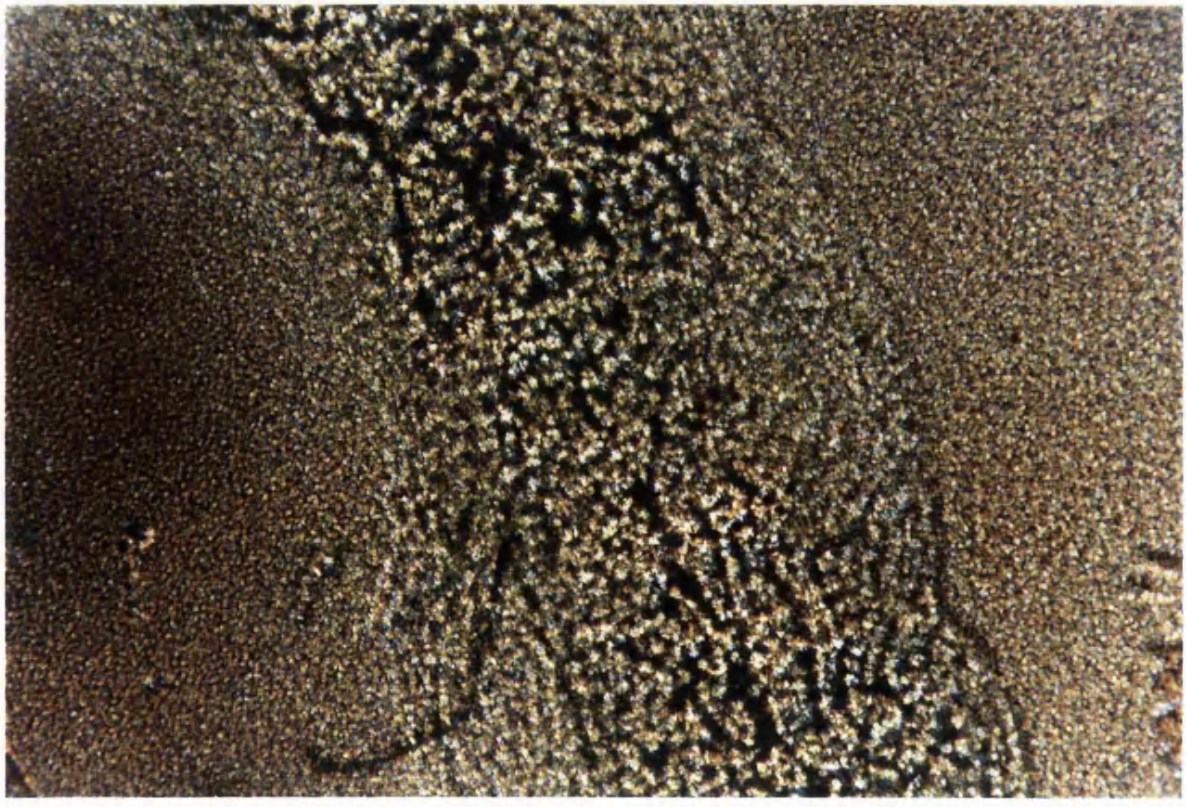
(a) 30°C



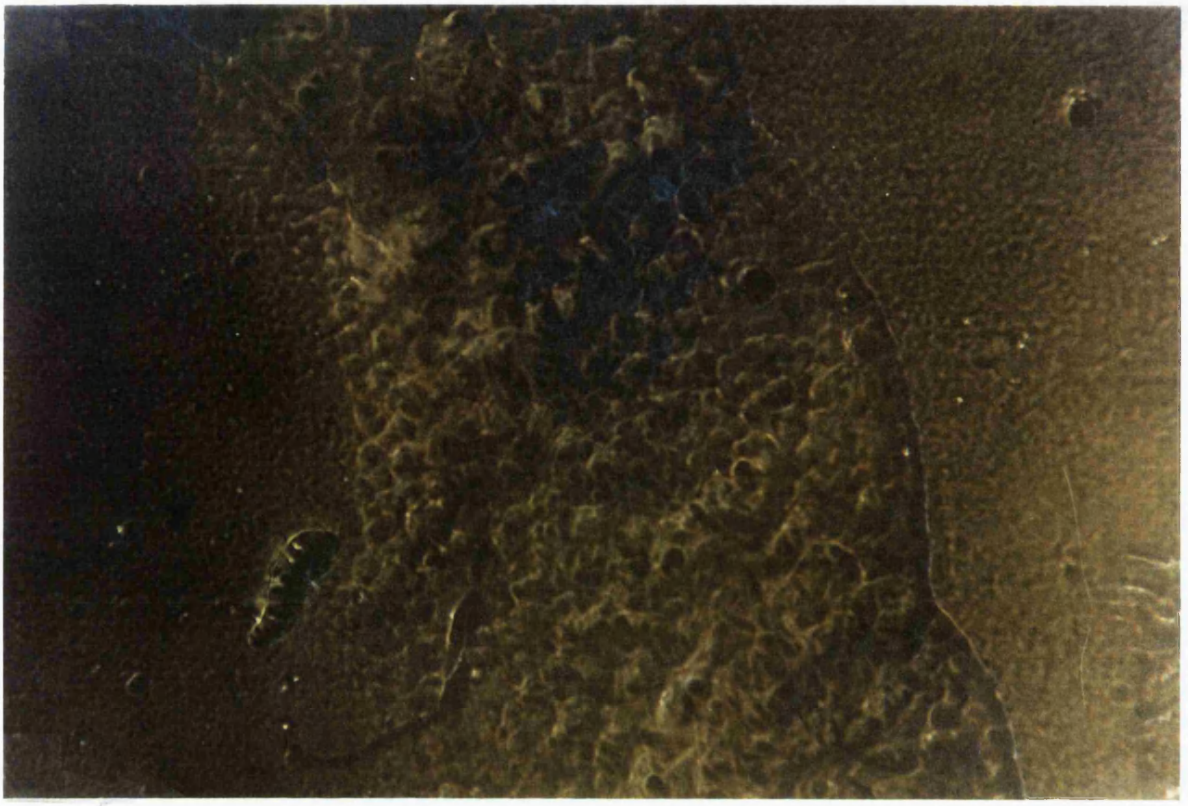
(b) 115°C



(c) 150°C



(d) 190°C



(e) 200°C

(f) 243°C

2.5.2. Separation of unentrapped material

There are several ways in which separation of free and entrapped drug may be achieved, including dialysis, centrifugation, ultrafiltration or gel filtration chromatography, as described in Chapter 1. The basis behind separation of free material by centrifugation is that liposomes containing entrapped drug material will be more dense than the supernatant fluid. Thus, when suspended in either of these media and subjected to a high gravitational field, these liposomes would be expected to sediment, whilst any free drug material should remain in the supernatant. This has been the method of choice for many authors (Taylor et al, 1990b; Ma et al, 1991). However, separation of free material by centrifugation may be impossible for small liposomes, since they are difficult to sediment (Tyrell et al, 1976). In addition, if the material to be entrapped has a large molecular weight, or is present in a high concentration, the density of the suspending medium may approach that of the liposome sample, thus resulting in sedimentation being difficult, if not impossible. This problem may be overcome by diluting the liposomes in a medium of much lower density, making them heavier than their surrounding medium and therefore easier to sediment. Alternatively, liposomes may be suspended in a medium of increased density, resulting in liposomes floating on top after centrifugation (Fraley et al, 1980). This was the rationale behind the investigation of the separation of unentrapped crystals of BDP from liposomally-entrapped material.

It was thought that centrifugation in ordinary water would produce sedimentation of liposomes containing drug, and would also cause large BDP crystals to sediment, making the removal of this unentrapped drug difficult. However, it was postulated that separation would be achieved on the basis of density differences between liposomes and crystals, if they were suspended in a medium denser than ordinary water, such as deuterated water. Whilst density differences are important, the rate of sedimentation of the crystals also depends on the dispersion medium, as well as on gravity (g), crystal radius (d) and the viscosity of the dispersion medium (η). According to Stoke's Law, the rate of sedimentation of a spherical particle, v, in a fluid medium is given by,

$$v = \frac{2 g d^2 (\rho_1 - \rho_2)}{9\eta} \qquad \text{Equation 2.1.}$$

where ρ_1 and ρ_2 are the densities of the particle and medium, respectively.

Since deuterated water is more dense than ordinary water, liposomes prepared in ordinary water would be predicted to float when suspended in D_2O, and any drug crystals should sediment (assuming that the density of BDP crystals is greater than that of the suspending medium). However, the medium in which the liposomes are prepared may affect the sedimentation of crystals. Therefore liposomes containing 10 mole % BDP were made in ordinary and deuterated water. In addition, the use of both ordinary and deuterated water as a suspending medium, was investigated.

2.5.2.1. Methods

A DPPC liposomal suspension containing approximately 10 mg/ml phospholipid and 10 mole % of BDP was made in 5 ml of H_2O (according to the procedure outlined in Section 2.3.2.2.) and then transferred to a 10 ml glass vial. Two 1 ml samples were then added to either 5 ml of H_2O or D_2O, in centrifuge tubes, which were then centrifuged for 1 hour at a setting of 6 (corresponding to approximately 2280 rpm) on a bench centrifuge (Minor S, M.S.E. Ltd., UK). This was repeated for the remainder of the liposomal sample. The liposome fraction following centrifugation in deuterated water was present as floating material, and was removed using a pasteur pipette, and observed under a DIC microscope. Similarly a liposome suspension containing 20 mg/ml DPPC and 10 mole % BDP was made in 5 ml of deuterated water, and 1 ml samples were added to either 5 ml of H_2O or D_2O, and centrifuged as above, and the resulting liposome fractions were also observed under a DIC microscope.

As described in Section 2.5.2.2., after liposomes made in deuterated water were centrifuged, small crystals within the liposome fraction were still detected. In order to remove these crystals the floating layer was removed and resuspended in fresh

deuterated water, and centrifuged at setting 6 for a further hour. In addition liposomes containing approximately 10 mg/ml DPPC and 10 mole % BDP were prepared in 5 ml of deuterated water, and 1 ml of the resulting liposomes was suspended in 5 ml of D_2O and centrifuged at a higher setting of 8 (corresponding to approximately 3040 rpm) on the bench centrifuge, for 1 hour. This was repeated three times and each fraction was removed in the same way as before and analysed for the presence of crystals.

2.5.2.2. Results and Discussion

Crystals were found to be present in liposomes containing 10 mole % BDP made in H_2O (Figures 2.13(a) and (b)) and D_2O (Figures 2.14(a) and (b)). DIC and cross-polarisation microscopy were used to detect the presence of these crystals. After centrifugation of these samples in either H_2O or D_2O (for 1 hour at setting 6 on a bench centrifuge), DIC was used to photograph the various fractions.

The liposomes made in H_2O and suspended in H_2O did not float at the surface after centrifugation was complete. The supernatant was found to contain liposomes and crystals, whilst the pellet contained a greater proportion of liposomes and crystals (Figure 2.15.). Liposomes made in H_2O and suspended in D_2O did produce a floating layer, which on microscopic examination was found to contain liposomes with a few small BDP crystals present too (Figure 2.16(a)). The supernatant also contained a few liposomes and crystals (Figure 2.16(b)), whilst the pellet contained many crystals and apparently no liposomes (Figure 2.16c)). Liposomes prepared using deuterated water did not float in a H_2O medium after centrifugation was complete, and the pellet was found to contain many liposomes and crystals (Figure 2.17.). However deuterated water liposomes when suspended in D_2O, did produce a floating layer consisting of liposomes and some smaller BDP crystals too (Figure 2.18(a)). A second fraction, just below the floating top layer, was analysed and found to contain some liposomes and crystals (Figure 2.18(b)). The supernatant was mostly clear (Figure 2.18(c)), but some smaller crystals and liposomes were also present, whilst the pellet consisted of BDP crystals and a few liposomes (Figure 2.18(d)).

In order to remove the small BDP crystals that still remained in the liposome fraction after centrifuging in D_2O, the top layer from the sample (made in D_2O) was removed, resuspended in 5 ml D_2O and centrifuged for a further 1 hour at the setting

Figure 2.13(a) DIC photograph of liposomal suspension made in H_2O, containing 10 mole % BDP (× 400).

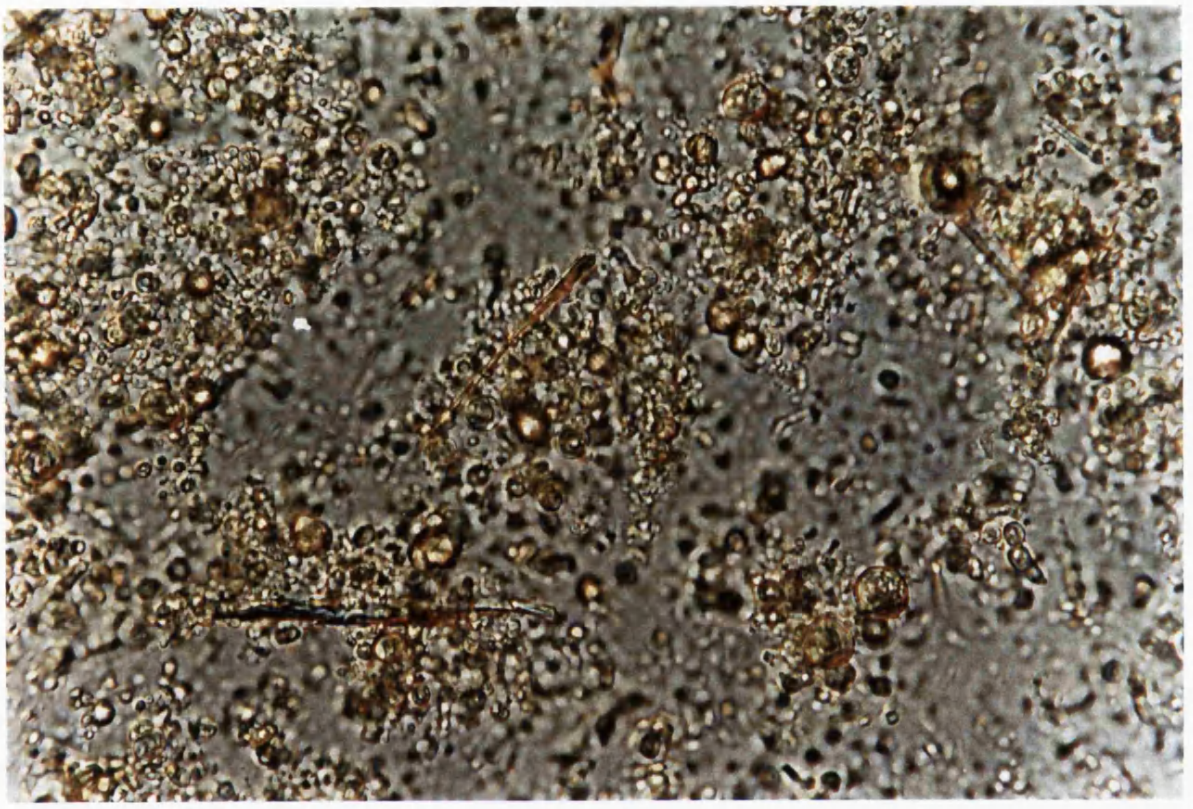


Figure 2.13(b) CP photograph of liposomal suspension made in H_2O, containing 10 mole % BDP (× 400).

Figure 2.14(a) DIC photograph of liposomal suspension made in D_2O, containing 10 mole % BDP (× 400).

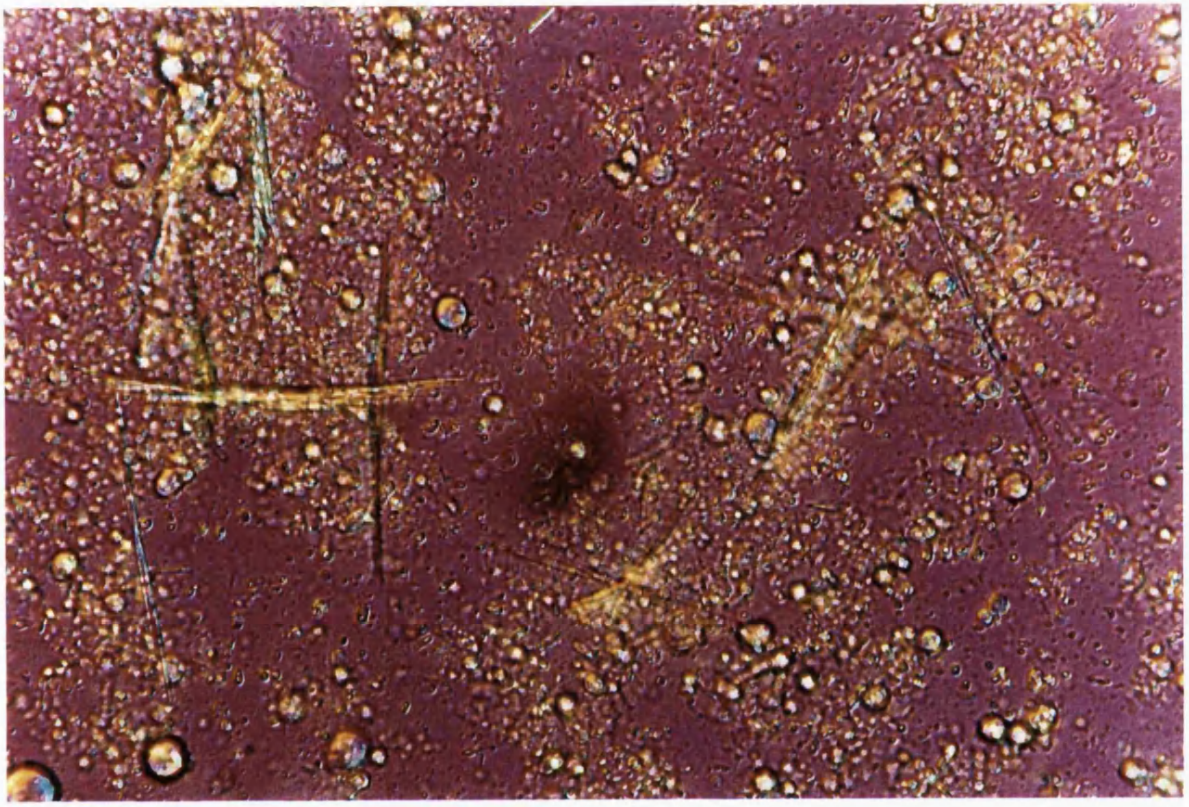


Figure 2.14(b) CP photograph of liposomal suspension made in D_2O, containing 10 mole % BDP (× 400).

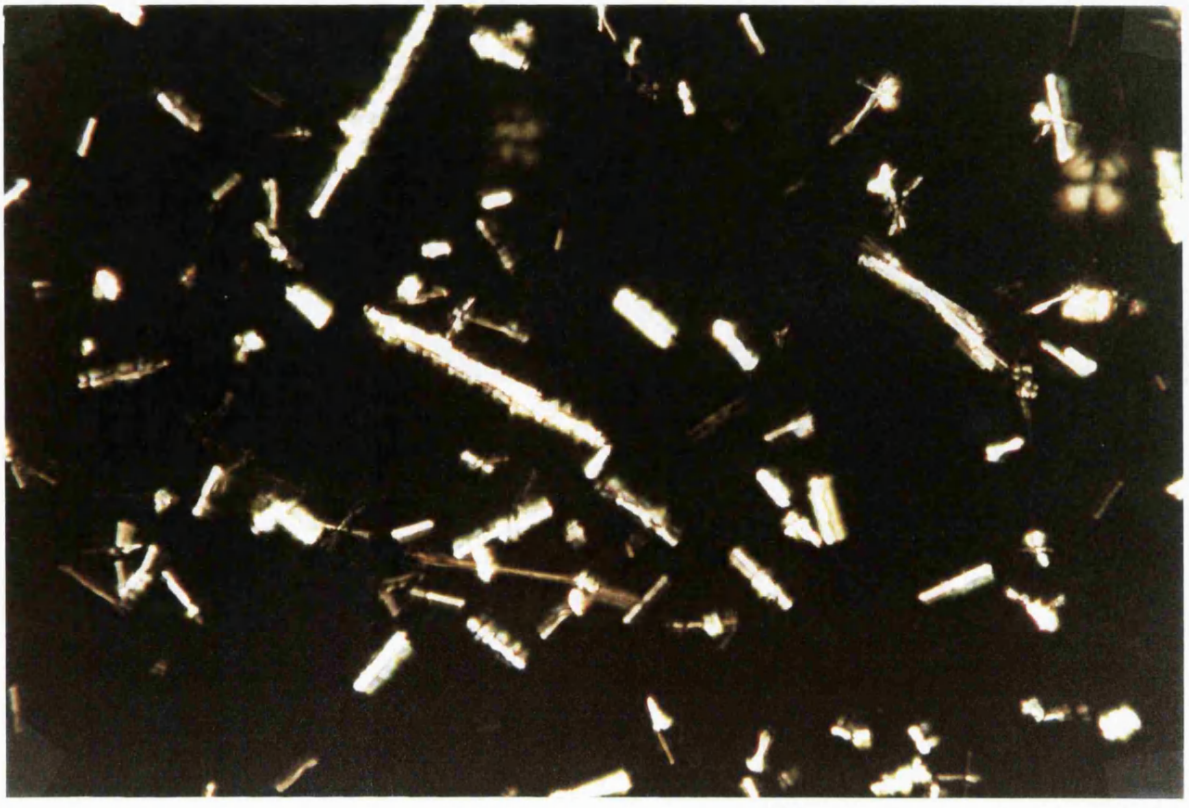


Figure 2.15. DIC photograph of pellet after centrifugation of H_2O liposomes, in H_2O (× 400).

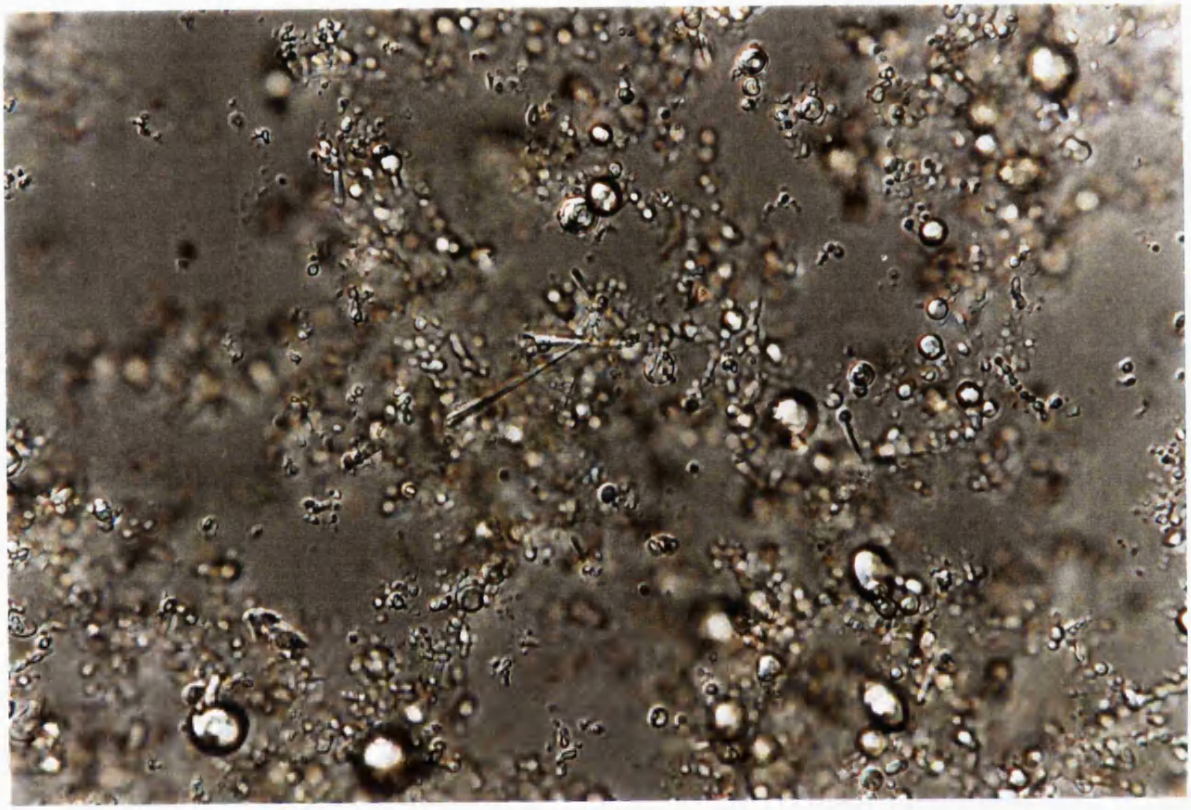


Figure 2.16(a) DIC photograph of top layer after centrifugation of H_2O liposomes, in D_2O (× 400).

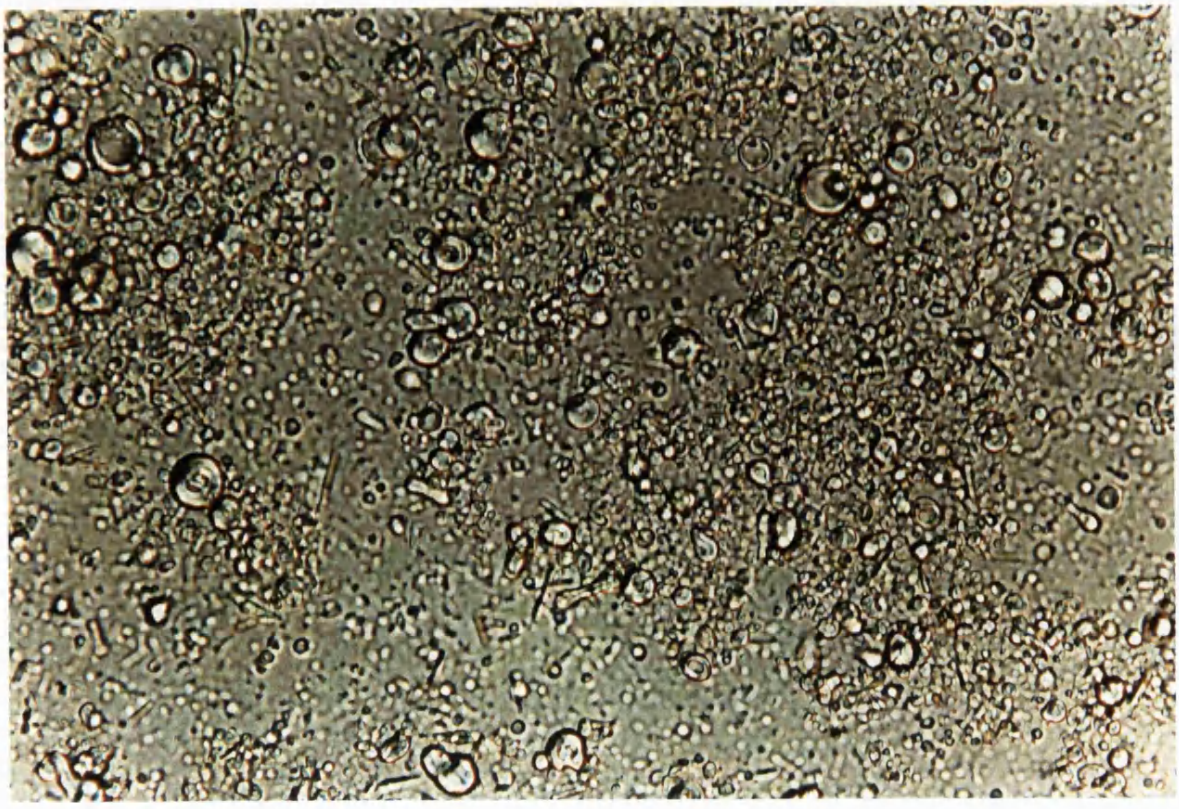


Figure 2.16(b) DIC photograph of supernatant after centrifugation of H_2O liposomes, in D_2O (× 400).

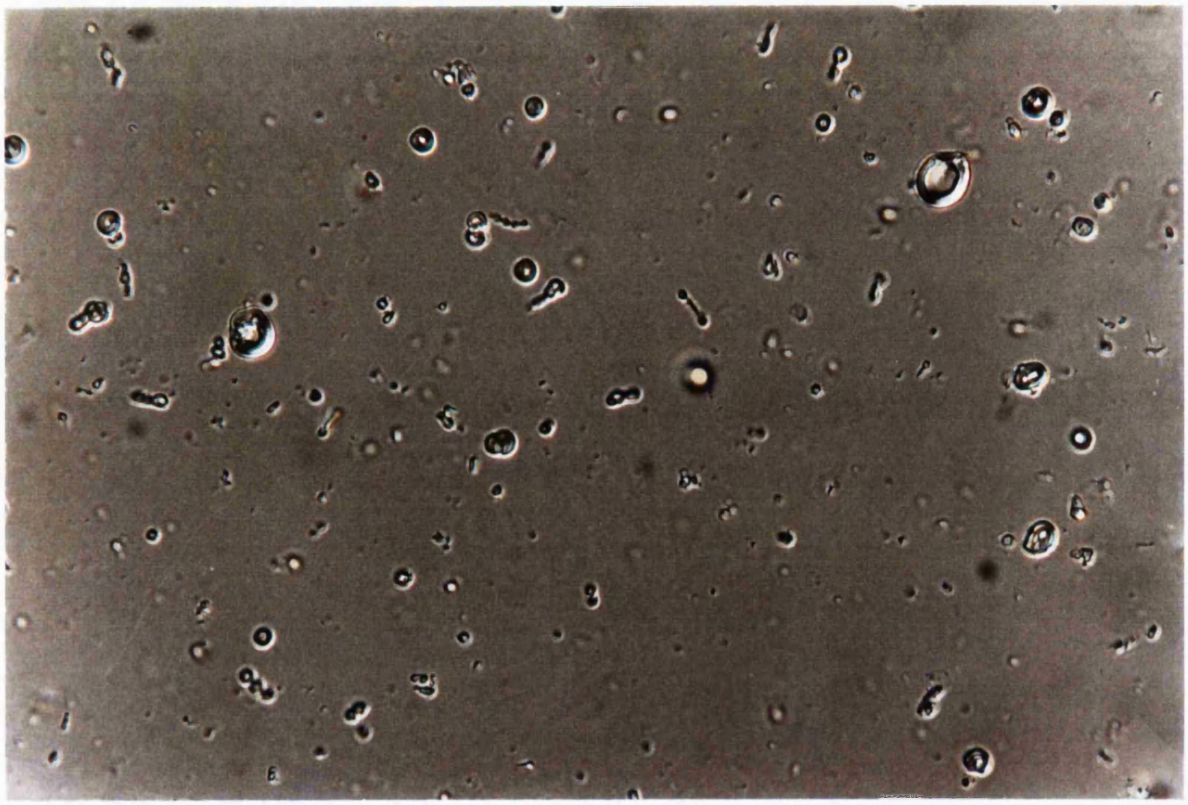


Figure 2.16(c) DIC photograph of pellet after centrifugation of H_2O liposomes, in D_2O (× 400).

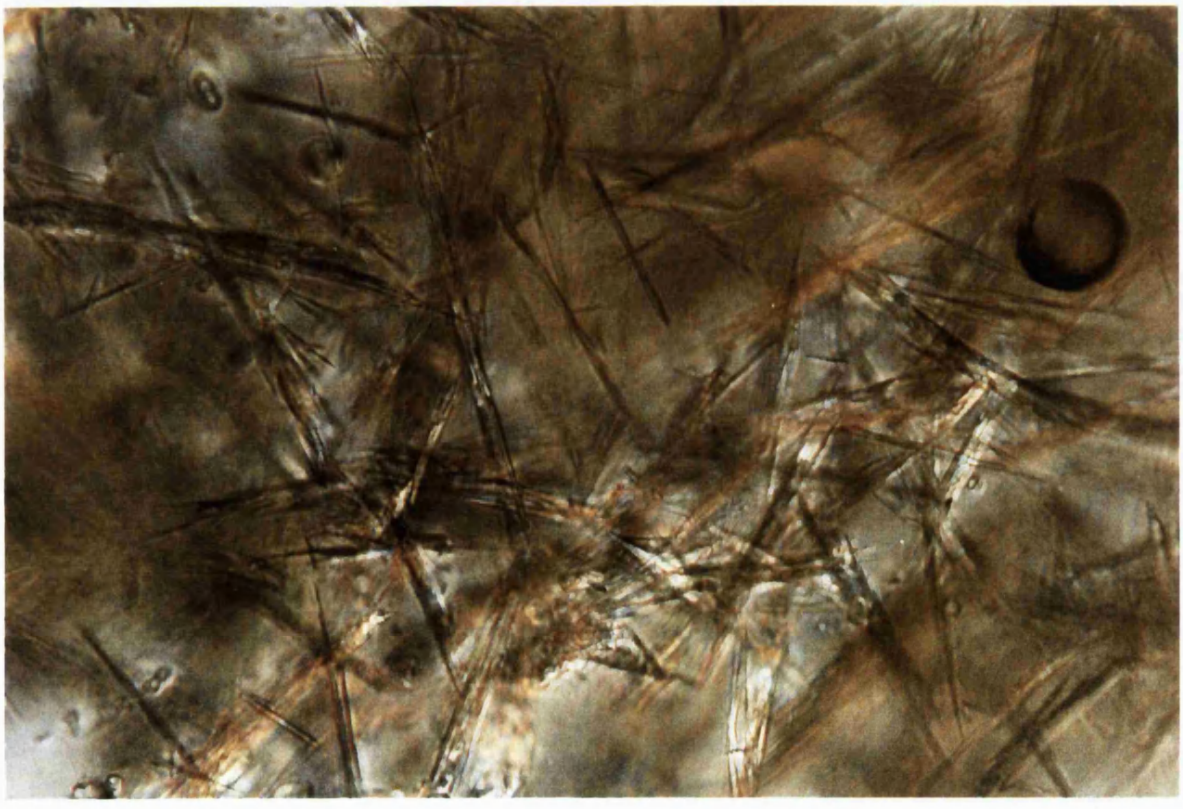


Figure 2.17. DIC photograph of pellet after centrifugation of D_2O liposomes, in H_2O (× 400).

Figure 2.18(a) DIC photograph of top layer after centrifugation of D_2O liposomes, in D_2O (× 400).

Figure 2.18(b) DIC photograph of second fraction after centrifugation of D_2O liposomes, in D_2O (× 400).

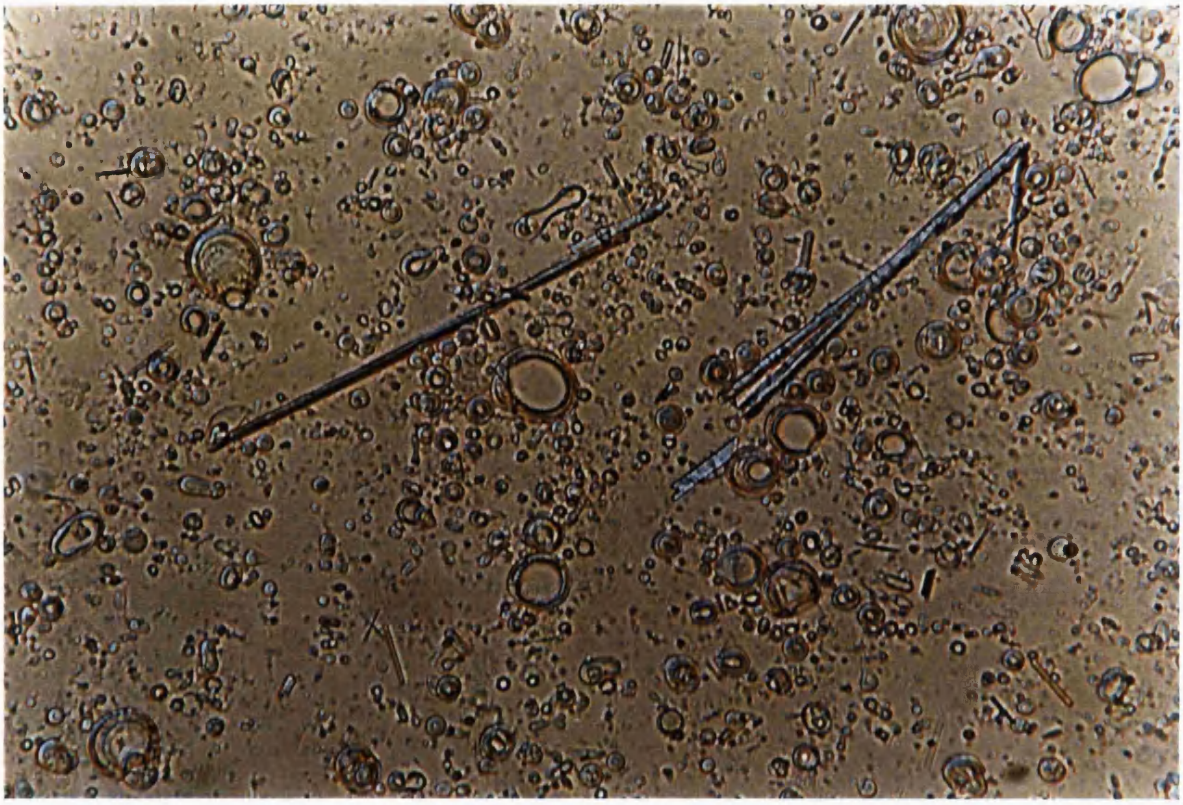
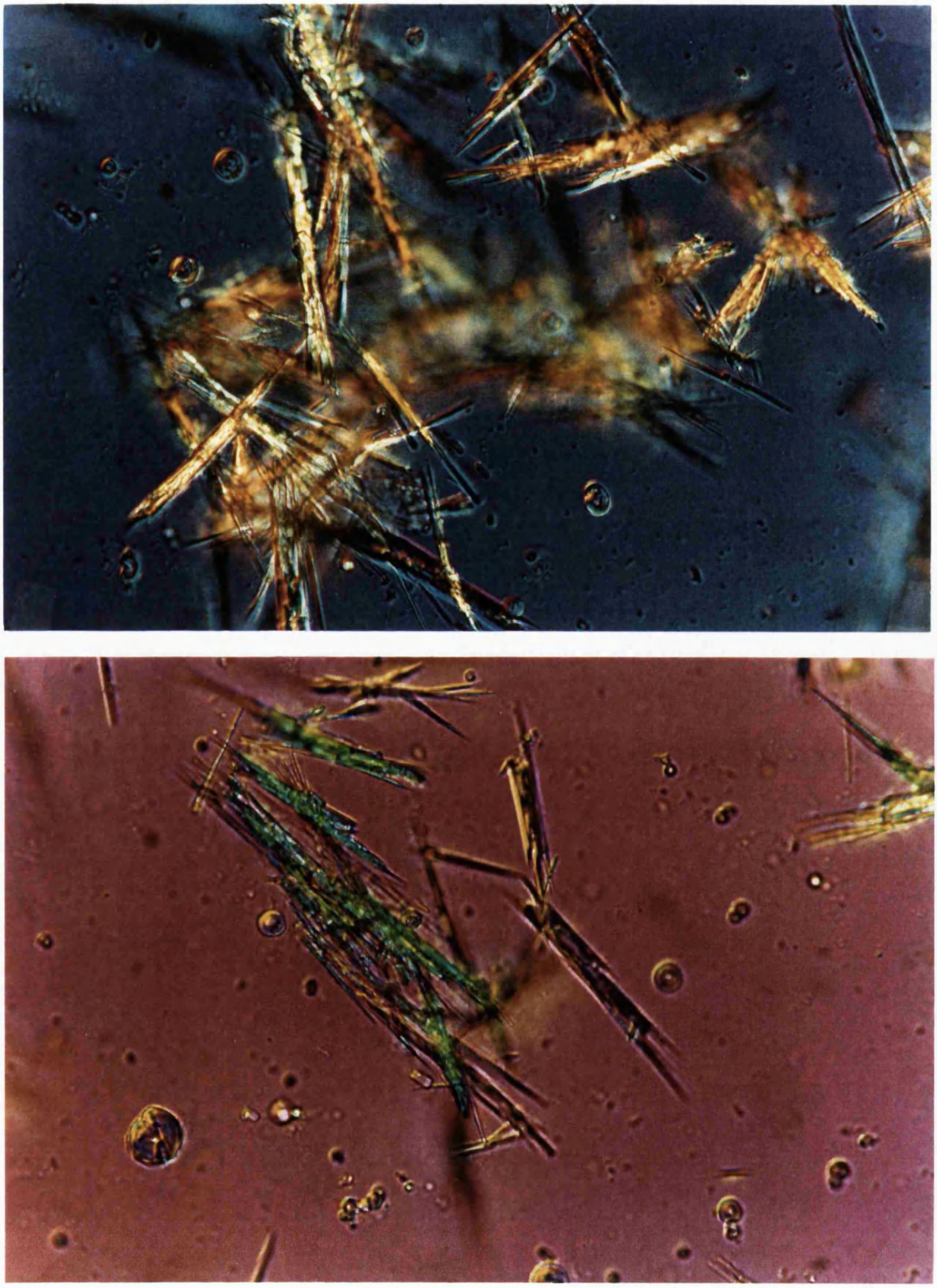


Figure 2.18(c) DIC photograph of supernatant after centrifugation of D_2O liposomes, in D_2O (× 400).

Figure 2.18(d) DIC photographs of pellet after centrifugation of D_2O liposomes, in D_2O (× 400).



previously used. However on microscopic examination (DIC and CP), a few crystals were still observed in the top fraction (Figures 2.19(a) and (b)). Alternatively, centrifugation of a liposomal sample containing 10 mole % BDP and made in D_2O, was performed at a higher setting (setting 8 on the bench centrifuge) for 1 hour. The liposome fraction was then found to contain no crystalline material (Figures 2.20(a) and (b)) whilst the pellet consisted of densely packed BDP crystals and liposomes (Figures 2.21(a) and (b)).

Whilst the advantages of separating unentrapped material by this technique include the fact that it is quick, simple, and particularly useful when small quantities of entrapped material is required for analysis purposes, the disadvantages of this technique are mainly concerned with the sampling technique (of the floating layer). It was found that the top layer was difficult to remove without also including some material from the underlying fraction. However, once centrifugation parameters for efficiently sedimenting the smallest BDP crystals was determined, sampling was not a significant problem. In fact, the absence of crystals at higher centrifugation settings suggests that BDP crystals are most probably absent from the fraction below the floating layer.

2.5.2.3. Conclusions

These results suggest that H_2O is not a suitable medium for efficiently separating BDP crystals from liposomes by centrifugation. D_2O was found to be a better suspending medium, and there was little difference as to whether liposomes were made in H_2O or D_2O. Sedimentation of the smallest BDP crystals was optimal when centrifugation was carried out at setting 8 for 1 hour. Also, DIC and CP microscopy were found to be useful techniques in visualising crystals.

2.5.3. HPLC assay for the determination of BDP in the presence of DPPC

High-performance liquid chromatography (HPLC) is a valuable technique with which a solution containing BDP may be analysed and separated into individual components, according to the attraction of BDP and the solvent in which it is dissolved, for the stationary and mobile phases. The time of BDP retention in the

Figure 2.19(a) DIC photograph of top layer after recentrifugation of D_2O liposomes, in D_2O (× 400).

Figure 2.19(b) CP photograph of top layer after recentrifugation of D_2O liposomes, in D_2O (× 400).

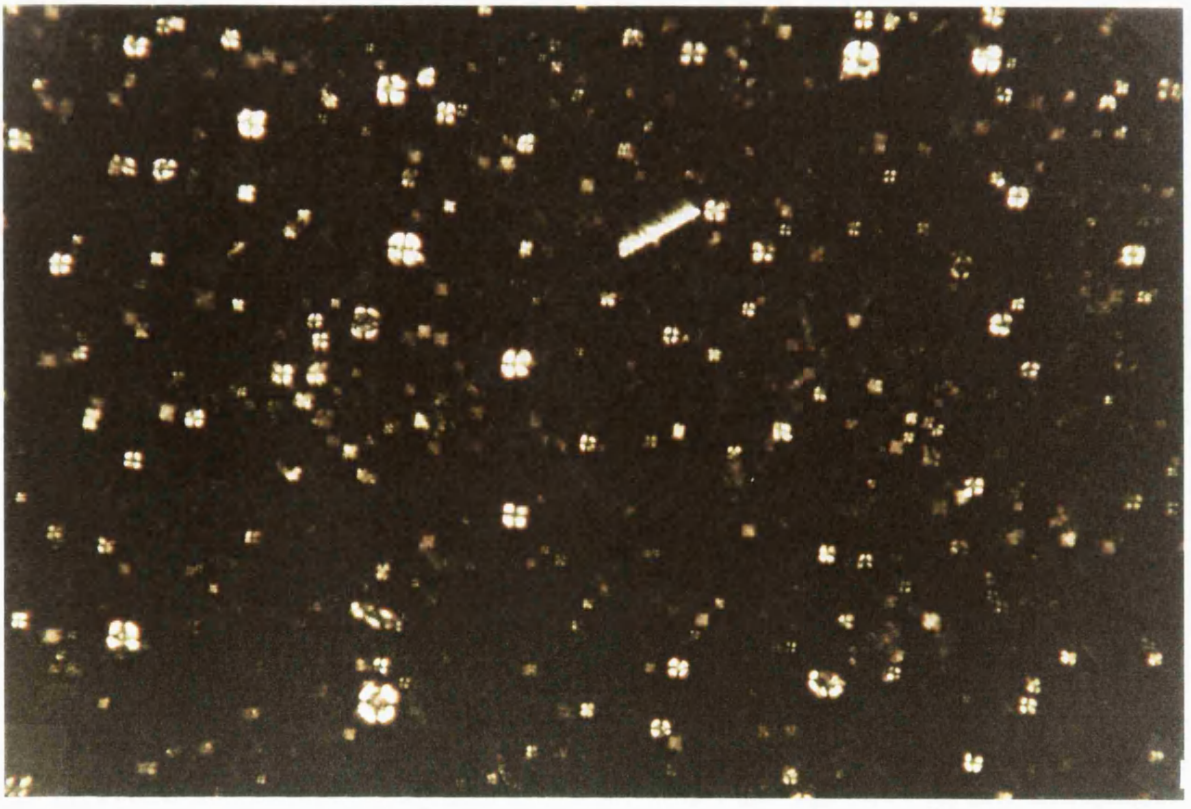


Figure 2.20(a) DIC photograph of top layer after centrifugation (at a higher setting) of D_2O liposomes, in D_2O (× 400).

Figure 2.20(b) CP photograph of top layer after centrifugation (at a higher setting) of D_2O liposomes, in D_2O (× 400).

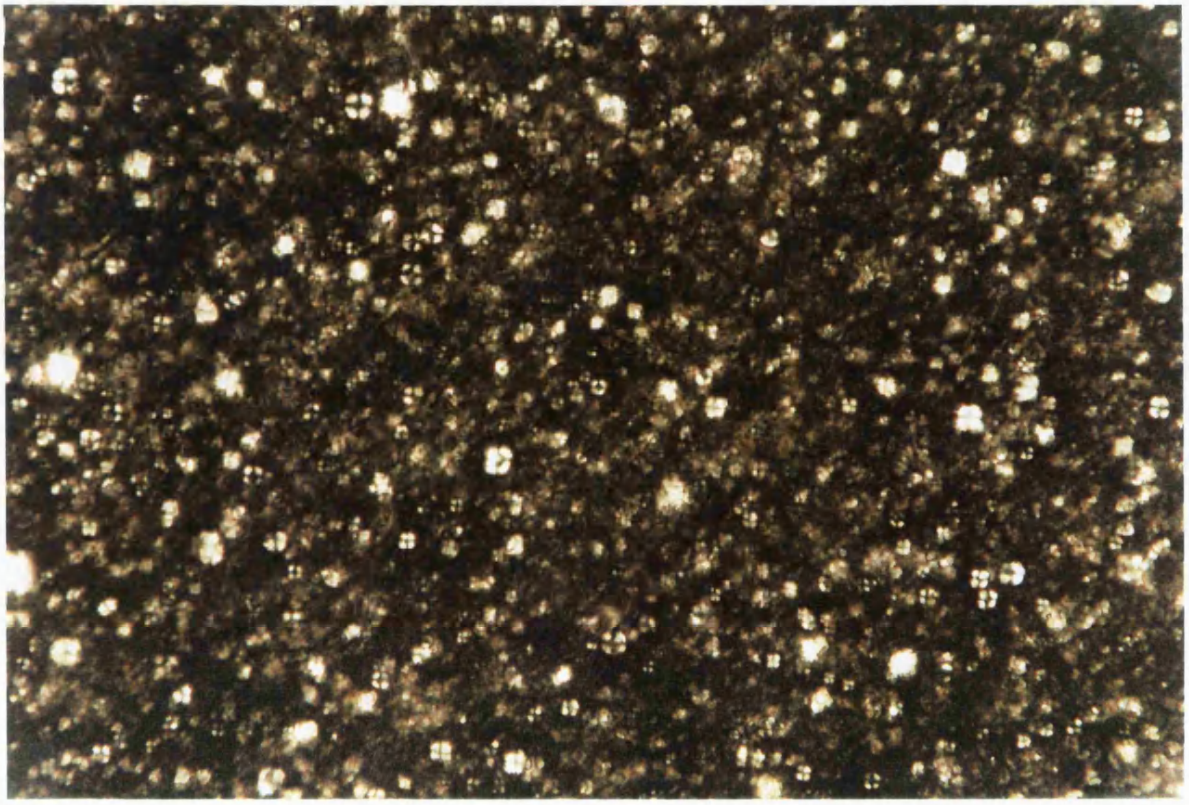
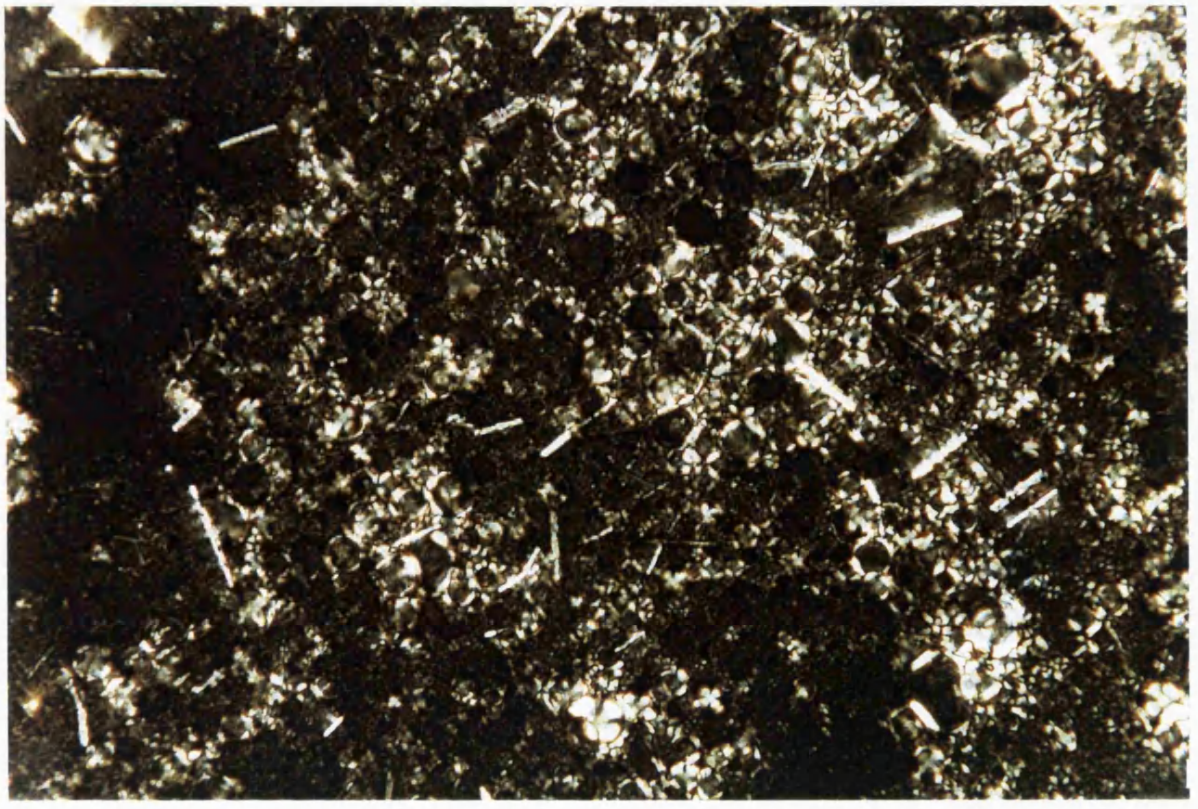


Figure 2.21(a) DIC photograph of pellet after centrifugation (at a higher setting) of D_2O liposomes, in D_2O (× 400).

Figure 2.21(b) CP photograph of pellet after centrifugation (at a higher setting) of D_2O liposomes, in D_2O (× 400).



column can be used as a qualitative analytical tool, whilst the measured amount of BDP can be used for quantitative analysis. Therefore the use of this technique may be extended to detecting and measuring the concentration of BDP in liposomal preparations, and thus quantitative information regarding the incorporation of this steroid into liposomes may be generated.

2.5.3.1. Materials and Methods

HPLC utilises a tightly packed column containing small particles of the stationary phase. The column used throughout these studies was a 5 μm Hypersil ODS (octyldecylsilane) column (Shandon HPLC, Runcorn, Cheshire), with a length of 20 cm, and an internal diameter of 4.6 mm. The creation of a pressure differential between the inlet and the outlet of the column (favouring efficient flow rates of mobile phase through the stationary phase), was achieved by means of a pump. The pump used in the HPLC apparatus (Gilson, Anachem, Beckenham) was designed to be as pulseless as possible so that there is little variation in pressure or flow rates. An injector unit was used to introduce the sample into the column. This is essentially a valve which allows the flow of mobile phase directly into the column when the sample is being loaded into the sample loop with a microsyringe. The size of the loop used was 50 μl (later substituted by a 20 μl loop) and was filled to overflowing with the sample solution. Switching the position of the valve from "load" to "inject" resulted in the diversion of the flow of mobile phase through the sample loop and into the column. Thus the sample was swept into the column along with the mobile phase where separation of the sample components took place. An ultraviolet absorption detector (Gilson, Anachem, Beckenham) continuously monitored the eluate leaving the column, and a plotter (Servogor 120, John Minster Instruments, Folkestone) recorded the developed chromatogram. The apparatus was left to equilibrate overnight, at a low flow rate to minimise the waste of solvent. All solutions were deaerated prior to use, to prevent the detector responding to air as it passes through the column. The deaeration was accomplished by sonicating the vessel containing the solution in an ultrasonic bath (Decon Ultrasonics Ltd., Sussex), for approximately 10 minutes.

Solutions not in use were stored in the refrigerator at 2-8°C, in stoppered flasks and sealed with laboratory film (Parafilm "M", Greenwich, USA).

Details for an HPLC assay for the determination of BDP were supplied by Glaxo Wellcome. The mobile phase used was a mixture of acetonitrile and deionised water, in a ratio of 6:4 (v/v). The mixed solvent in which BDP was dissolved was prepared using methanol and deionised water, in a ratio of 7:3 (v/v). To 1 litre of this mixed solvent was added 1 ml glacial acetic acid. The HPLC apparatus was set at a mobile phase flow rate of 2 ml/minute, variable sensitivity at ambient temperature, a sample injection volume of 50 µl and UV detection at 238 nm. The chart recorder parameters were set at 10 mv with a chart speed of 3 cm/minute. All samples were assayed a minimum of four times, and the column was rinsed with injections of mobile phase between each assay.

With this set of conditions, calibration solutions of BDP were prepared in order to carry out HPLC assays and construct calibration curves (of peak area versus BDP concentration) at sensitivities of 0.2, 0.05 and 0.01 AUFS. From the resultant chromatograms, peak areas were measured manually by two methods, known as Peak area 1 and 2.

Peak area 1 = peak height × half base width,
Peak area 2 = peak height × peak width at half height.

However, problems were encountered when further use of the HPLC apparatus resulted in unreproducible and inconsistent results at these sensitivities. A steady baseline was eventually produced at 0.5 AUFS, and therefore a calibration curve was constructed at this sensitivity. Further apparatus problems resulted in the installation of a new injection valve with a sample volume of 20 µl, and recalibration of this system was then carried out.

In order for the Glaxo Wellcome HPLC assay to be valuable in the determination of the amount of BDP entrapped within liposomes, the assay required validation in the presence of phospholipid. DPPC was used in validation studies, as it is the phospholipid most commonly used throughout in the production of liposomes containing BDP. Therefore these studies involved the addition of varying amounts of

DPPC to a known amount of BDP, and analysing the resulting chromatograms. Validation studies were performed once calibration curves had been plotted. Initially, validation of the assay was carried out when the apparatus was only efficient at a sensitivity of 0.5 AUFS, but further validation studies were required when the injection valve of the HPLC apparatus was replaced. Varying amounts of DPPC were dissolved in mixed solvent (methanol and water in a 7:3 v/v ratio) by means of prolonged sonication, and an appropriate quantity of this solution was added to a solution of BDP (in mixed solvent) containing a known amount of drug. All solutions were then assayed to determine if the presence of DPPC affected the assay for BDP concentration.

The opportunity arose for the original assay to be optimised, since it was thought that alternatives could easily be investigated. To eliminate the use of different solvents in the mobile phase and the mixed solvent, an acetonitrile system was investigated where the mobile phase used was identical to the one previously used (acetonitrile and deionised water in a 6:4 v\v ratio), and the dissolving solvent was acetonitrile alone. An alternative system using methanol was also investigated, since BDP was found to dissolve in methanol and produced consistent peaks with mobile phases of acetonitrile and water (6:4 v\v ratio), and methanol and water (7:3 v\v ratio). Acetonitrile and glacial acetic acid could now be eliminated from the conditions outlined in the original assay, since methanol was used in both the mixed solvent and the mobile phase. This optimised system required calibration and solutions were prepared and assayed to construct a calibration plot. A stock solution containing 40 μg/ml BDP was diluted to produce further solutions containing 20, 16, 10, 8, 6.7 and 5 μg/ml BDP.

The optimised system required validation in the presence of DPPC and this was achieved by first assaying a solution of BDP in methanol, which gave an average peak area (calculated using peak area 2), after four assays, of 0.973 cm^2 (standard deviation =0.106). From Equation 2.1(b) generated from the constructed calibration curve (Figure 2.26.), this area was found to correspond to a BDP concentration of 11.009 μg/ml. To produce a solution containing approximately a 99:1 molar ratio of DPPC:BDP, 7.4 mg DPPC was dissolved in 5 ml of this BDP solution. This solution was then sonicated and assayed. Peak areas were determined from the chromatograms

obtained, in order to conclude how much BDP was calculated to be in the solution. In addition, a solution of DPPC in methanol, also containing 1.48 mg/ml DPPC, was assayed to determine if DPPC in the absence of any BDP, had any effect on the HPLC assay.

2.5.3.2. Results and Discussion

The assay previously used by Glaxo Wellcome showed reproducibility for assaying BDP at sensitivities of 0.01, 0.05 and 0.2 AUFS, and later at 0.5 AUFS. Recalibration of the system (due to the fitting of a new injection valve) also produced a reproducible calibration plot. The plots for each of these calibrations can be seen in Figures 2.22(a), (b), (c), (d) and (e).

The initial validation studies involved assaying solutions containing known amounts of BDP and varying amounts of DPPC. However, the peak areas obtained were unreproducible, inconsistent and therefore no distinct relationship between DPPC concentration and peak areas could be identified. Whilst it was thought that the observed inconsistencies may have been due to the effect of DPPC on BDP, they could also have been due to the effect of vesicle formation by DPPC in the presence of water (in the mixed solvent). A possible solution to this problem may have been found in the use of Triton X-100, a detergent frequently employed in the lysis of liposomes to release any entrapped BDP. Whilst BDP (2 μg/ml in mixed solvent) and Triton X-100 (0.25 % v/v in mixed solvent) have retention times of approximately 10 and 13 minutes, respectively, the latter displays broader peaks on a chromatogram which completely overlap with those of BDP (Figure 2.23.) making it unsuitable for use in this study.

Optimisation studies found that when acetonitrile was used as the dissolving solvent alone (and the mobile phase was acetonitrile and deionised water in a 6:4 v\v ratio), the peaks obtained were shouldered (Figure 2.24.) with retention times of approximately 12 minutes. DPPC does not dissolve in acetonitrile, even after prolonged sonication and therefore validation in the presence of this phospholipid would have been impossible. However, DPPC was found to dissolve in methanol, and so studies using this solvent as the dissolving solvent were carried out. The mobile

Figure 2.22(a) Calibration of HPLC assay at 0.01 AUFS (n=x±SD)

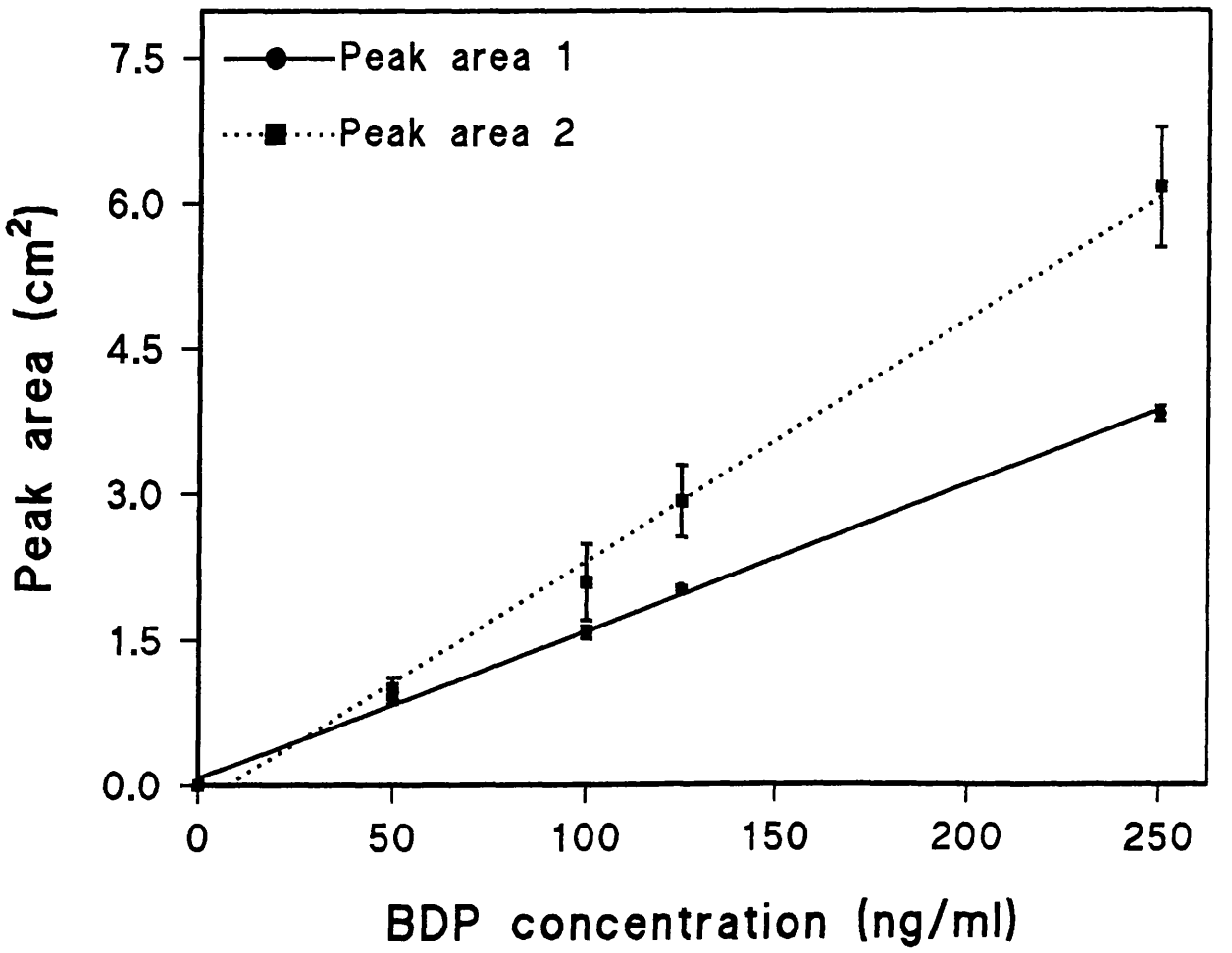


Figure 2.22(b) Calibration of HPLC assay at 0.05 AUFS (n=x±SD)

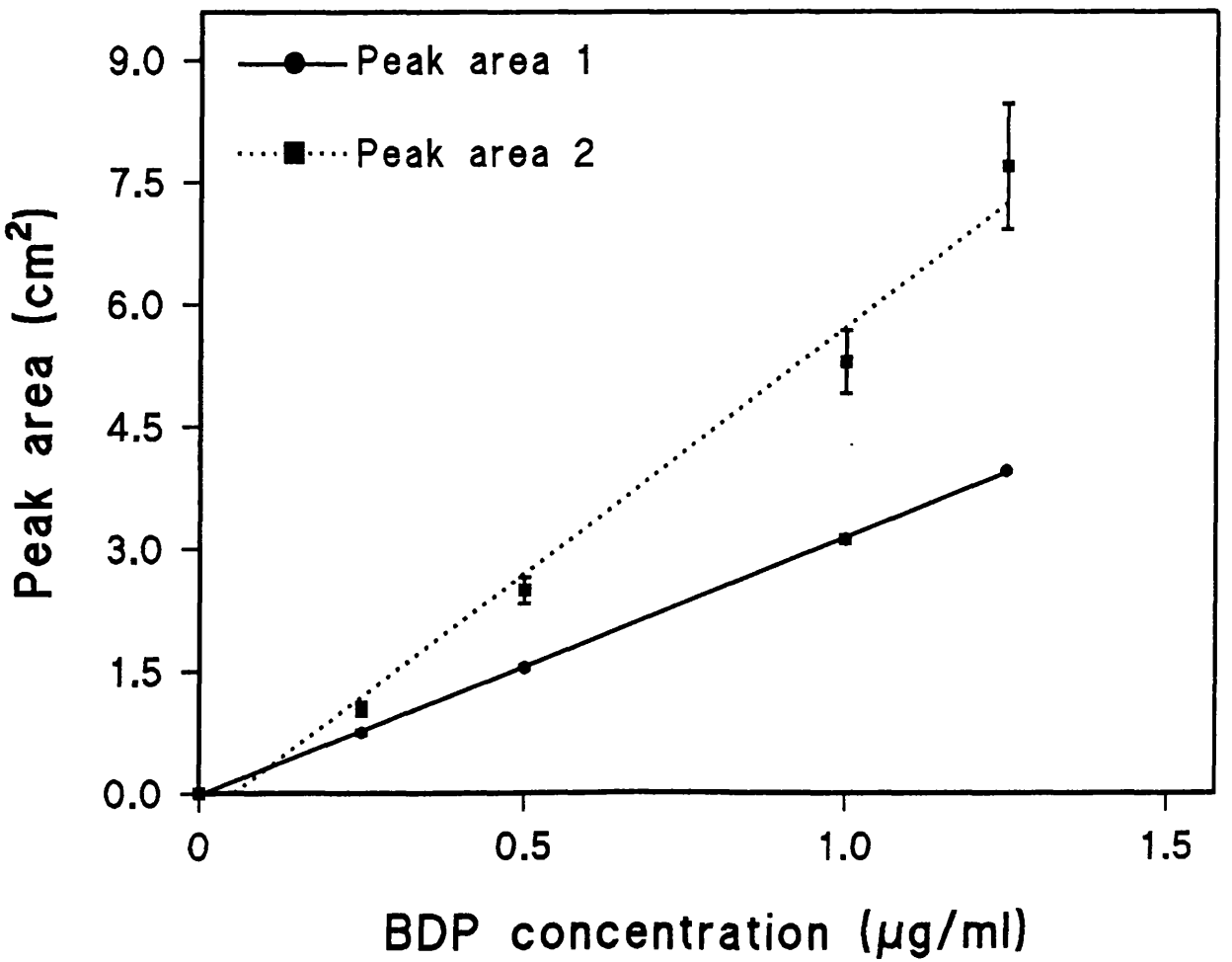


Figure 2.22(c) Calibration of HPLC assay at 0.2 AUFS (n=x±SD)

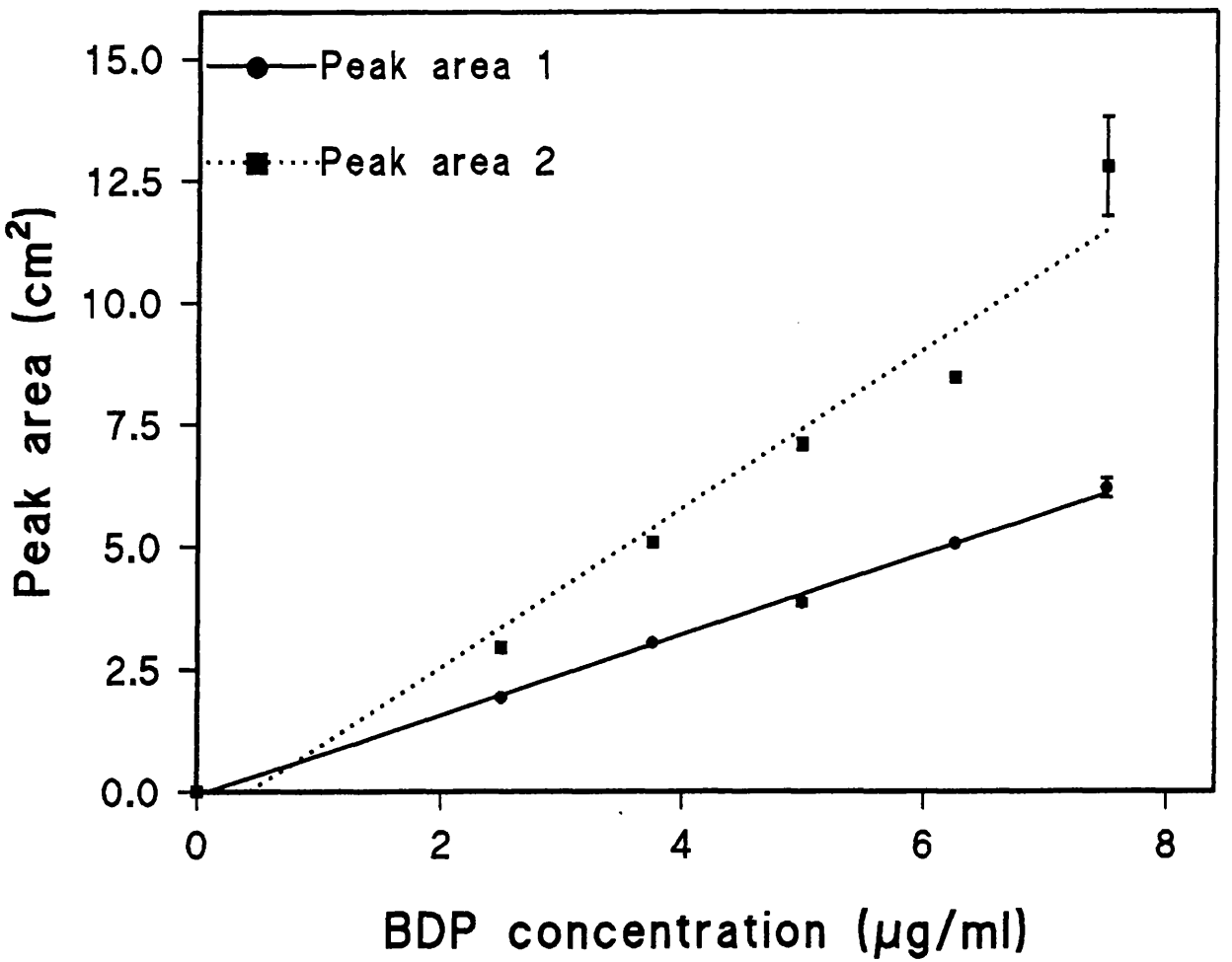


Figure 2.22(d) Calibration of HPLC assay at 0.5 AUFS (n=x±SD)

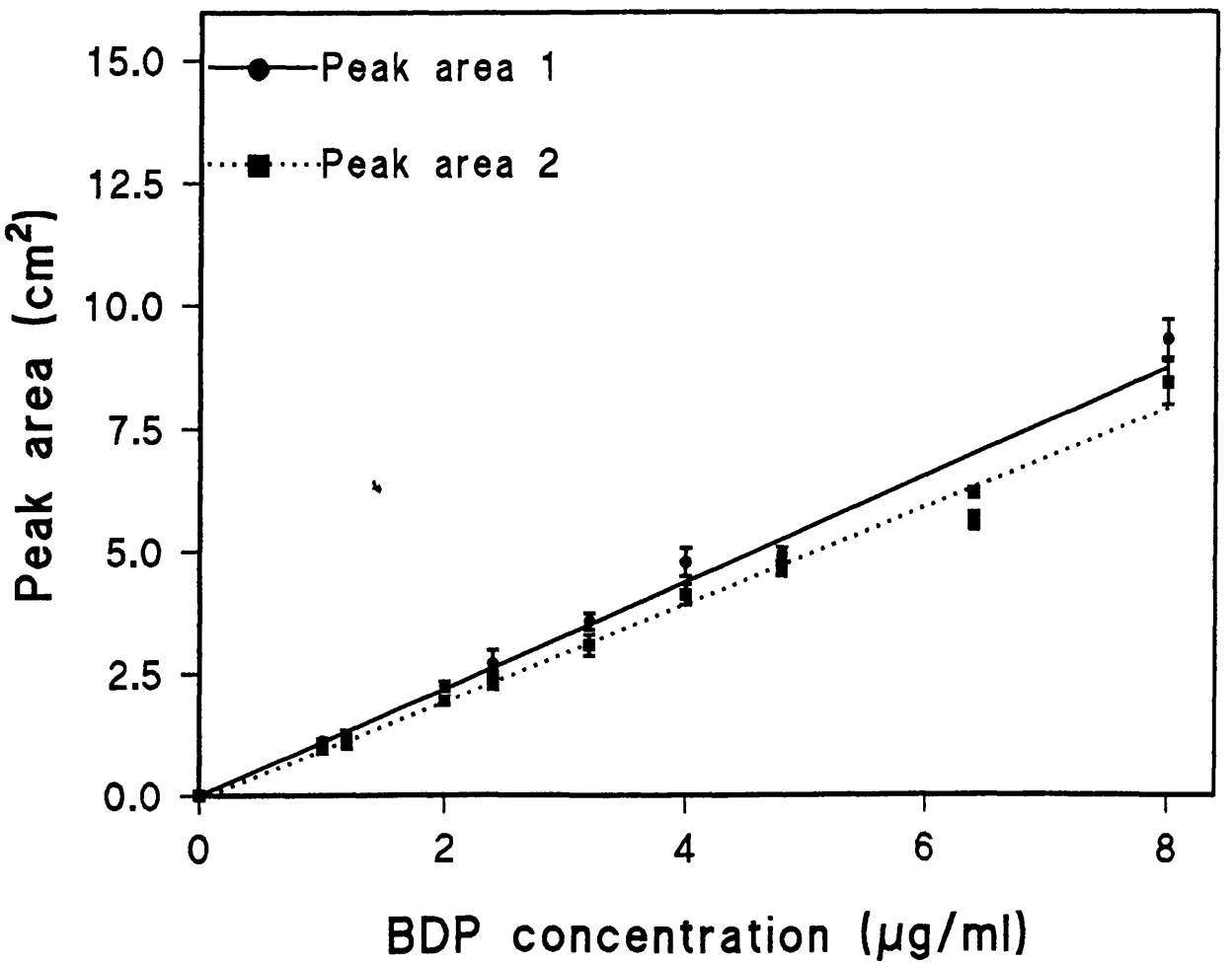


Figure 2. 22(e) Calibration of HPLC assay at 0.5 AUFS, with new injection valve (n=x±SD)

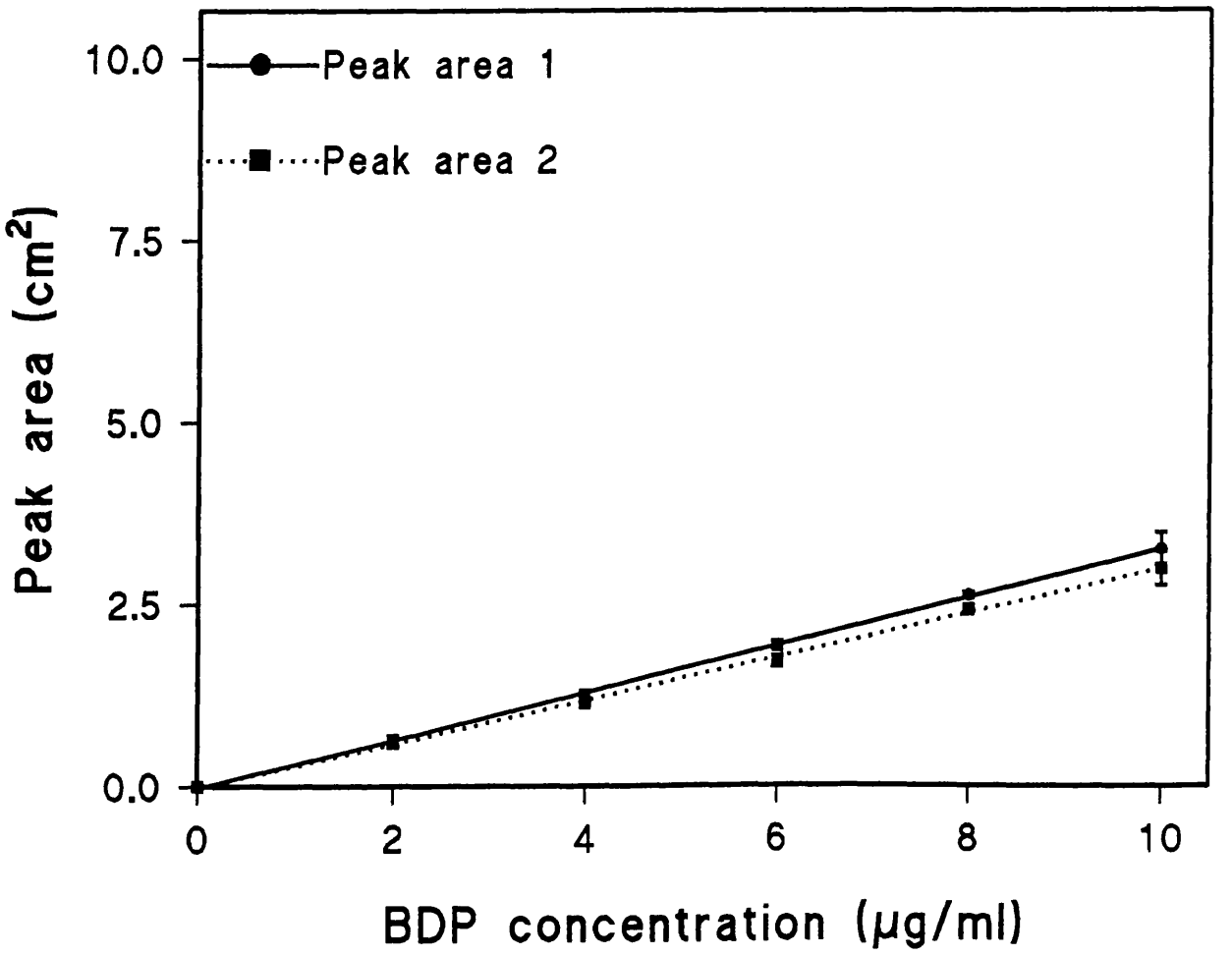
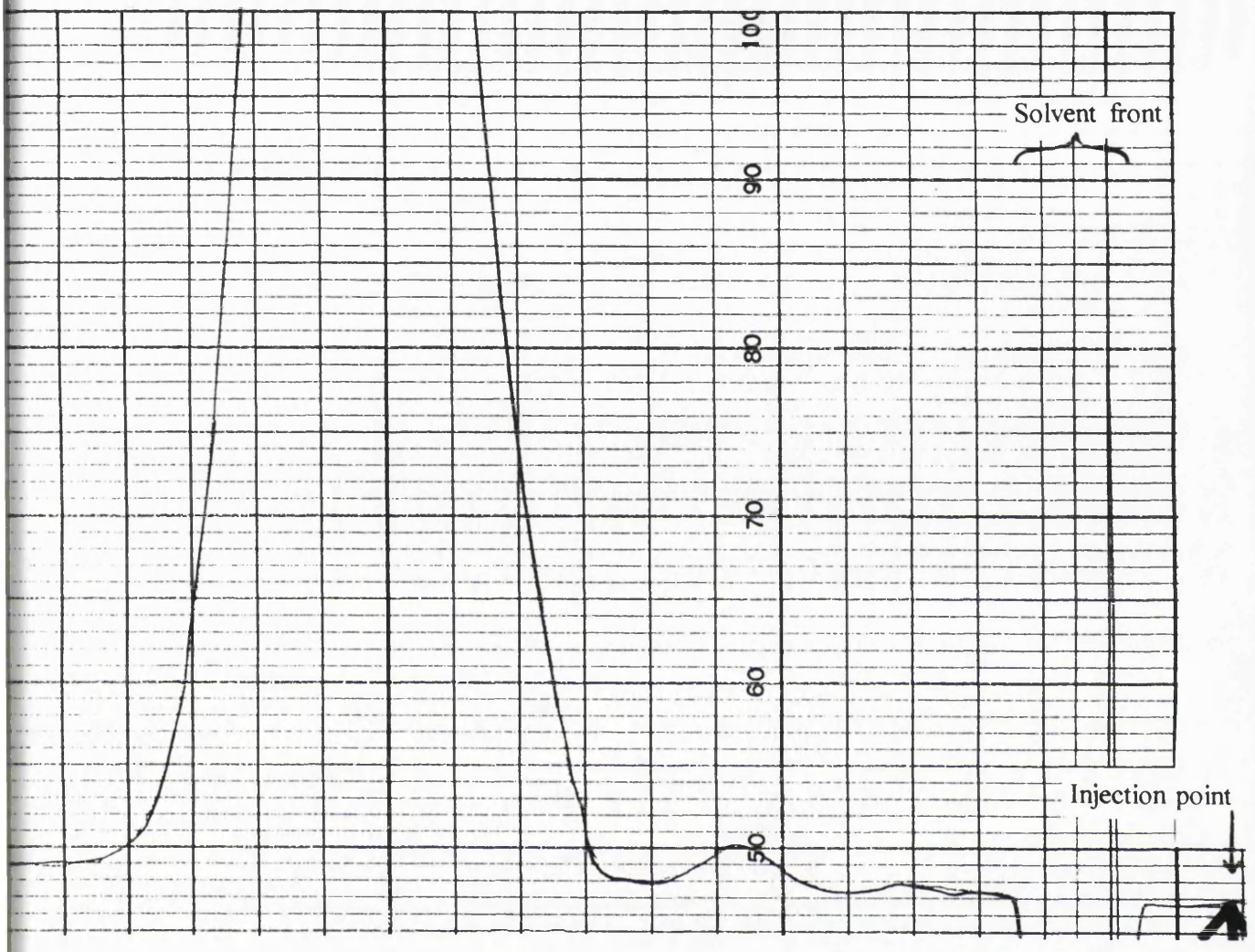


Figure 2.23. Chromatogram of Triton X-100 (0.25 % in methanol:water, 7:3 v/v).



Chromatogram of BDP (2 µg/ml in methanol:water, 7:3 v/v).

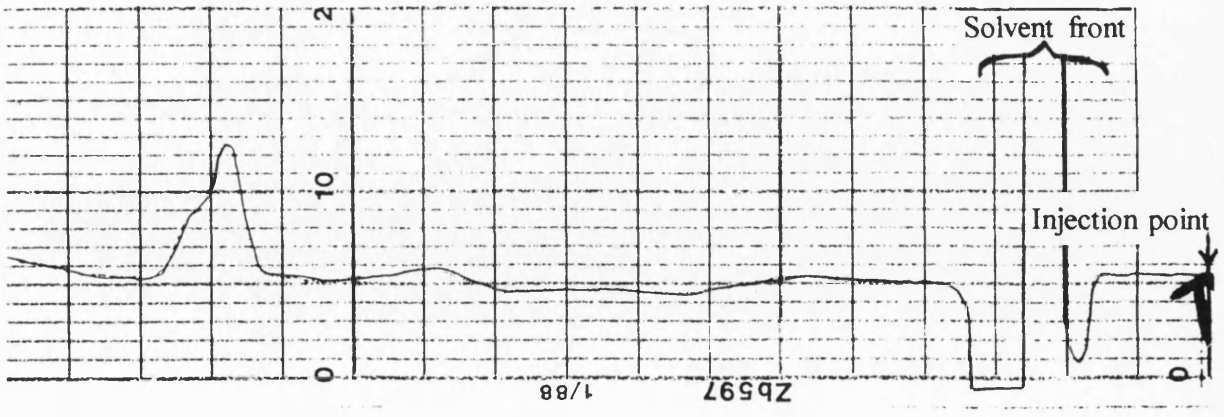
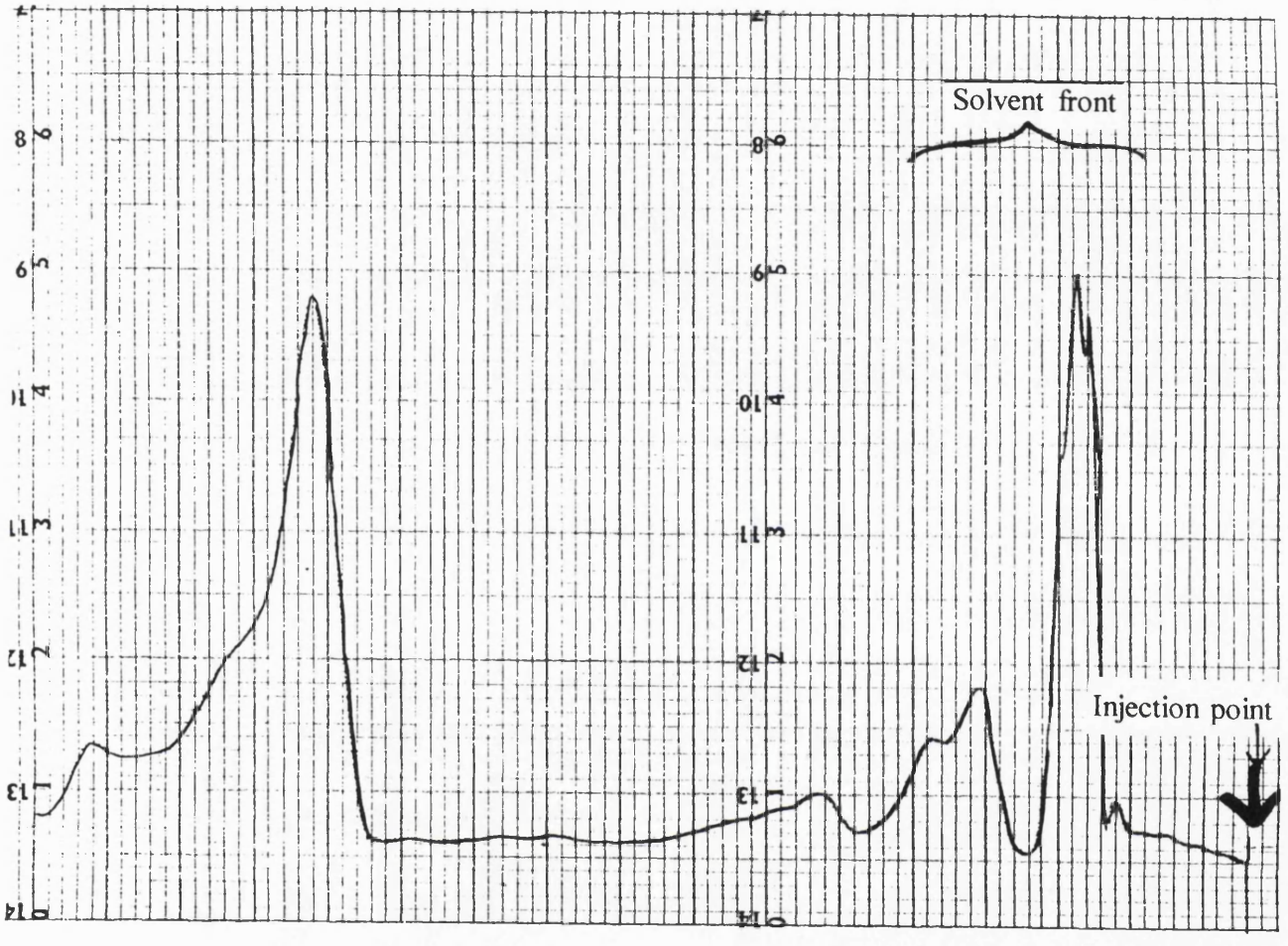


Figure 2.24. Chromatogram of BDP dissolved in acetonitrile, with a mobile phase of acetonitrile and water (6:4 v/v).



phase used was identical to the one used previously, and the peaks obtained for solutions of BDP were reproducible and consistent (Figure 2.25.) with retention times of approximately 12 minutes, but again they were shouldered. This was thought to be due to the presence of a possible impurity, and this was confirmed by unpublished work (Glaxo Wellcome in-house data) as a common observation.

The elimination of acetonitrile from the system, and its subsequent optimisation was achieved by using methanol as the dissolving solvent, and a mixture of methanol and deionised water (7:3 v\v ratio) as the mobile phase. All peaks were reproducible and consistent, with retention times reduced to approximately 8.5 minutes. A calibration plot was constructed (Figure 2.26.), which followed the equations below.

(a) Peak area 1	$y = 0.086180(x) + 0.095874$	Equation 2.2(a).
(b) Peak area 2	$y = 0.080049(x) + 0.091722$	Equation 2.2(b).

In addition, peaks were found to be symmetrical with no signs of the presence of any impurities (Figure 2.27.). Validation of this optimised system was performed by assaying a solution of BDP in methanol. The average corresponding peak areas obtained were used to calculate that the solution contained 11.009 μg/ml BDP (from Equation 2.2(b)). Appropriate amounts of DPPC were then added to this solution, such that the molar ratio of DPPC:BDP was 99:1. The assay of this solution produced peak areas which were used to calculate the corresponding BDP concentrations, from Equations 2.2(a) and (b). The values obtained are presented in Table 2.3., and these show that the BDP concentrations calculated in the absence and presence of DPPC are not significantly different. Assay of a solution of 1.48 mg/ml DPPC in methanol (the same concentration as above) produced chromatograms with no peaks (Figure 2.28.), indicating that DPPC alone has no effect on the HPLC assay, whilst BDP chromatograms in the presence of DPPC are shown in Figure 2.29. Therefore these results indicate that at a concentration of 99 mole %, DPPC does not affect the HPLC assay for the determination of BDP, and any lower concentration of phospholipid is therefore also unlikely to show any effects. In addition, BDP retention times in the presence of DPPC were similar to that of BDP alone (Table 2.3.) confirming that the assay is valid in the presence of DPPC.

Figure 2.25. Chromatogram of BDP dissolved in methanol, with a mobile phase of acetonitrile and water (6:4 v/v).

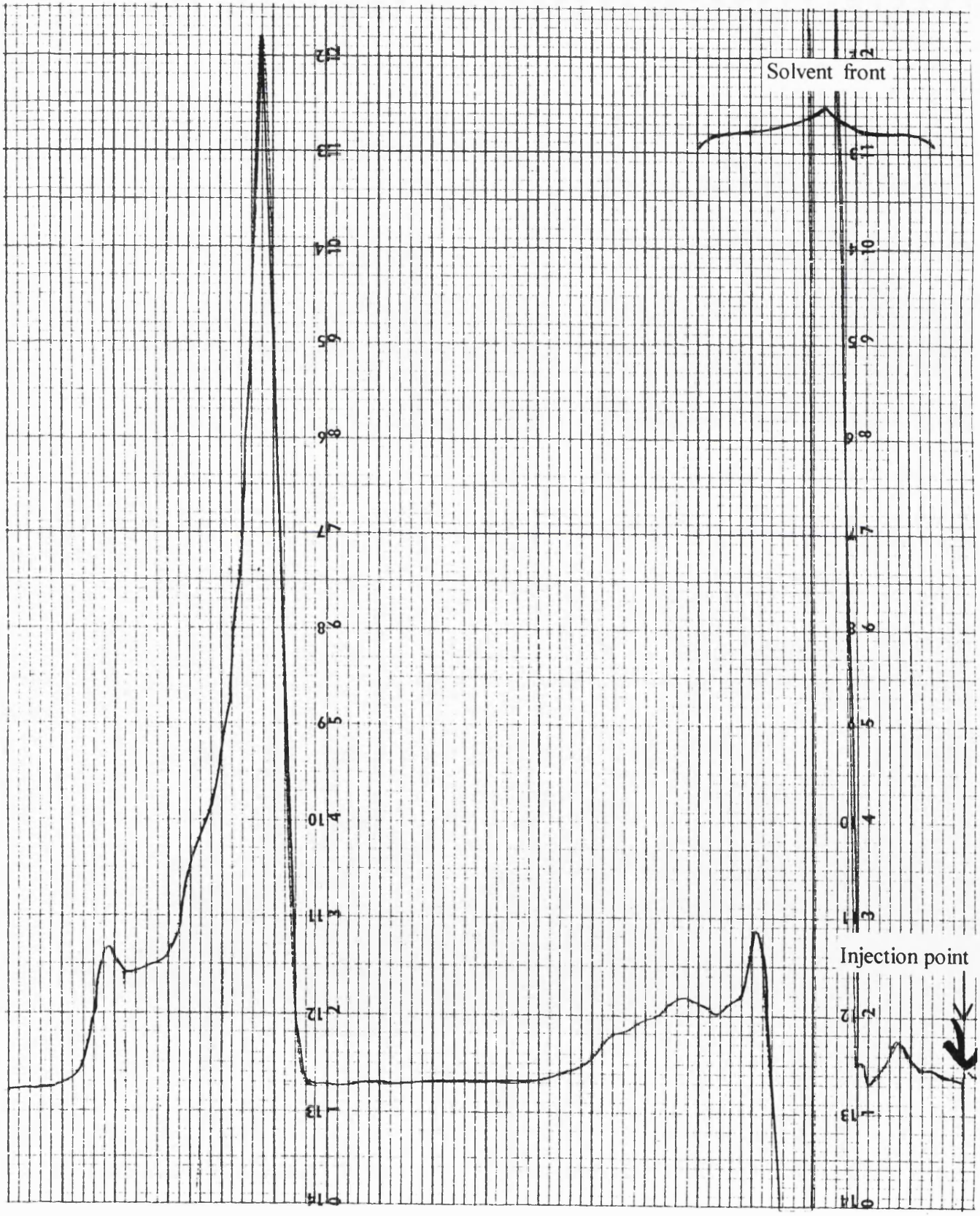


Figure 2.26 Calibration of optimised HPLC assay at 0.5 AUFS (n=x±SD)

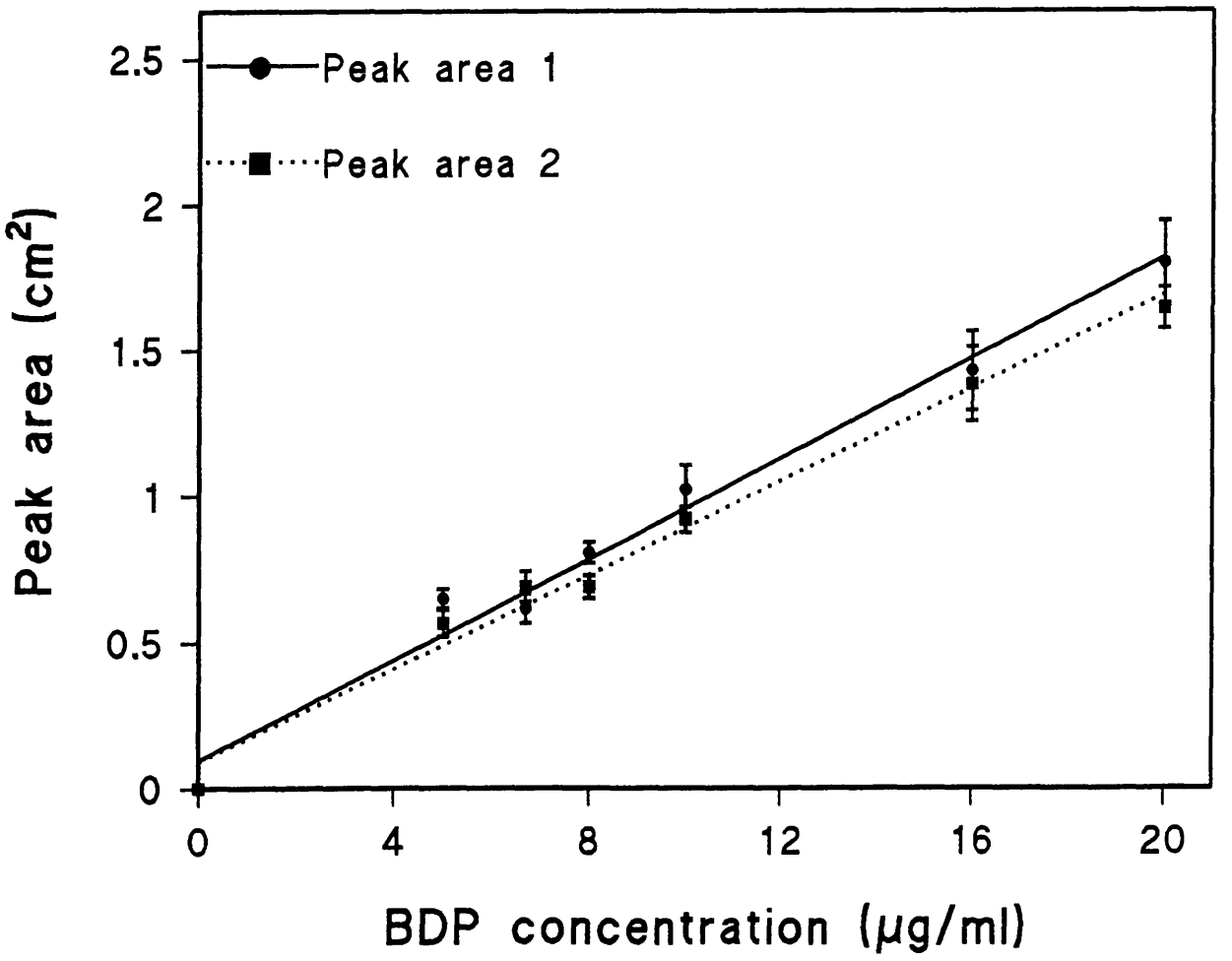


Figure 2.27. Chromatogram of BDP dissolved in methanol (11.009 μg/ml), with a mobile phase of methanol and water (7:3 v/v).

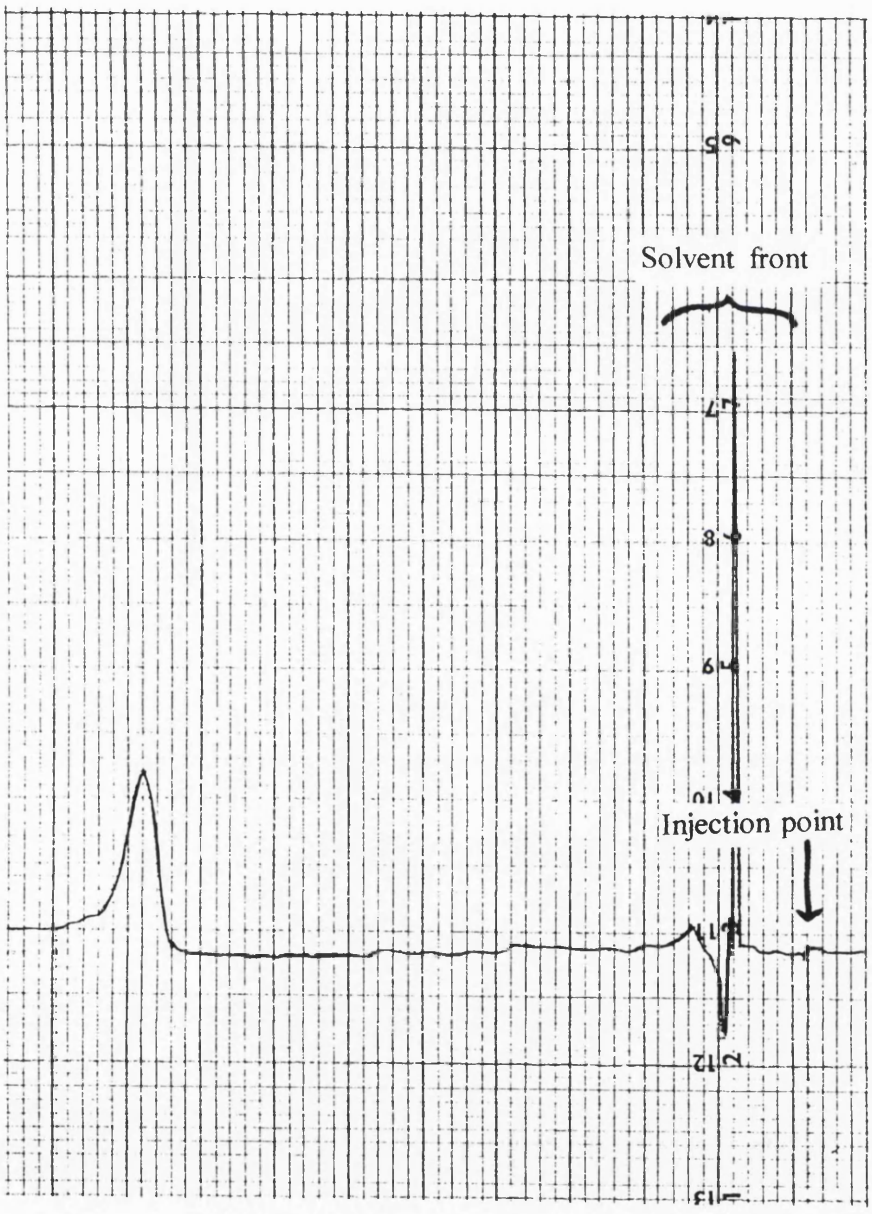


Figure 2.28. Chromatogram of DPPC dissolved in methanol (1.48 mg/ml), with a mobile phase of methanol and water (7:3 v/v).

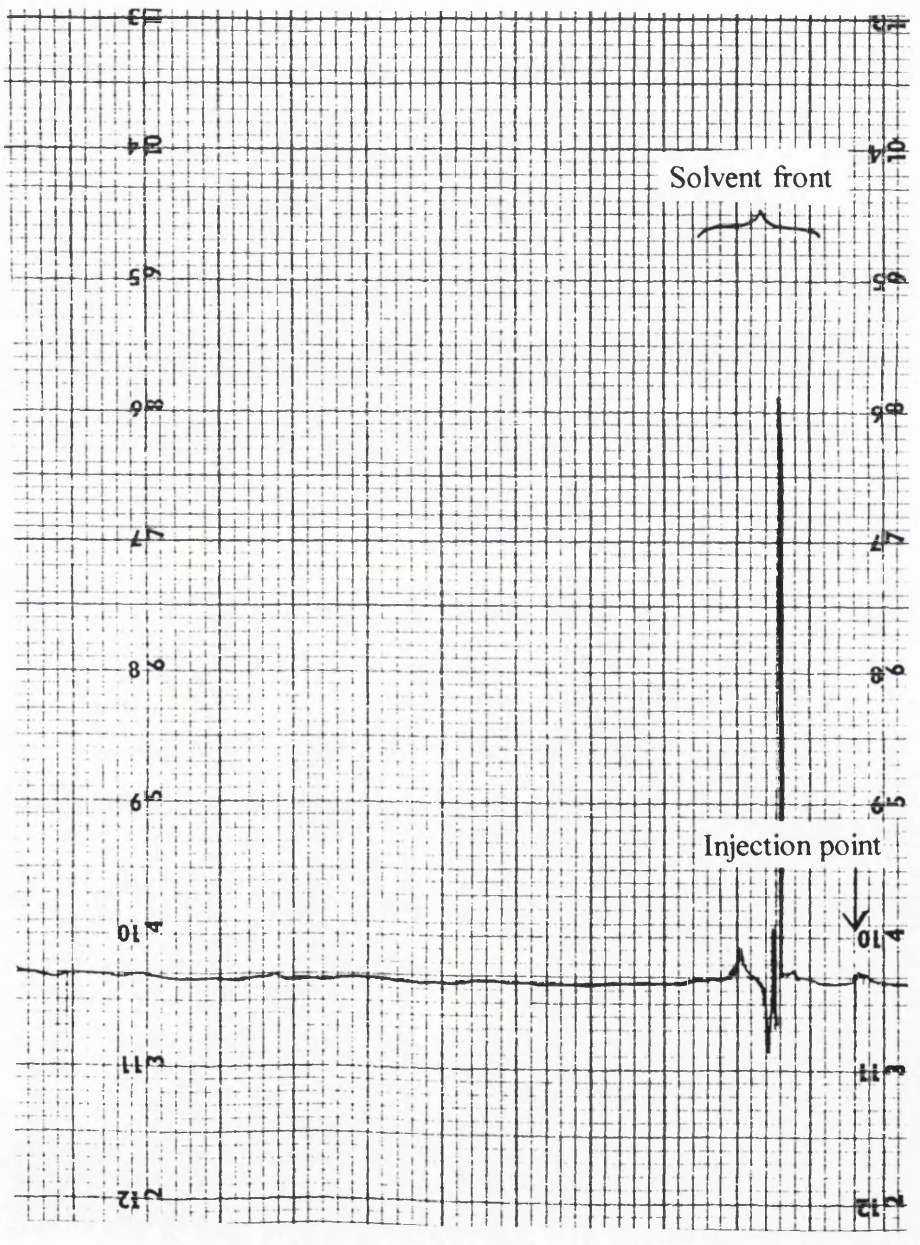


Figure 2.29. Chromatogram of BDP dissolved in methanol (11.009 μg/ml) in the presence of 99 mole % DPPC, with a mobile phase of methanol and water (7:3 v/v).

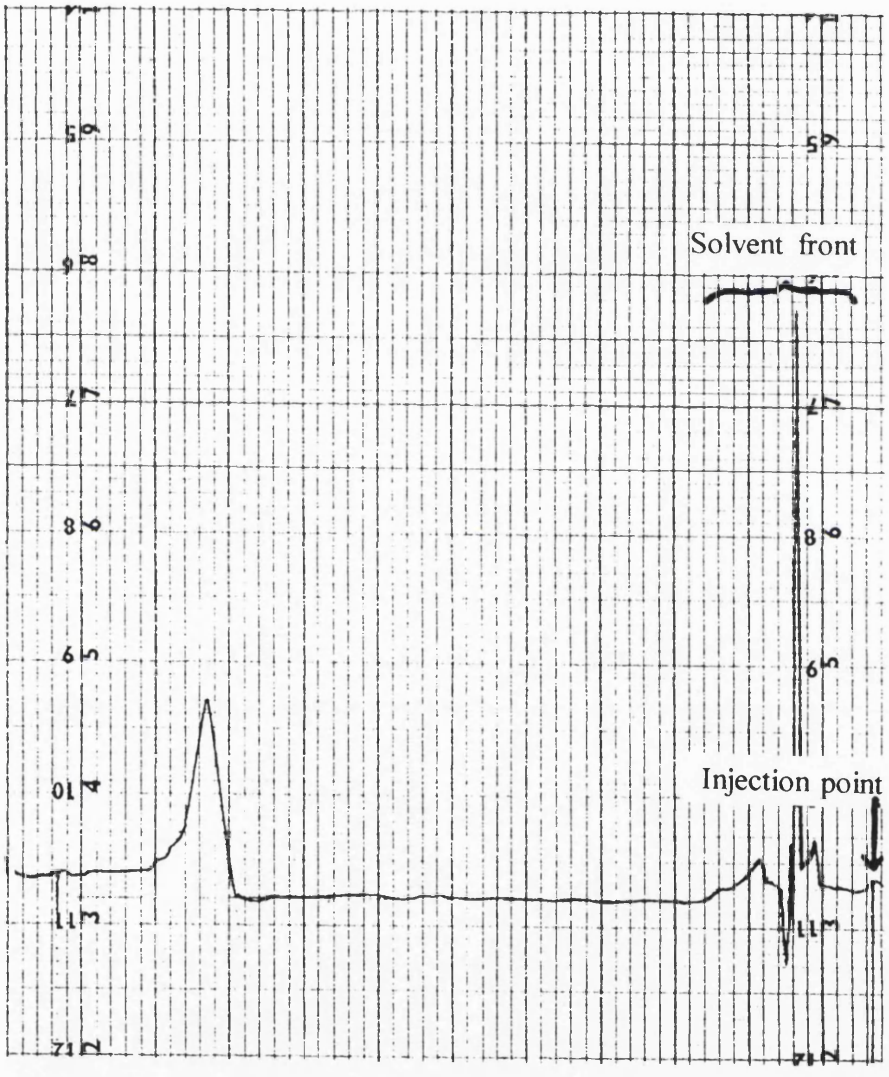


Table 2.3. Peak areas obtained for validation of BDP assay in the presence of DPPC.

Solution	BDP alone (11.009 μg/ml)	+ 99 mole % DPPC
Mean peak area 1 (cm^2)	1.144 ± 0.088	1.141 ± 0.035
Mean peak area 2 (cm^2)	0.973 ± 0.106	0.974 ± 0.068
Mean retention time (minutes)	8.663 ± 0.111	8.625 ± 0.050

2.5.3.3. Conclusions

The original Glaxo Wellcome assay, whilst capable of producing consistent and reproducible results, was adapted by using methanol as the dissolving solvent, and a mixture of methanol and deionised water (in a 7:3 v\v ratio) as the mobile phase, permitting the elimination of acetonitrile and glacial acetic acid from the assay. Validation of this system in the presence of 99 mole % DPPC found that this did not affect the determination of BDP concentration within that sample, or the retention of BDP. Therefore this assay may be used to determine the amount of BDP entrapped in liposomes composed of DPPC.

2.5.4. Determination of the maximum amount of BDP entrapped in DPPC liposomes

2.5.4.1. Introduction

Previous work in this chapter has identified the conditions required to efficiently remove BDP crystals from liposomal suspensions. In addition, an assay to accurately determine the amount of BDP entrapped within liposomes has been calibrated and validated in the presence of DPPC. The aims of this study was to incorporate BDP into DPPC liposomes, at a concentration exceeding that of crystal formation, and to subsequently remove these crystals and determine accurately the maximum amount of BDP that can be entrapped by DPPC liposomes.

2.5.4.2. Methods

Liposomes containing 50 mg/ml DPPC and approximately 5 mole % BDP were prepared by the method described in Section 2.3.2.2. This suspension was checked microscopically for the presence of crystals, which were sedimented by centrifugation in deuterated water, according to the procedure outlined in Section 2.5.2.1. The top layer of concentrated liposomes was then removed and deposited onto a tared glass slide. This was microscopically checked for the absence of crystals and placed in an oven at 50°C. The weight of the sample was monitored until no further weight loss was observed, indicating that all the water in the sample had been removed. The dried sample was weighed, dissolved in and made up to 10 ml with methanol and then assayed by HPLC to determine the concentration of BDP that had been entrapped by the DPPC liposomes.

2.5.4.3. Results and Discussion

DPPC liposomes having approximately 5 mole % BDP added, were found to contain drug crystals, and these together with other unentrapped material (such as non-liposomal phospholipid) were removed by centrifugation. The concentrated liposomal sample was then dried in an oven at 50°C overnight, to yield a powder. This dry sample (17.463 mg) was dissolved in and made up to 10 ml methanol, and subsequently assayed by HPLC to determine the concentration of BDP entrapped. The average peak area (calculated using peak area 1) obtained was 2.841 cm^2 (standard deviation =0.429). From Equation 2.2(a), this corresponds to a BDP concentration of 31.853 μg/ml. Therefore the solution contained 0.319 mg BDP in 10 ml, corresponding to 5.92×10^{-7} moles of BDP. By subtraction from the weight of the original powder, the weight of DPPC present was calculated as 17.144 mg, which is equivalent to 2.33×10^{-5} moles of DPPC. This means that the resulting molar ratio of DPPC:BDP of entrapped liposomes was 39.6:1. This value suggests that the maximum amount of BDP entrapped into DPPC liposomes is approximately 2.5 mole % BDP.

2.5.4.4. Conclusions

The maximum amount of BDP that was completely entrapped into DPPC liposomes was found to be 2.5 mole %. This value is close to the one obtained from microscopy studies (Section 2.3.4.), which suggested that above a concentration of 1.5-2 mole %, excess BDP forms crystals.

2.6. General Discussion

The results obtained from these investigations can be used to conclude that firstly, BDP forms a solvate with chloroform during the manufacture of liposomes. However, whether this solvate remains stable on hydration of the film, was not determined since studies on the state of the steroid molecule in the presence of water could not be undertaken.

Secondly, BDP forms crystals when present in excess in liposomal suspensions. This has also been reported by other authors (Radhakrishnan, 1991, Waldrep et al, 1994). These crystals were found to form upon the addition of water, rather than forming in dry phospholipid films prior to hydration. As mentioned in Section 2.3.1. hydrophobic drugs in aqueous environments cause structuring of water and promote self-association into crystals (Arrowsmith et al, 1983a). Therefore this result was not unexpected. However, the presence of solvated BDP may account for the fact that BDP forms crystals easily when hydrated. For example, other authors have reported the use of solvents such as tertiary butanol (Waldrep et al, 1994), and chloroform (Radhakrishnan, 1991) in the preparation of liposomes. Glaxo Wellcome in-house data has indicated that both these solvents produce a solvate with BDP, and both authors reported the presence of BDP crystals in liposomal suspensions, suggesting a relationship between low entrapment values and solvate behaviour. If future work required the optimisation of BDP entrapment into liposomes, then a possible step by which this may be accomplished, might lie in the use of solvents that do not form solvates with BDP.

The third conclusion that can be drawn from these studies is that the concentration at which these crystals form in DPPC liposomal suspensions, lies

between 1.5 and 2 mole % BDP, as determined by microscopic methods. This indicates that the incorporation of less than 1.5 mole % BDP into liposomes, would be expected to result in 100 % entrapment efficiency. This was confirmed by the results of Waldrep et al (1994), who found that the addition of 0.05 mole % BDP resulted in 100 % entrapment efficiency. As has been reported (Taylor et al, 1990a), BDP is entrapped into liposomes to a certain extent, but in excess of this limit, BDP separates as a discrete crystalline phase. This suggests that the steroid molecule may interact with phospholipid chains. However, the ease by which BDP crystals increasingly appear in liposomal samples, only days after preparation, suggest that BDP entrapment probably involves a steric component, as suggested by Radhakrishnan (1991).

It was recognised that unentrapped crystalline material requires removal from liposomal suspensions for a variety of reasons (outlined in Section 2.1.). Centrifuging samples in deuterated water was identified as an efficient technique to accomplish this, since BDP crystals formed a pellet, whilst liposomes (containing drug) floated on top. A fourth conclusion can be drawn that liposomes are less dense than deuterated water, whilst BDP crystals are more dense. Therefore the centrifugation of samples in ordinary water, would have sedimented both liposomes and crystals, resulting in inefficient separation.

The HPLC assay was adapted from the original assay supplied by Glaxo Wellcome, and proved to be a useful technique for the determination of BDP, as others have reported (Devoiselle et al, 1992; Waldrep et al, 1994). This assay was found to be unaffected by the presence of DPPC, and dehydrated samples tested for entrapment values of BDP in DPPC liposomes yielded values (2.5 mole %) similar to those determined earlier by microscopy. The small discrepancy between the entrapment values obtained by microscopy methods and drug assays, reflects differences in the two methods of determining the entrapped BDP concentration. Radhakrishnan (1991) has reported that phospholipid liposomes generally only allow 1-3 mole % BDP encapsulation. Therefore the entrapment values of BDP obtained from these studies, are encompassed in this range. The main aim of these studies has therefore been fulfilled by the work undertaken in this chapter. Namely, two methods by which the concentration of liposomally entrapped BDP may be accurately determined, have been successfully developed and found to yield similar entrapment values.

3. INCORPORATION OF BDP INTO PHOSPHOLIPID MONOLAYERS

3.1. Introduction

Phenomena typical of 3-dimensional systems are paralleled in monolayers, since both can exist in different states of matter, and undergo well-defined phase transitions. Whilst the 3-dimensional and 2-dimensional states of monolayers and bilayers are not identical, the transitions they undergo are achieved by similar changes of conditions, such as temperature. When comparing both states, a high monolayer surface pressure such as 30 mNm^{-1}, is most indicative of bilayer behaviour (Blume, 1979), since the absolute molecular area and the change in molecular area accompanying the main phase transition in gel phase bilayers is similar to that of their equivalent monolayers. In other words, the behaviour of the high pressure region in monolayers resembles that of bilayers, and this is where phase separation is most likely to occur. The advantage that monolayers hold over other model membrane systems is that molecules in the monolayer may be manipulated and the areas they occupy at the interface may also be altered in a controlled way. However, each model has its own advantages and disadvantages, and the information obtained can be complementary. For example, monolayers can act as a model for the interactions affecting the external part of a membrane, whilst liposomes can act as a model for bilayer disturbance (Reig et al, 1992).

Monolayers provide a convenient model system for studying the interactions of ordered molecules with drugs. A number of studies have been conducted into the effects of drugs on phospholipid monolayers, including studies using hydrocortisone (Cleary and Zatz, 1973), insulin (Birdi, 1976), antihistamines (Attwood and Udeala, 1975), and skin penetration enhancers (Lewis and Hadgraft, 1990). Whilst the above workers interpreted their results in terms of the penetration of the monolayer by the drug molecules, in practice it is difficult to determine the exact extent of penetration since the factors determining the mechanism and extent of incorporation are not yet fully understood. In addition the interpretation of phase behaviour is difficult even in simple binary mixtures, because of the co-existence of separate domains by monolayer components, a phenomenon that will be described later in Sections 3.4.5. and 3.4.6.

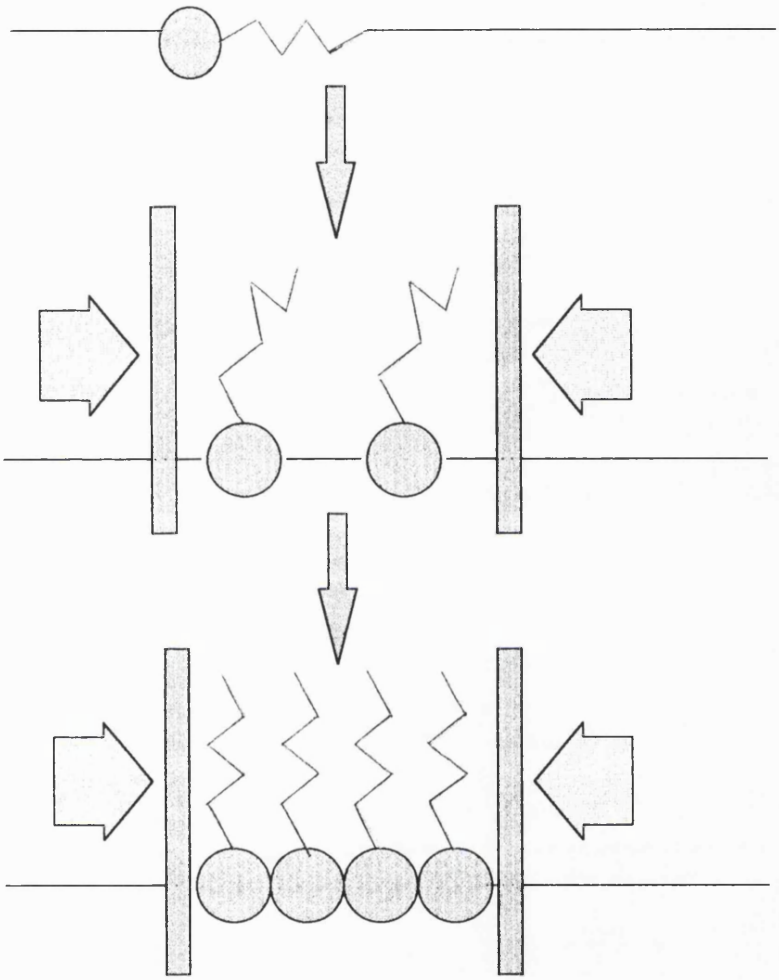
Alkanoic acids and their salts, and long chained alcohols are some of the materials which have been extensively studied as monolayers (Langmuir Users Manual, 1992). With the exception of hydrocortisone (Cleary and Zatz, 1973), little work has been carried out on the interaction of corticosteroids with phospholipid films. Therefore there is considerable potential for extending the use of this technique to characterise the interactions between BDP and phospholipid monolayers.

3.1.1. The nature of Langmuir films

When amphiphilic molecules become trapped at the interface between two dissimilar phases, either liquid-liquid or liquid-gas, a Langmuir film is produced. The molecules become trapped because amphiphiles possess two different types of bonding within one molecular structure. The forces acting on the hydrophilic group are predominantly coulombic, whilst the forces acting on the hydrophobic region are predominantly van der Waals.

When surfactants are dissolved in a non-polar, volatile solvent and this solution is spread onto a polar liquid, the solvent evaporates to leave the surfactant molecules at the liquid-gas interface. The orientation of the molecules is such that hydrophilic head groups "pull" the molecule into the bulk of the polar liquid, whilst the hydrophobic tail groups point into the air and away from the surface of the polar liquid. If a barrier is swept over the surface of this liquid, then the surface area that the deposited molecules occupy is reduced. Inevitably the molecules come closer together and eventually an ordered, compressed monolayer is formed. This monolayer is known as a Langmuir film, and its formation is schematically represented in Figure 3.1. A monolayer will only form if the balance between the hydrophilic and the hydrophobic moieties is correct. So, for example, if the length of the hydrophobic tail is relatively short in comparison to the size of the head group, there is a possibility that the molecule will be dragged into the polar liquid. Alternatively if there is no hydrophilic polar head group present, then the molecules will form a thick multilayer or simply evaporate.

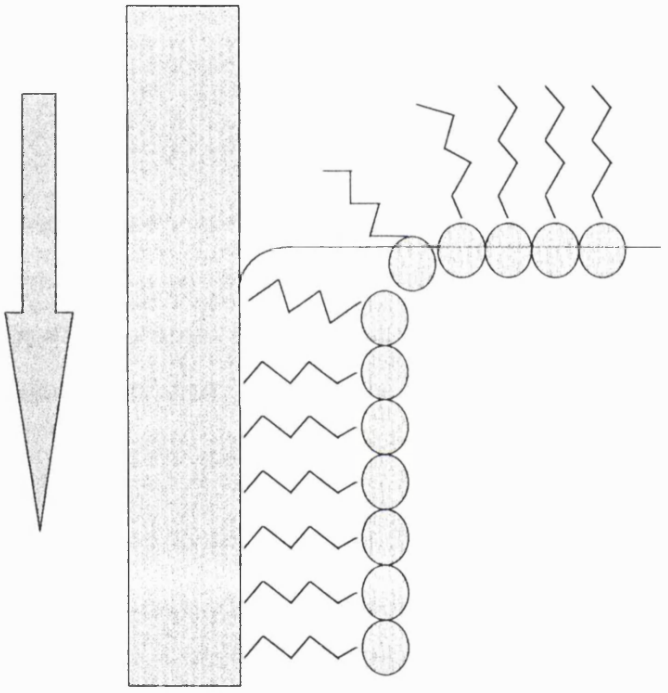
Figure 3.1. Schematic representation of the formation of a Langmuir film.



Langmuir films differ from Langmuir-Blodgett films, which are constructed by the transfer of monolayers (Langmuir films) from an aqueous subphase surface onto a solid substrate, by dipping the substrate through the monolayer. This technique has been used to prepare films of fatty acids on glass (Blodgett, 1934). The substrate used may be a glass microscope slide, the surface of which may be rendered hydrophobic or hydrophilic, depending on the type of Langmuir Blodgett film required. For example, for a substrate with a hydrophilic surface, there is no ordered deposition until the first up-stroke because hydrophobic tails are repelled by the hydrophilic substrate on immersion. But for substrates which have a hydrophobic surface, monolayers are deposited on the first down-stroke (Figure 3.2.). One layer of molecules is picked up

for every pass, in or out, and therefore multilayers can be deposited. The thickness of this film is the product of the individual molecule's chain length and the number of times the substrate has crossed the interface between the polar liquid and air. The substrates can be examined, microscopically or otherwise, to study monolayer (and multilayer) phenomena that cannot be studied on a liquid surface.

Figure 3.2. Ordered deposition of a monolayer onto the surface of a hydrophobic substrate.



3.1.2. Surface tension and pressure

Molecules in a solution are subject to attractive forces, which are equal in the bulk of the liquid. However at surfaces, or interfaces, the forces are not equal and the net effect is that the peripheral molecules are pulled into the bulk of the solution. This effect gives rise to surface tension, which can be defined as the force required to increase the surface area isothermally by a unit amount. The units of surface tension are mNm^{-1}. Amphiphilic molecules such as surfactants tend to collect at the interface

in order to remove their hydrophobic group(s) from an aqueous environment, and hence achieve a minimum free energy state. This results in the hydrophilic groups of these amphiphiles replacing water molecules at the surface. The intermolecular attraction of water molecules at the surface is effectively reduced by the presence of the intruding surfactant molecules, and therefore amphiphiles reduce the surface tension of water. The surface tension of a pure, clean water surface is 72.8 mNm^{-1}, and may be depicted by γ'. When a monolayer is present on the surface, the surface tension is decreased to a value depicted by γ. The difference in these values is the surface pressure, π. The relationship between surface tension and pressure may be expressed by the Equation 3.1.

$$\pi = \gamma' - \gamma \qquad \text{Equation 3.1.}$$

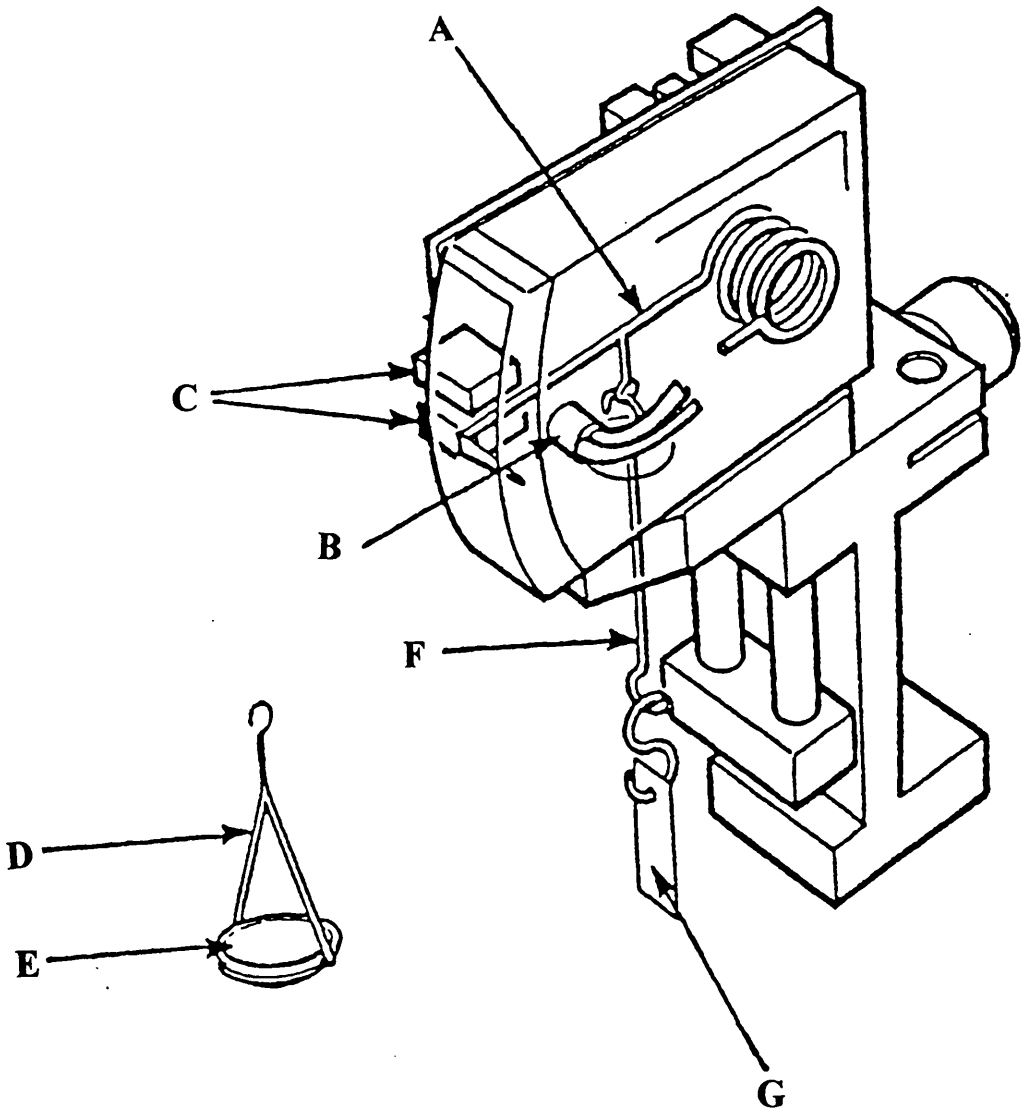
Surface pressure represents the force required to contain the monolayer within a certain area. Therefore it is possible to monitor the surface pressure of a monolayer as a function of the area occupied per molecule within the monolayer (provided that the number of molecules deposited at the surface is known). This useful measurement can indicate whether penetration of the monolayer by other molecules has taken place (Attwood and Udeala, 1975).

The basis for pressure measurement in the Langmuir trough is the Wilhelmy plate, as recommended by International Standard ISO 304. The Wilhelmy plate is a strip of chromatography paper (Whatman Grade 1) with a width of 10 mm, which when suspended at an air-water interface, is pulled down into the bulk of the subphase by the surface tension of the water. The forces which act on the plate are gravity, surface tension (acting downwards into the subphase) and buoyancy (due to displaced water acting upwards). These forces are measured by means of an electronic micro-balance.

Figure 3.3. depicts the pressure sensor of a Langmuir trough which works by directly measuring the force required to suspend a Wilhelmy plate at the liquid-gas interface. It has a magnetic coil, the moving arm of which (A) is illuminated by an infra-red diode (B). The shadow cast by the arm falls on two infra-red detectors (C), and the control electronics hold the arm in place, so that the shadow falling on each

detector is always the same. Hence the arm remains at a constant position under different loads, and the force required to hold the arm steady is read off by measuring the current through the coil. For a 10 mm width Wilhelmy plate, an accuracy of 0.1 mNm^{-1} is achievable. To calibrate the sensor, a weighing pan (D) and pan holder (E) were attached to a moving arm via a hook (F) and a known balance weight was added to the pan. A 100 mg calibration weight is equivalent to 49.1 mNm^{-1} for a 10 mm width plate, taking g=9.81 ms^{-2}. To make surface pressure measurements, the Wilhelmy plate (G) was then attached in place of the weighing pan. The apparatus was interfaced with a PC equipped with software, allowing the processing and storage of results.

Figure 3.3. Schematic diagram of the surface pressure sensor of the Langmuir trough.



3.1.3. Pressure-area isotherms

Langmuir troughs, or "thin film balances", are used for the compression of molecules in order to produce isotherms. As described in Section 3.1.1., when a solution of amphiphilic molecules in a volatile solvent is deposited onto an aqueous subphase surface, the solvent evaporates to leave the molecules oriented at the surface. On compression of these molecules, plots of surface pressure versus area occupied per molecule are constructed simultaneously. Molecular areas may be calculated in terms of $Å^2$ or nm^2, depending on the available computer software. These plots are known as "pressure-area isotherms" because compression takes place at constant temperature. For example, a typical isotherm of stearic acid usually consists of three distinct regions (Figure 3.4.), namely the gaseous, liquid-expanded (liquid) and liquid-condensed (solid) phases.

After initial deposition onto the subphase, when no external pressure is applied, the molecules in the monolayer behave as a two-dimensional gas, which can be described by Equation 3.2.

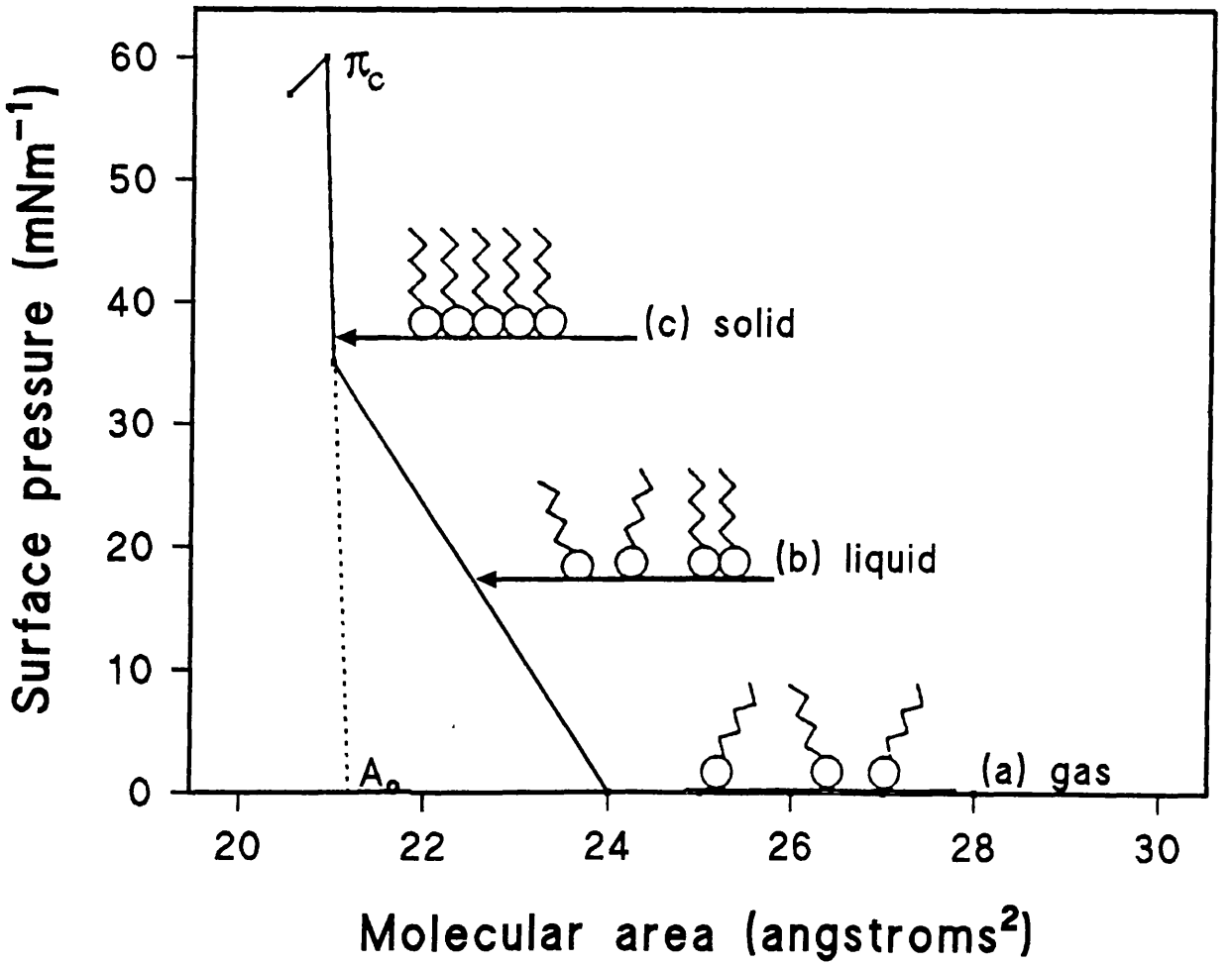
$$\pi A = kT \qquad \text{Equation 3.2.}$$

where π is the surface pressure, A is the molecular area, k is the Boltzmann constant and T is the thermodynamic temperature. This gaseous state is schematically represented in Figure 3.4(a).

On further compression, some ordering of the film takes place and molecules behave as a two-dimensional liquid, seen in Figure 3.4(b). This phase is commonly referred to as the "liquid-expanded" phase. There is now a degree of interaction between the hydrocarbon chains of different molecules. The flexible chains adopt conformations that try to minimise their interaction with air.

With continued closing of the barriers, the increase in pressure causes additional ordering, with the monolayer behaving as a quasi-solid. This solid or "liquid-condensed" state is characterised by a steep and usually linear relationship between surface pressure and molecular area, as seen in Figure 3.4(c). Eventually the

Figure 3.4. An idealised stearic acid pressure–area isotherm showing molecular orientations during compression.



forces exerted upon the film become too strong for its confinement in two dimensions, and the film irretrievably loses its monomolecular form and collapses. The collapse pressure, π_c (Figure 3.4.), is the maximum to which a monolayer can be compressed without the detectable expulsion of molecules from the Langmuir film. At the collapse pressure molecules are ejected out of the monolayer plane into either the subphase or the superphase (the region above the plane of the monolayer), resulting in the formation of bilayers or multilayers. However, collapse is not uniform across the monolayer, but is usually initiated near the leading edge of the barrier, or at discontinuities in the trough such as corners, or the Wilhelmy plate. Usually a collapsed film will consist of large areas of uncollapsed film interspersed with "mountain ridges" where monolayers have been crushed together to form bulky aggregates. The pressure to which a monolayer can be compressed before it collapses depends upon the details of the experimental procedure used, such as the rate at which the film is compressed, the temperature of the subphase, the chemical substances used and so on. The collapse behaviour of multicomponent systems has been used as a guide to the miscibility of components in monolayer and bulk states (Lewis and Hadgraft, 1990).

It is generally assumed that when two amphiphilic components are mixed, the minor one is dispersed evenly within the major one. However when one component is hydrophobic in nature, then this species may be banished to the alkyl chain region, as is the case with the incorporation of hydrophobic drugs into liposomal systems. There may be an actual interaction between steroids and hydrocarbon chains of phospholipid molecules in monolayers, or they may be immiscible and exist as separate domains (Yamauchi et al, 1993). If two immiscible components have well-defined and different collapse pressures (determined on separate monolayers), the resultant monolayer should start to collapse at the lower value. Once all the domains of the first material have collapsed, the surface pressure increases until the higher collapse pressure is reached. If the components are miscible then only a single collapse would be observed (Yamauchi et al, 1993). Handa and Nakagaki (1979) found a 2-stage collapse in isotherms of mixed monolayers of dimyristoylphosphatidylserine and cholesterol acetate. From surface pressure values, they concluded that the two components are miscible in the monolayer when applied as premixed solutions, but immiscible in the

bulk phase as demonstrated by a constant value of a higher collapse pressure with varying composition.

In addition, some inferences can be drawn from the onset of collapse. For example, Lewis and Hadgraft (1990) studied mixed monolayers of oleic acid and DPPC and found that as the mole fraction of oleic acid within the monolayer was increased, so the surface pressure characterising the onset of collapse was decreased, compared to DPPC alone. This relationship suggested a degree of miscibility of the two components in the monolayer. Furthermore, the collapse pressure of the mixed monolayer remained below that of the pure DPPC film, indicating that there was no phase separation of DPPC and enhancer occurring

Quantitative information can be obtained from pressure-area isotherms on the molecular dimensions and shape of the molecule under study. Molecules in the liquid-condensed phase are relatively well oriented (hydrocarbon chains are parallel to each other and lie perpendicular to the subphase surface) and closely-packed, and the zero-pressure molecular area (A_o) can be obtained by extrapolating the slope of the solid phase to zero pressure (Figure 3.4.). For a fully saturated alkanoic acid such as stearic acid, the molecular area determined in such a manner is 22-25 $Å^2$, corresponding to the cross-sectional area of a hydrocarbon chain.

The shape of the isotherm is also characteristic of the molecules in the film, and may indicate whether penetration of the film by drug molecules has occurred. For example, Attwood and Udeala (1975) demonstrated that monolayers spread onto an aqueous antihistamine subphase solution showed an increase in surface pressure compared to monolayers formed on a pure water subphase. This implies that antihistamine molecules have penetrated the monolayer. The increase in surface pressure indicated an approximate area per molecule for the drug molecule in the film, which was confirmed by calculation of the molecular area using the Gibb's adsorption equation.

3.1.4. Langmuir studies of phospholipid films

The hydrophobic-hydrophilic balance of lipids (Demel et al, 1967; Phillips and Chapman, 1968; Villalonga, 1968) is such that stable films are readily formed. Phospholipid monolayers are suitable experimental models of biomembranes, and the production of DPPC monolayers are particularly well documented (Cadenhead and Kellner, 1974; Perez-Gil et al, 1992; Nag and Keough, 1993). DPPC is well suited to film balance studies since it shows no tendency to dissolve in the subphase under compression, and undergoes a number of well-defined phase transitions during compression. A typical isotherm for DPPC is shown in Figure 3.5.

The plateau region observed for DPPC is interpreted as a transition between the liquid-expanded and the liquid-condensed phase (Weis, 1991). This transition at approximately 10 mNm^{-1} is characteristic of DPPC (and can be used as an indication of it's purity), and corresponds to a change in the conformation of the alkyl chains from a situation where there is an abundance of gauche conformers, to a situation where alkyl chains adopt a predominantly trans conformation (Figure 3.6.).

This phase transition is therefore analogous to the thermotropic gel-liquid-crystalline phase transition of DPPC liposomes (Blume, 1979), and both crystalline and liquid-crystalline domains co-exist at the transition (Weis, 1991). The phase transition begins when the hydrocarbon chains start to move away from the interface. The chains are flexible and adopt conformations that try to reduce their interaction with air. The glycerol backbone of DPPC lies perpendicular to the plane of the bilayer, so that the distance between the negative and positive charges of the zwitterionic head-group is kept to a minimum. As DPPC monolayers undergo further compression they form a so-called solid (or liquid-condensed) phase, in which alkyl chains become tightly packed and the monolayer is highly resistant to further compression. Solid phase monolayers collapse at very high surface pressures, when portions of the monolayer are no longer able to remain adsorbed at the air-water interface, and slide over one another to form a multi-layered structure. This type of monolayer collapse is usually accompanied by a sharp decrease in surface pressure (Langmuir Users Manual, 1992), but in practice the surface pressure converges to a constant value.

Figure 3.5 Pressure–area isotherm of DPPC.

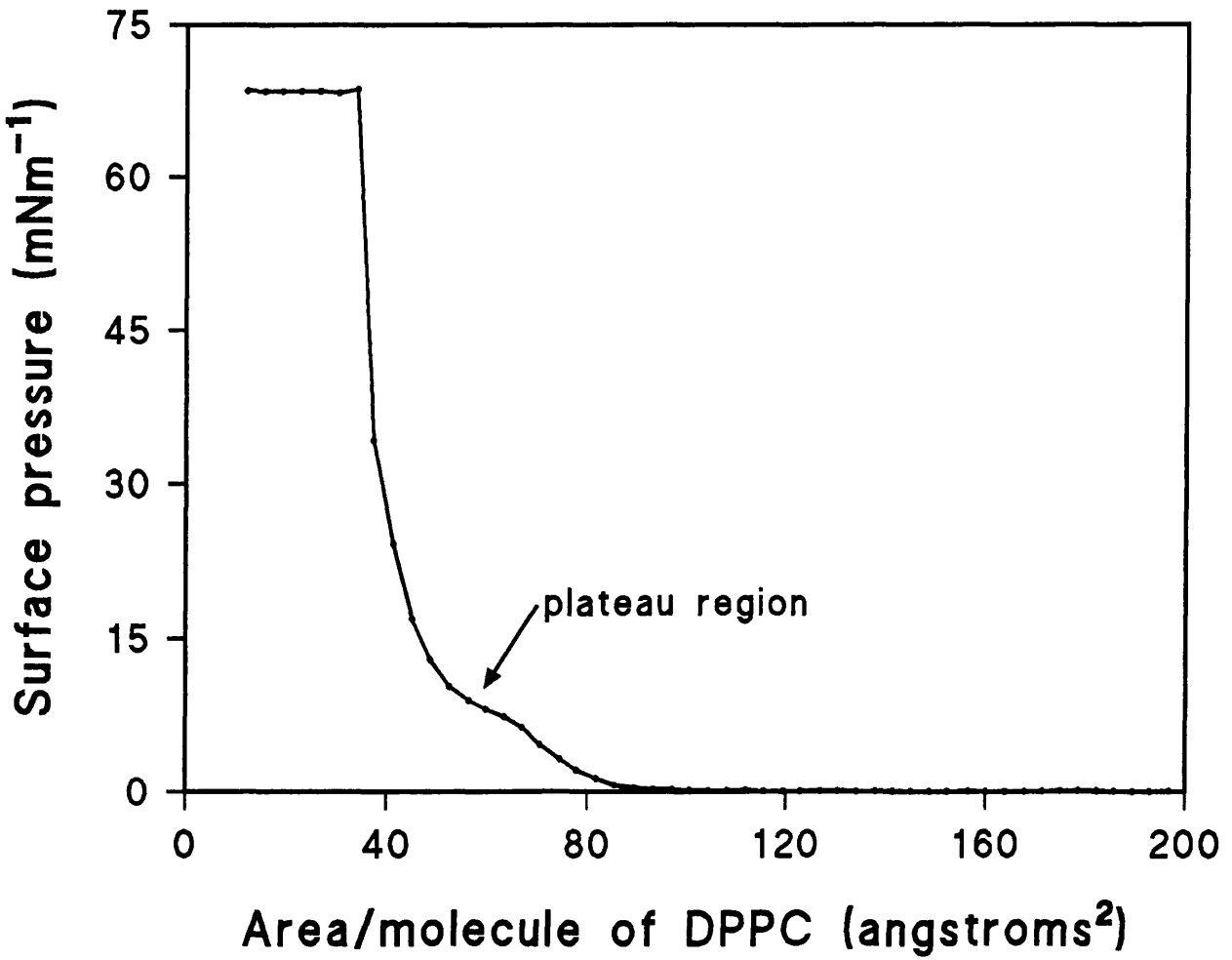
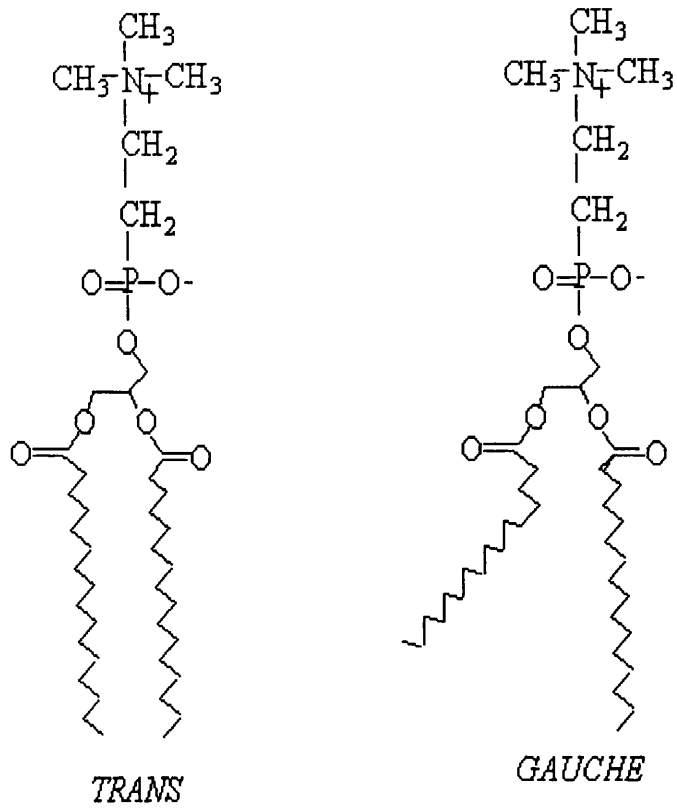


Figure 3.6. Rotational isomerism in alkyl chains of DPPC.



Monolayers of DPPC have also been studied using fluorescence microscopy (Weis, 1991; Nag and Keough, 1993), whereby monolayers have been formed containing small concentrations of a fluorescent dye. During compression DPPC molecules start to crystallise and form solid domains (Ahlers et al, 1990). Generally fluorescent dyes are only weakly soluble in the solid phase, and so are excluded from the domains which then appear dark (Möhwald, 1986). During the transition, DPPC exists in the solid and fluid states simultaneously (Weis, 1991). If drugs or other materials are added to the DPPC monolayer containing fluorescent dyes, domain shapes, sizes and numbers would be expected to be affected (Möhwald, 1986; Ahlers et al, 1990).

To conclude, any increase in the surface pressure of phospholipid films containing BDP (when compared to films of pure phospholipids) will indicate the presence of the drug in the plane of the monolayer. In describing the interactions of compounds with phospholipids, emphasis will be placed on the effects on the transition

from the liquid-expanded to the liquid-condensed phase, which indicates the ability of BDP to promote structural changes in the phospholipid alkyl chains.

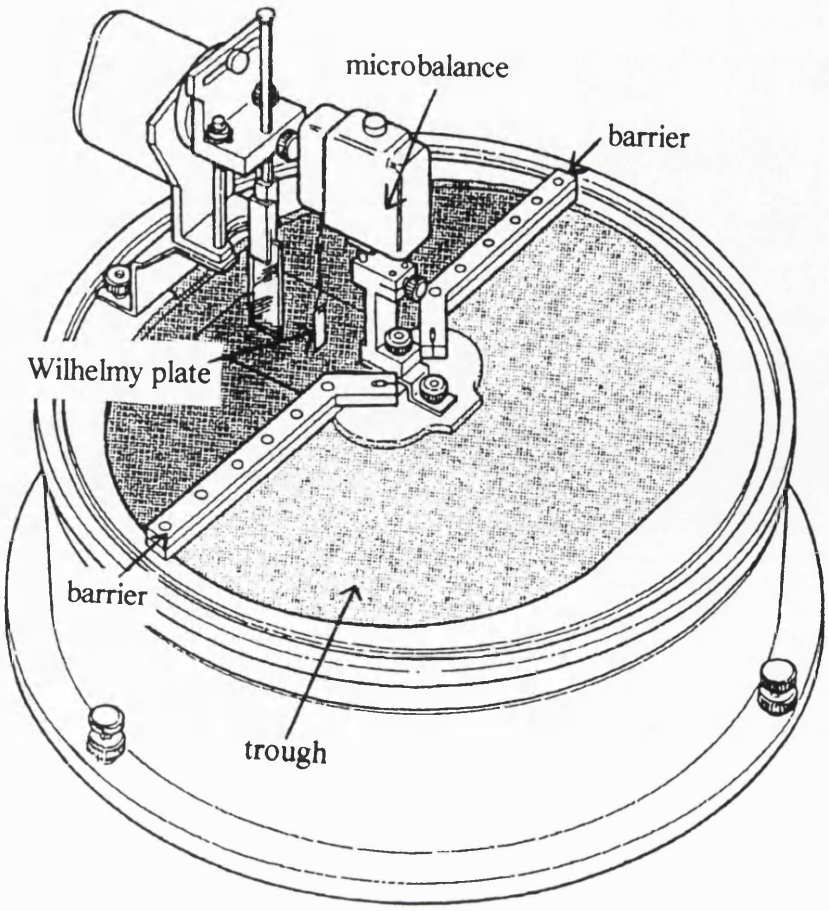
3.2. Materials and Methodology

3.2.1. Apparatus

Langmuir troughs, or "thin film balances", are used for the compression of amphiphilic molecules on a liquid surface, to measure their pressure-area isotherms. The term "balance" originates from the Langmuir-Adam trough, which used a floating barrier to separate the monolayer from a clean, reference sample (Grunfield, 1993). The force exerted by the film on the barrier was measured by means of a torsion balance.

The Nima trough, Model 2011 (Nima Technology, Coventry) used in this work (Figure 3.7.), is made from a round sheet of PTFE with two rigid, removable PTFE barriers.

Figure 3.7. The Nima trough, Model 2011.



The latest Langmuir Blodgett troughs are designed so that the pressure sensor (to measure surface pressure) and dipper mechanism (to construct Langmuir Blodgett films) are easily interchangeable, making the trough suitable for either application. These troughs are constructed from bulk polytetrafluoroethylene (PTFE), a material which can be subjected to rigorous cleaning procedures and will minimise the contamination of the subphase by mineral ions and surfactants. Also, this polymer, the most hydrophobic known, will not leach plasticiser and is essentially chemically inert. Other troughs have been constructed from stainless steel and aluminium (both of which can oxidise in the humid environment around the trough) and nylon (which is not capable of withstanding solvents such as chloroform).

A trough specifically for the fluorescence microscopy of monolayers on the subphase surface was first designed by Losche and Möhwald (1984). The objective lens of the microscope was actually incorporated into the subphase, which improved the optical collection efficiency and the reliability of data capture. The fluorescence trough (Zeiss, Germany) used in these investigations was similar to the Nima one described above, but of a smaller size, with a ring in the centre made from PTFE. The trough was set on the stage of a fluorescence microscope, and the microscope was mounted directly above the ring in such a way that the formation of the monolayer within this PTFE ring, was observed without any physical disturbance. The microscope was fitted to a video monitor (Hamamatsu) and a video graphic printer (Sony, UP-850).

3.2.2. Materials

The trough used contained approximately 1 litre of water. Ideally the water used should be as pure as possible so that the surface properties of monolayers remain unaffected. For consistent results, deionised water (WP 700, Whatman) was further purified using an Elgastat UHQ-PS (Ultra High Quality Water Purification System), which produced water of a quality of approximately 18 m Ω-cm resistivity. The temperature of the water used was 20°C.

The phospholipids used in the production of pure phospholipid monolayers and mixed monolayers containing BDP, were DPPC (approximately 99 % pure), dilauryl-

phosphatidylcholine (DLPC) (approximately 99 % pure), distearoylphosphatidylcholine (DSPC) (approximately 99 % pure), dipalmitoylphosphatidylglycerol (DPPG) (approximately 98 % pure) and dipalmitoylphosphatidic acid (DPPA) (approximately 98 % pure), the structures of which are depicted in Section 1.1.5. All phospholipids were obtained from Sigma Chemicals (Poole, Dorset), and stored below 0°C. Chloroform, acetone and 96 % ethanol were AnalaR grade, and obtained from BDH (Poole, Dorset). Beclomethasone dipropionate was obtained from Glaxo Wellcome (Ware, Herts.) as a micronised powder of the monohydrate.

Fluorescence studies were carried out at the University of Mainz, and the fluorescent dye used, Texas Red (MW=1382), was kindly supplied by those laboratories.

3.3. Methodology

Before and after each experiment the trough was thoroughly cleaned, and any leftover monolayer material was removed from the subphase surface before the trough was emptied. The trough surface was cleaned using a Kimwipe (Kimberly-Clarke), a surfactant-free wipe, soaked with chloroform (since other solvents may be harmful to the PTFE surface). Excess solvent was wiped off as pools of chloroform may evaporate to leave contaminants behind. The barriers were also removed and wiped with a chloroform-laden Kimwipe tissue. Other trough components, such as the hooks of the sensor apparatus, were wiped with a Kimwipe soaked in 96 % ethanol, in order to remove loose dust.

Usually the subphase employed for the study of monomolecular films is ultra-pure water, but other high surface tension liquids have been used as subphases including ethylene glycol, glycerol and mercury (Langmuir Users Manual, 1992). The trough was filled with approximately 1 litre of clean water, obtained fresh from an Elgastat UHQ-PS unit, until the trough was just over-filled. The temperature of this water was approximately 20°C. The prime criteria for establishing the cleanliness of the subphase is a high value of surface tension. The surface tension of pure, clean water at 293 K is 72.8 mNm^{-1} (Langmuir Users Manual, 1992). This value will decrease with an increase in temperature. A minimal change in surface tension should

be seen on ageing and on compression of the surface. The surface tension of the water subphase was measured by lifting the Wilhelmy plate out of the water after having zeroed the pressure sensor. If a reading of approximately 70 mNm^{-1} was not observed, the pressure sensor was recalibrated. Then an isotherm was produced by compressing the surface. The plots were almost flat but slight "tails", observed at high compression, indicated the presence of dirt on the water surface. To clean the surface, an aspirator pump with a pipette attached was used to suck off any floating material such as dust and amphiphilic contaminants. For a "clean" surface, the change in surface pressure on compression should be less than 0.5 mNm^{-1} (Langmuir Users Manual, 1992). Once the surface was prepared for deposition, a perspex lid was used to protect the water surface from air-borne particles, and this transparent lid was kept on the trough whenever it was filled with water.

The appropriate amount of each material under investigation was weighed (AD-4 Autobalance, Perkin Elmer, Beaconsfield) and made up to volume with solvent in a volumetric flask. Each solution was sonicated for a few minutes in an ultrasonic bath (Decon Ultrasonics Ltd., Sussex). When solutions were not in use, the stopper of each solution flask was sealed using laboratory film (Parafilm "M", Greenwich, USA) and kept in the refrigerator, in order to minimise any evaporation. A 50 µl microsyringe (Hamilton, Nevada, USA) was cleaned by sucking up and expelling a little of the solvent, several times. It was then rinsed out with the solution under investigation and 50µl was drawn up. The solution was deposited drop by drop onto the water subphase, from a few mm above the surface. Each drop was allowed to spread fully before the next was deposited. The solvent was left to evaporate from the subphase surface. The barrier speed was adjusted to 100 cm^2/minute, since this speed is a compromise between a good isotherm (that is one that is not distorted by dynamic effects) and the time available. A monolayer was formed by compression of the surface, and a pressure-area isotherm was simultaneously produced. After isotherms were stored for processing, the surface was cleaned using the suction pump ready for deposition of the same material. All runs were repeated a minimum of three times, and where possible all isotherms have been plotted. However, when isotherms of different concentrations are being compared, only one isotherm, representative of each concentration has been plotted. When monolayers of different materials were

produced, the subphase was replaced with fresh water and a new Wilhelmy plate was attached. The cleanliness of the surface was checked in the usual way prior to the next experiment.

3.3.1. Solvent effects on monolayer formation

Most materials are deposited onto the subphase in a solution of a volatile solvent. However before choosing a suitable solvent a number of factors must be considered since the solvent may affect the film-forming properties of the solute. The solvent must be able to dissolve a substantial quantity of the material under investigation, and must be chemically inert and relatively pure. The cleanliness of the solvent can be verified by spreading it on the subphase, allowing it to evaporate, then running an isotherm of the remaining surface material. Any increase in surface pressure will be due to surface active contaminants present in the solvent. This technique is known as "blank spreading". Before each set of experiments was performed, an isotherm was obtained of the solvent used, to ensure that all recorded isotherms were true representations of the pressure-area relationships, and not related to any contaminants that may have been present in the solvent. The volatility of the solvent must be such that the evaporation time is fairly short. It is important that all the solvent evaporates, but it has been reported that if the evaporation time is too long, the deposited films are streaky (Langmuir Users Manual, 1992). The boiling point of the solvent should lie between 40 and 80°C. For example, solvents used in this study were chloroform and acetone, with boiling points of 61°C and 56°C respectively. Organic solvents which are very soluble in water should be avoided as they will tend to carry amphiphilic material into the subphase and precipitate it out. To counteract this effect, a high concentration of material must be dissolved in the water miscible solvent. Solvate formation may affect the incorporation of beclomethasone dipropionate into monolayers, as solvates have different physical properties and solubilities (James, 1986).

With the above factors in mind, experiments were performed to determine the effects of solvents on monolayer formation, namely those of solvent purity and evaporation. A microsyringe was filled with 50 μl of chloroform, which was blank

spread onto a water subphase surface. The solvent was allowed to evaporate before the surface was compressed. The resulting shape of the isotherm was used to check for the presence of contaminants. The length of the evaporation time was only 30 seconds, since it was the purity of the solvent that was under investigation.

The effect of solvent evaporation on monolayer formation was investigated by preparing monolayers of DPPC, chosen because they are relatively easy to prepare and also have a characteristic shape. A solution of 1 mg/ml of DPPC in chloroform was prepared in a volumetric flask, and 50 µl of this solution was deposited onto the surface of a water subphase, by means of a microsyringe. The solvent was allowed to evaporate for a period of 30 seconds before the start of compression. Similarly, other solutions were deposited and allowed to evaporate for 1 minute and 4 minutes.

3.3.2. Preparation of beclomethasone dipropionate films

In order to compare isotherms of mixed monolayers of phospholipid and BDP, it was first necessary to construct films of pure BDP on a water subphase. It was not known whether this compound would produce a monolayer, or a stable film of any sort, or which solvent would be suitable for spreading BDP. Therefore, solutions of BDP in acetone, 96% ethanol and chloroform were prepared, each containing 1 mg/ml BDP. 50 µl of each solution was spread onto a water subphase and isotherms were constructed. For reasons outlined in Section 3.4.2. chloroform was chosen as the dissolving solvent, and subsequently solutions of BDP in chloroform were prepared, with final concentrations of 0.012 mg/ml, 0.108 mg/ml and 1.017 mg/ml. Different volumes of each solution were spread in order to produce isotherms.

3.3.3. Preparation of dipalmitoylphosphatidylcholine monolayers

In order to compare the isotherms of mixed monolayers, solutions of DPPC were deposited and compressed into monolayers and their isotherms plotted. Solutions in chloroform were prepared each containing 1.032 mg/ml and 0.089 mg/ml DPPC. Different volumes of each solution were spread and the resulting isotherms compared.

3.3.4. Preparation of DPPC monolayers containing BDP

The object of this experiment was to study the incorporation of BDP molecules into monolayers of DPPC. The two methods by which this was achieved were firstly by the injection of a solution containing BDP under a DPPC monolayer, and secondly by the compression of premixed solution of BDP and DPPC. The incorporation techniques were compared, and the BDP content of mixed films was altered in order to examine the effects of concentration on monolayer penetration.

For the first method, DPPC monolayers were constructed by compressing 50μl of a solution containing 1 mg/ml of DPPC in chloroform. BDP solutions were prepared to produce final concentrations of 0.125, 0.25. 0.5, 1 and 4 mg/ml BDP in chloroform. 50 μl of each BDP solution was injected underneath an expanded DPPC monolayer using a microsyringe, and the monolayer was subsequently recompressed to produce isotherms.

For the second method, DPPC and BDP were dissolved together in chloroform to produce three solutions containing concentrations used in the previous experiment. These solutions contained 1 mg/ml DPPC and 0.25, 1 or 4 mg/ml BDP, corresponding to approximately 25, 58 and 85 mole % BDP, respectively. 50 μl of each solution was spread and compressed to form isotherms. In addition, DPPC and BDP were dissolved together in chloroform to produce solutions containing 1 mg/ml DPPC and 1.5, 10 and 57 mole % BDP. Fifty μl of each solution was spread and compressed to produce isotherms.

3.3.5. Fluorescence studies of DPPC monolayers containing BDP

Fluorescence microscopy can be used to visually observe the liquid-expanded and liquid-condensed phases exhibited by DPPC monolayers. However this requires the monolayers to contain a small amount of fluorescent probe which partitions preferentially into the different phases. Therefore pure DPPC monolayers and those containing BDP have been studied using Texas Red as a fluorescent dye. Interactions in monolayers have been studied using this technique (Weis, 1991), which is particularly useful if the interaction affects the phase transition. In this study, the

experimental arrangements that have been used to observe monolayer fluorescence, consist of a miniature trough set on the stage of a fluorescence microscope. For visual observation of the formation and growth of domains of DPPC, the monolayers were compressed in steps. At each step video images of the appearance of the monolayer were recorded. However it was not possible to record simultaneously the pressure-area curves along with the monolayer images, to compare events.

Stock solutions of DPPC (1 mg/ml), DPPC and BDP (containing approximately 2.5 and 10 mole % BDP), BDP (1 mg/ml) and Texas Red (0.5 mg/ml) were prepared in chloroform. Although the fluorescent probe is considered to be an "impurity", and can therefore theoretically influence transition properties, any effects were minimised using a low concentration of the probe (Ahlers et al, 1990). Therefore, to construct monolayers of pure DPPC, 1 µl of Texas Red stock solution was added to 1 ml of DPPC stock solution, so that the molar concentration of dye was 0.026 %. Twenty-five µl of the resulting solution was spread, and photographs of domain formation obtained. To construct "films" of BDP, 1 µl Texas Red stock was added to 1 ml BDP stock, and 25 µl of the resulting solution was spread. To construct mixed monolayers, 1 µl Texas Red stock was added to 1 ml of each solution (2.5 and 10 mole %), and 25 µl of the resulting solutions were spread.

3.3.6. Langmuir studies of other phospholipid monolayers

The aim of this study was to investigate the action of BDP on individual monolayers of DLPC, DSPC, DPPA and DPPG. Stock solutions in chloroform containing 1 mg/ml of each phospholipid were prepared. Appropriate amounts of BDP were added to each stock solution to prepare further solutions containing 2.5, 10 and 55 mole % BDP. Fifty µl of each solution was deposited and compressed to produce isotherms.

3.4. Results and Discussion

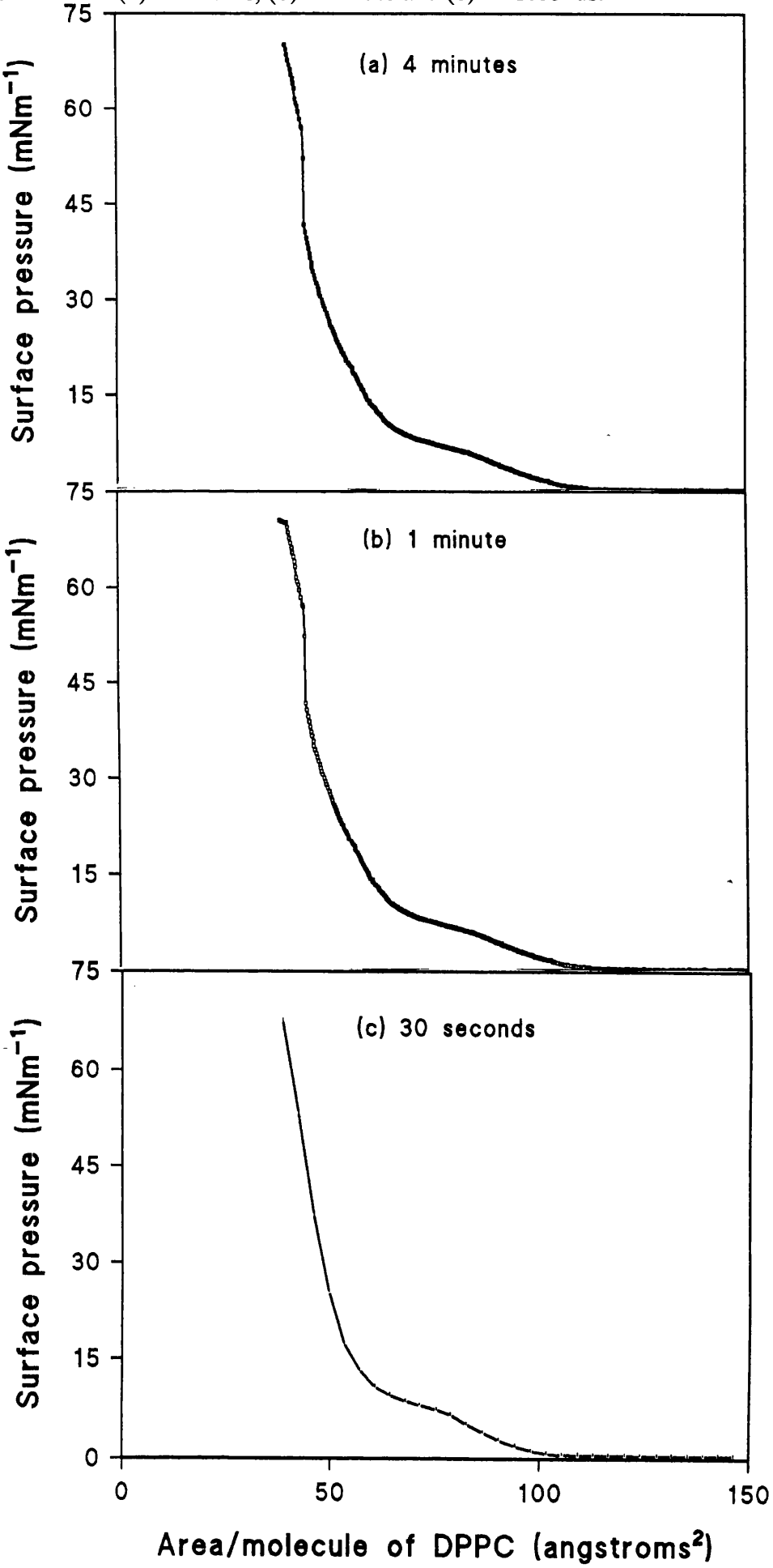
3.4.1. Solvent effects on monolayer formation

The isotherm obtained on blank spreading of chloroform was a flat line with no increase in surface pressure, even at high compression. This confirmed that all the solvent had evaporated from the subphase surface after only 30 seconds. It also confirmed the cleanliness of the solvent, since contaminants within the solvent would have been left behind on the surface once the solvent had evaporated, and this would have caused an increase in surface pressure. Blank spreading was performed before each set of experiments.

Figures 3.8(a), (b) and (c) show the effect of leaving a solution of DPPC in chloroform to evaporate for different lengths of time (4 minutes, 1 minute and 30 seconds, respectively) prior to compression. From the initial blank spread, chloroform was found to evaporate from the subphase surface after only 30 seconds, but the presence of chloroform on the film-forming characteristics of DPPC were unknown. The molecular area of a DPPC molecule was found to be approximately 50 $Å^2$ for all solutions left to evaporate for different lengths of time. Street (1993) found the molecular area of DPPC to be approximately 70 $Å^2$, whilst Lewis and Hadgraft (1990) found this value to be nearer to 60 $Å^2$. However, the observed results were consistent and therefore indicate that for these evaporation times, chloroform had not affected the formation of the condensed phase of DPPC monolayers.

Cadenhead and Kellner (1974) found that within 5 minutes of spreading a solution of DPPC in chloroform, surface pressure values approached zero, indicating evaporation was complete. However, leaving the solvent to evaporate for such a long time can lead to problems such as a slow accumulation of contaminants on the subphase surface, or even material evaporation. It has also been reported that leaving solutions to evaporate for a long time may result in the production of streaky films (Langmuir Users Manual, 1992) which may be significant when producing mixed monolayers. Because of time constraints a long evaporation time was deemed inappropriate, and therefore an evaporation time of 1 minute was chosen as a compromise.

Figure 3.8. Pressure-area isotherms of DPPC in chloroform (50 µl, 1 mg/ml) after evaporation times of (a) 4 minutes, (b) 1 minute and (c) 30 seconds.



3.4.2. BDP films

Figures 3.9(a), (b) and (c) show the shapes of the isotherms obtained when BDP was dissolved in acetone, 96 % ethanol and chloroform, respectively. Solution concentrations were constant (1 mg/ml), and hence the number of molecules on the surface was the same. However, the shapes of the three isotherms were different, indicating that the behaviour of BDP molecules is not consistent with a true film-forming material, which would be expected to show the same shape of isotherm upon deposition in different solvents. The differences may be attributable to the effect of solvent retained within the film, suggesting solvate formation by the BDP molecules, as described in Section 2.2. Methanol has been shown to not form a BDP solvate (Glaxo Wellcome in-house data). However, the ability of ethanol and methanol to lower the surface tension of a water subphase might result in a false expansion of the film being observed, which may be significant when studying the effect of BDP on phospholipid monolayers. Therefore these solvents were avoided and chloroform was selected as the solvent of choice because it did not lower the surface tension of water once evaporated. In addition, chloroform is widely available, has desirable solubility profiles for BDP and DPPC, and has been used successfully in previous monolayer studies. The phenomenon of BDP solvate formation by chloroform was taken into account, but since all liposome studies utilised this solvent during manufacture, solutions for monolayer studies were also prepared using chloroform, so that comparisons of the two systems may be carried out.

Figure 3.10. shows isotherms of different volumes and different concentrations of BDP solutions in chloroform. Film formation by deposition of the highest concentration of BDP (approximately 1 mg/ml) produced an isotherm characteristic of "overspreading". In other words, the concentration or the volume of solution deposited was too high, and therefore the surface pressure at the beginning of compression was not 0 mNm^{-1}. The large number of molecules deposited tended to spread and form a surface film without the need for any compression. The Wilhelmy plate inevitably registered an initial increase in surface pressure produced by the presence of this "monolayer".

For materials such as phospholipids, which are ideal film-formers, monolayer collapse is usually characterised by a sharp decrease in surface pressure. This is

Figure 3.9. Pressure-area isotherms of BDP (1 mg/ml) in (a) acetone, (b) 96 % ethanol and (c) chloroform.

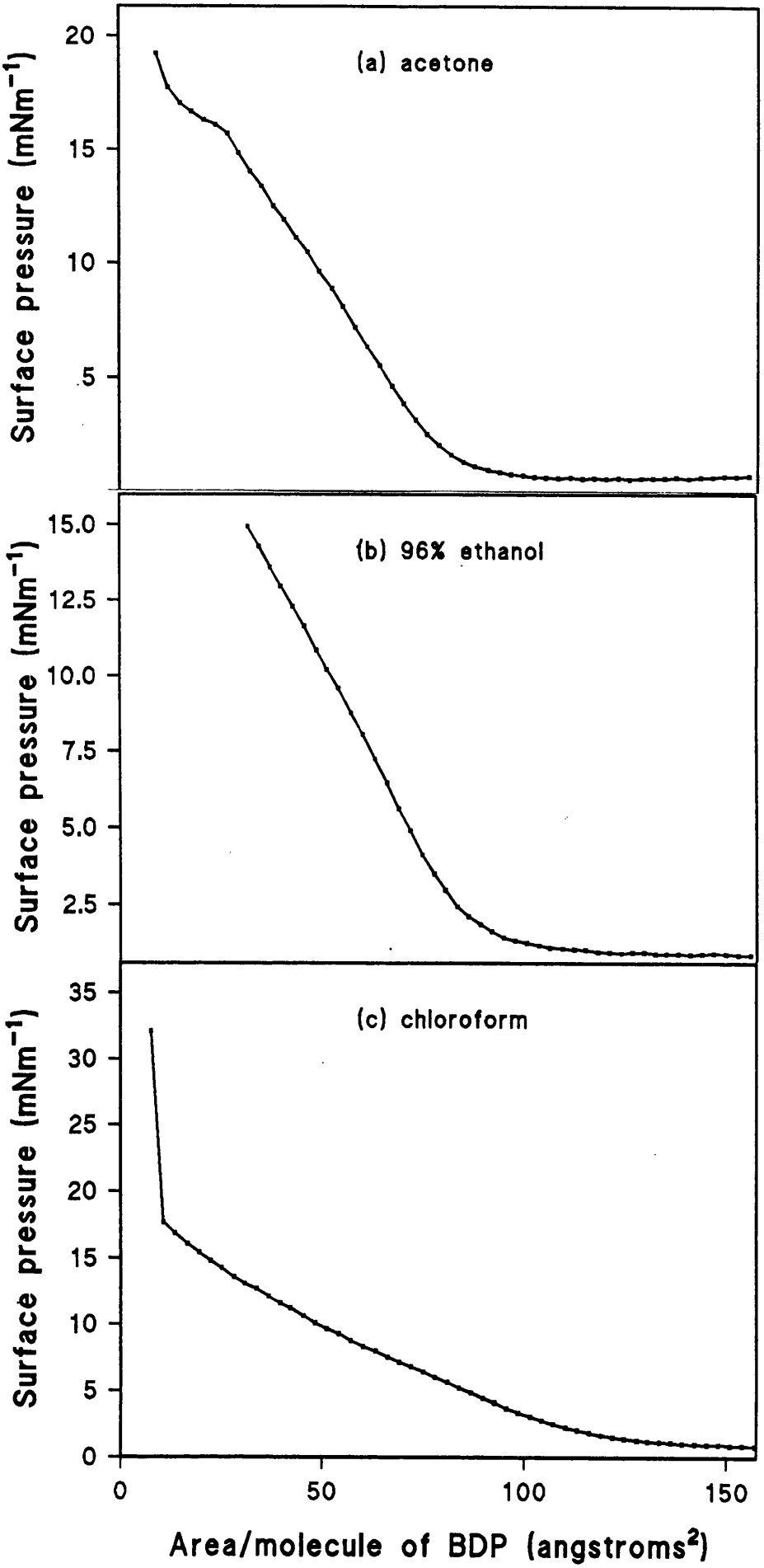
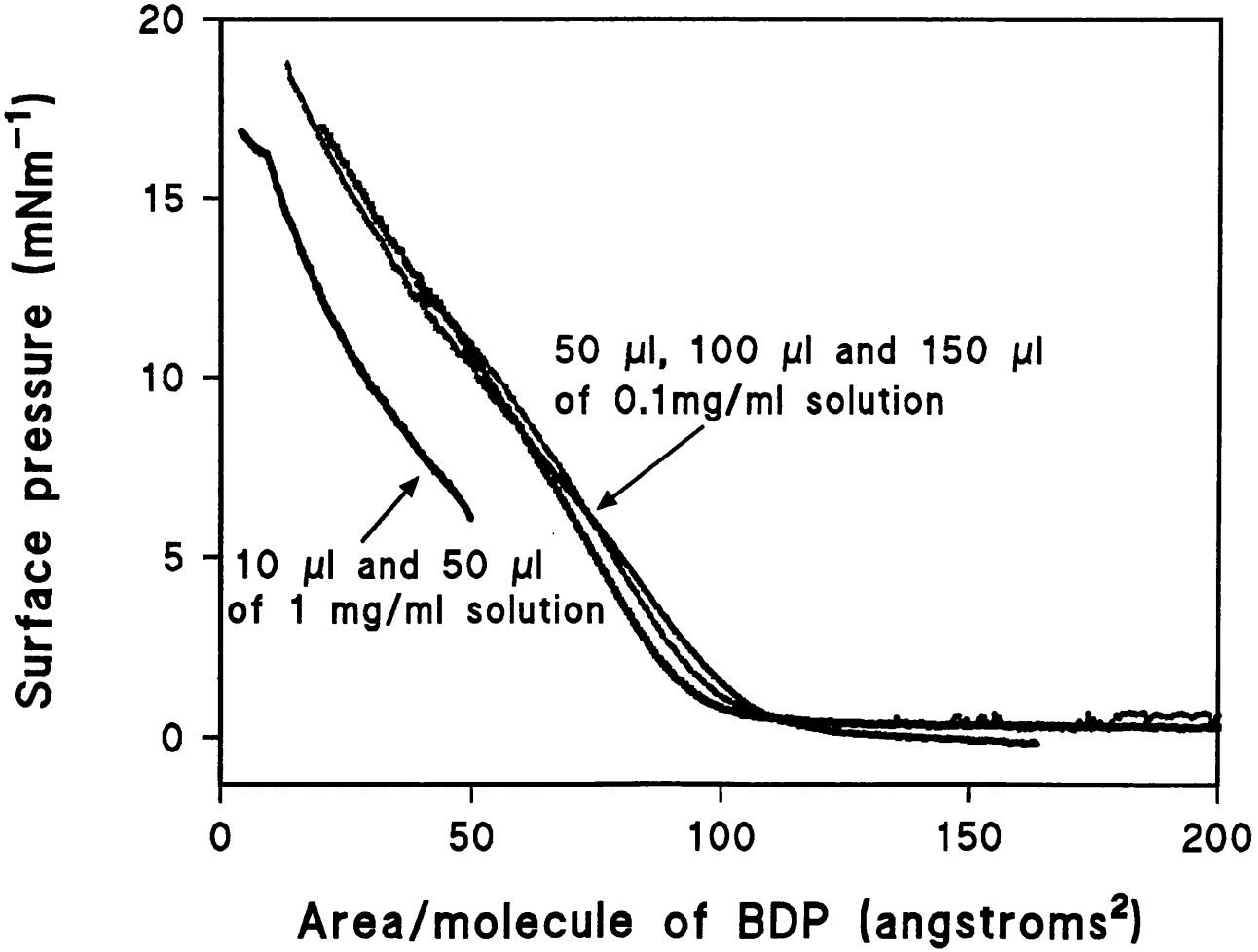


Figure 3.10 Pressure–area isotherms of different volumes and concentrations of BDP.



because further compression of the closely packed, rigid monolayer causes a sudden ejection of molecules into either the subphase or the superphase. However for BDP, collapse of the film probably occurs near the start of compression, but cannot be characterised by a sharp decrease in surface pressure due to the nature of the film produced. The nature of the BDP molecule does not make it an ideal film-forming material, being a "typical" hydrophobic material. It is thought that compression may lead to randomly oriented molecules of BDP "sliding" over one another, effectively forming aggregates and multilayers on compression, and disguising "collapse". The fact that the spreading of the higher concentration solutions actually resulted in smaller areas/molecule being observed confirms the theory that BDP molecules shear under pressure, and the formation of aggregates actually reduces the measured molecular area.

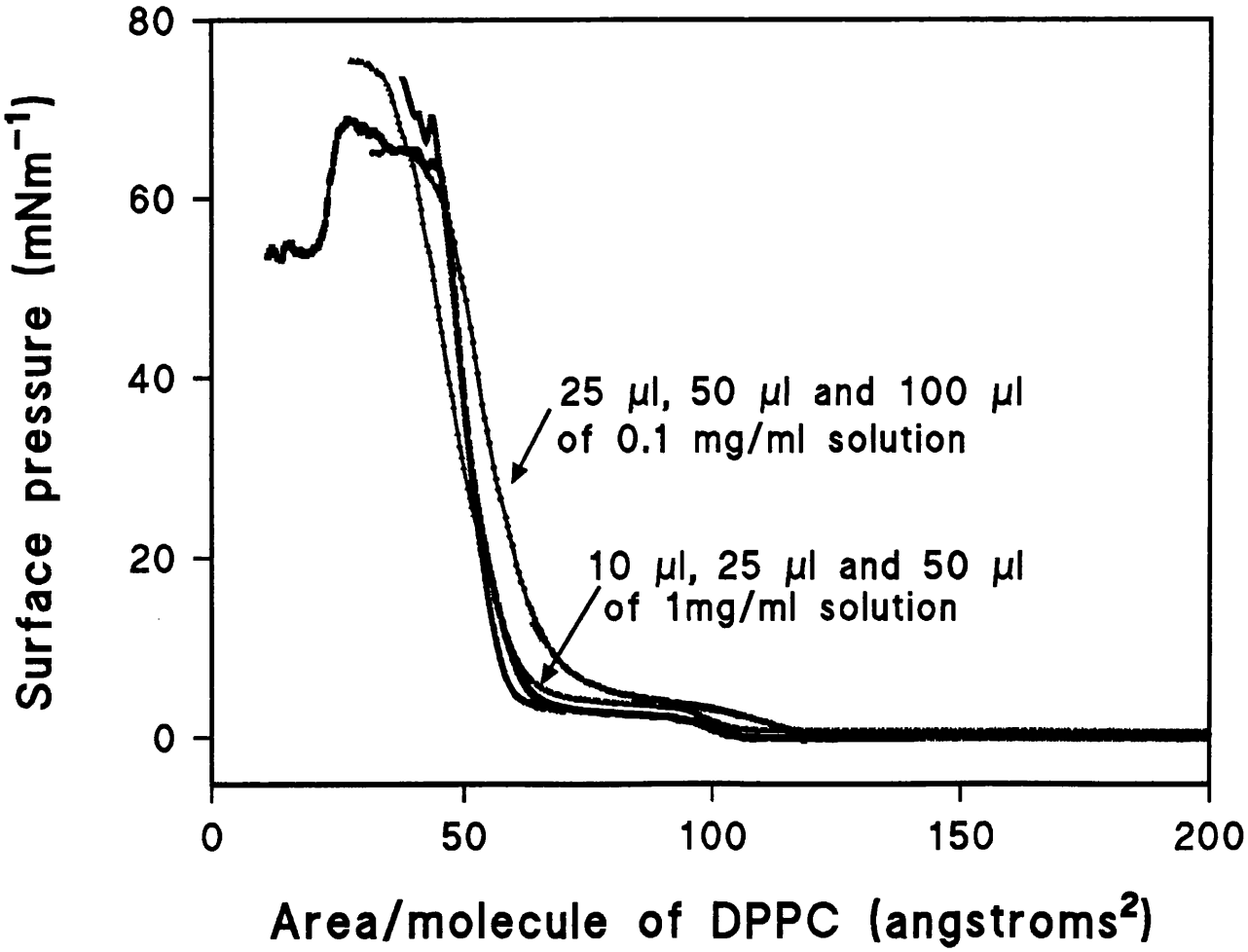
Isotherms of the compression of more dilute concentrations (approximately 0.1 mg/ml) do not show any collapse at all, indicating that BDP alone does not form a coherent monolayer. Therefore the miscibility of BDP and phospholipids within monolayers may not be measured by their collapse behaviour because BDP is incapable of forming a coherent monolayer and as such does not show a well-defined collapse pressure.

3.4.3. DPPC monolayers

Cadenhead and Kellner (1974) found that spreading DPPC in four different solvents (including chloroform) produced small variations in their resulting isotherms, and all showed a well-defined phase transition.

Figure 3.11. shows the isotherms obtained by compressing different volumes containing different concentrations of DPPC in chloroform. The highest concentration (approximately 1 mg/ml) produced a more pronounced plateau than the lower concentration (approximately 0.1 mg/ml). Compression of different volumes of the former solution were all reproducible, whilst compression of the more dilute solution resulted in larger areas/molecule being observed in the condensed phase. This may indicate that the sparsely distributed DPPC molecules on the subphase surface are subject to a degree of hydration and thus an increase in area/molecule (Bois and Albon,

Figure 3.11 Pressure–area isotherms of different volumes and concentrations of DPPC.



1985). The lack of overlap between the two isotherms also suggests that there are concentration-dependent factors involved.

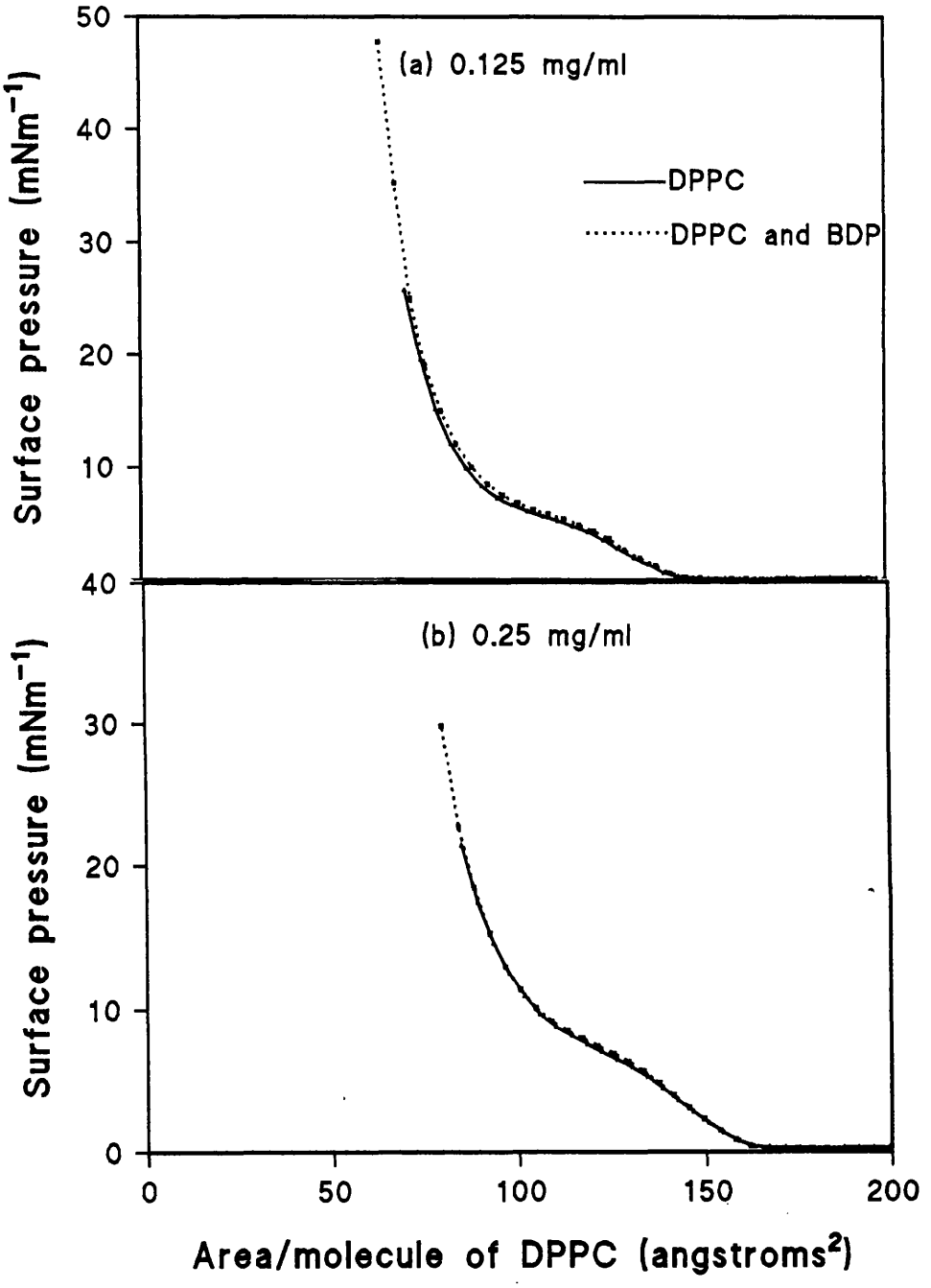
3.4.4. DPPC monolayers containing BDP

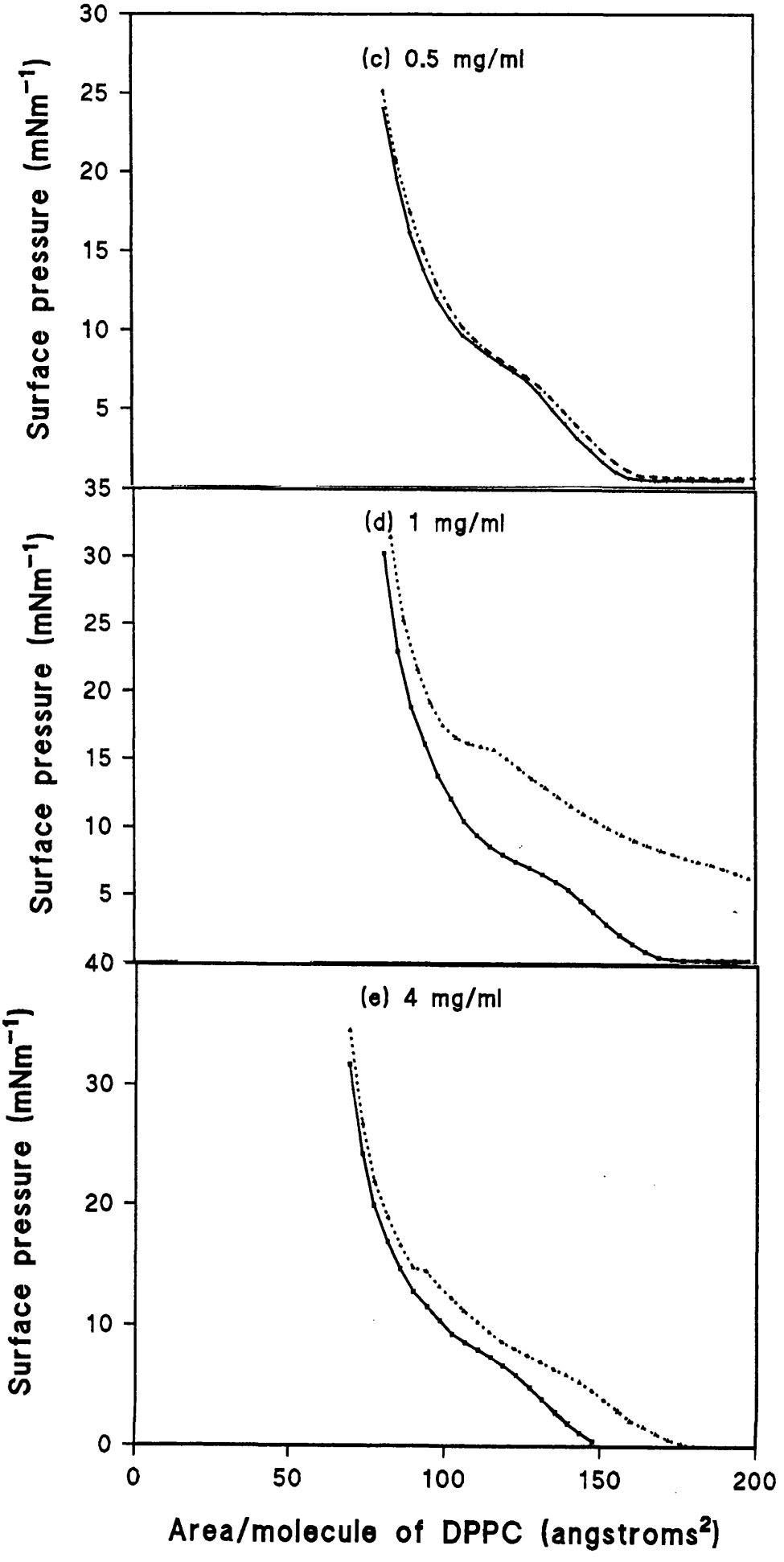
While plots are shown in terms of the area occupied by the phospholipid molecules, it must be noted that this area actually represents the sum of the area occupied by the phospholipid molecule and any surrounding BDP molecules. An increase in surface pressures relative to pure monolayers indicates the presence of BDP in the plane of the monolayer. However, when comparing molecular areas of mixed monolayers to those of pure phospholipid monolayers, simple additivity of molecular areas may be consistent with either ideal mixing or complete immiscibility. The presence of the characteristic DPPC phase transition indicates that the phospholipid molecules exist in a domain, and are capable of undergoing a co-operative transition.

A minimum of four reproducible isotherms were produced for each solution, and Figures 3.12(a), (b), (c), (d) and (e) are representative isotherms obtained when 50 µl of each BDP solution was injected under a DPPC monolayer. The injection of the lowest concentrations of BDP (0.125 and 0.25 mg/ml) produced no measurable increase in the surface pressure of DPPC monolayers, indicating that little or no penetration has occurred (Figures 3.12(a) and (b)). On increasing the concentration of BDP (0.5 and 1 mg/ml) the surface pressure increases at regions corresponding to those above the transition (expanded monolayers) (Figures 3.12(c) and (d)). At the highest concentration used (4 mg/ml) (Figure 3.12(e)) the increase in surface pressure was less than that found for the 1 mg/ml BDP injection. With all the concentrations however, condensed monolayers show similar surface pressures, and all films exhibit the characteristic phase transition of DPPC molecules. The monolayers containing 1 mg/ml BDP (Figure 3.12(d)) also showed a reproducible deflection at approximately 15 mNm^{-1}, the cause of which was unknown.

The effects of injecting BDP under DPPC monolayers were more pronounced when all the monolayers were in the expanded state. The higher surface pressure values observed (1 mg/ml) suggest the presence of BDP molecules in the plane of the monolayer. At the highest concentration used (4 mg/ml), the smaller effect exerted on

Figure 3.12. Pressure-area isotherms of DPPC (1 mg/ml) and injected solutions of BDP at concentrations of (a) 0.125 mg/ml, (b) 0.25 mg/ml, (c) 0.5 mg/ml, (d) 1 mg/ml and (e) 4 mg/ml.




(c) 0.5 mg/ml
(d) 1 mg/ml
(e) 4 mg/ml
Surface pressure (mNm^{-1})
Surface pressure (mNm^{-1})
Surface pressure (mNm^{-1})
30
25
20
15
10
5
35
30
25
20
15
10
5
40
30
20
10
0
0
50
100
150
200
Area/molecule of DPPC (angstroms^2)

expanded monolayers may be due to excess BDP forming secondary regions above or below the monolayer. The occurrence of domain formation by BDP molecules in phospholipid bilayers was suggested by Taylor et al (1990), and the incidence of this phenomenon in monolayers can be confirmed by the presence of the characteristic DPPC phase transition, observed with all the isotherms. The phospholipid transition is a co-operative event, and can therefore be abolished by the presence of BDP molecules between those of DPPC. However, since the transition is apparent at all concentrations, an assumption can be made that penetration of the monolayer by BDP molecules is such that the presence of BDP has not altered the behaviour of the phospholipid on compression. This indicates that BDP molecules have formed a separate discrete phase. In addition the deflection seen at 15 mNm^{-1} is characteristic of a BDP "film", since this is also seen in isotherms of 1 mg/ml BDP (Figure 3.10.), and this confirms the presence of a BDP domain.

The consistency of the condensed state surface pressures indicate that at these high surface pressures, the molecular areas occupied by each monolayer are approximately the same, regardless of the concentration of BDP injected underneath. This suggests that whilst drug molecules are present in the expanded state, as suggested by increases seen in surface pressures at low compression, they are expelled out of closely packed monolayers. This has been described for other drugs as a "squeeze out" effect (Cleary and Zatz, 1973; Doisy et al, 1995). Gershfield and Muramatsu (1971) confirmed that some steroid molecules do form mixed films with insoluble monolayers, but are completely ejected out of the condensed films.

The isotherms in Figures 3.13(a), (b) and (c) were obtained with the premixed solutions of BDP and DPPC, and show that at these concentrations (approximately 25, 58 and 85 mole % BDP), no phase transitions were observed, suggesting that BDP molecules are located between those of DPPC, sufficient to prevent any co-operative events such as a phase transition. Isotherms of the solutions containing lower concentrations (1.5 and 10 mole % BDP) in Figure 3.14. show that the plateau region representing the phase transition is still apparent. For the lowest concentration (1.5 mole % BDP) this phase transition is slightly displaced to higher molecular areas indicating the presence of BDP in the plane of the DPPC monolayer (Figure 3.14.). But for the solution of higher concentration (10 mole % BDP), the effects of this amount of BDP becomes apparent, as regions of the isotherm resemble that of pure

Figure 3.13. Pressure-area isotherms of DPPC (1 mg/ml) and mixed solutions of BDP at concentrations of (a) 0.25 mg/ml, (b) 1 mg/ml and (c) 4 mg/ml.

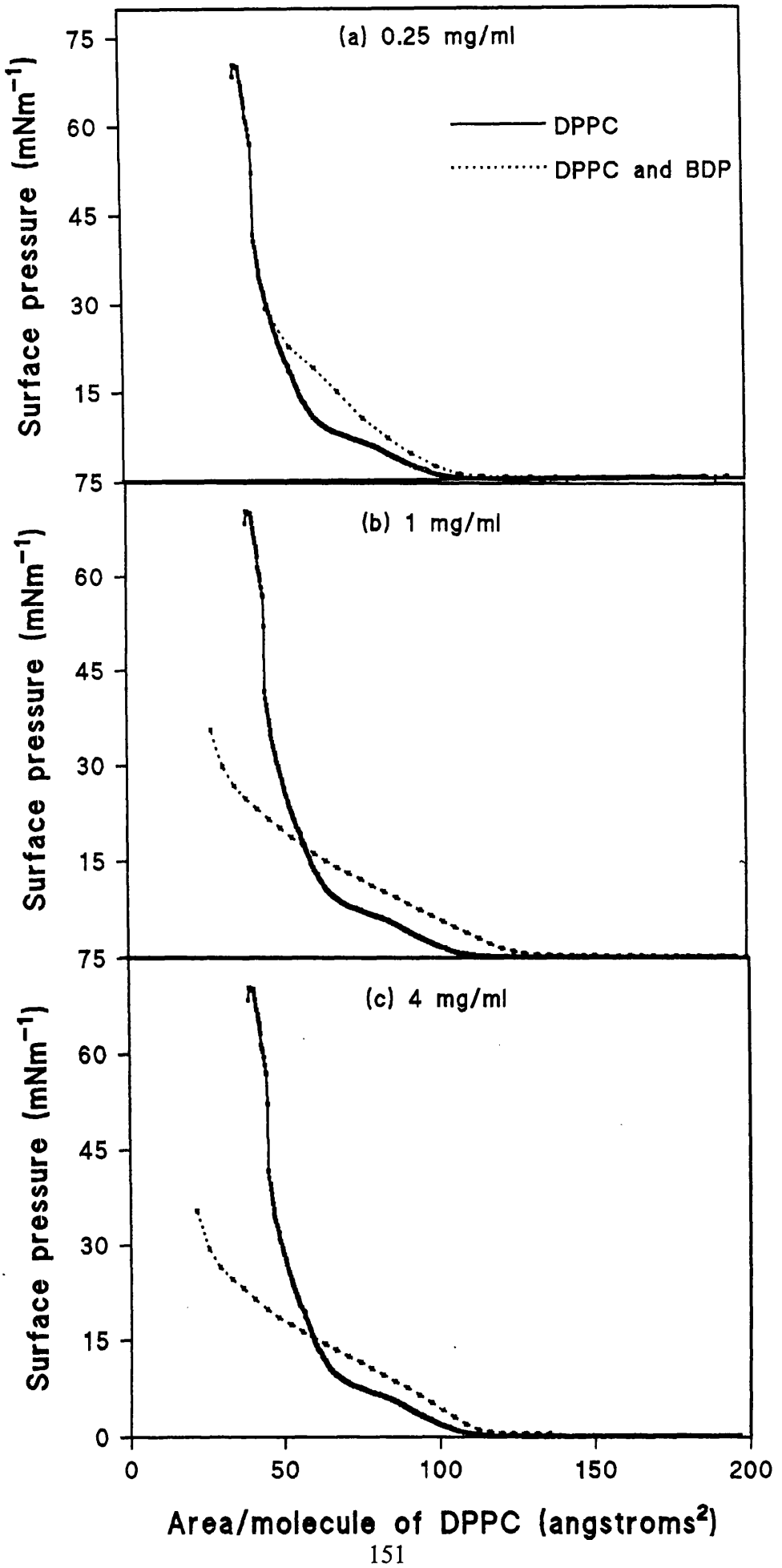
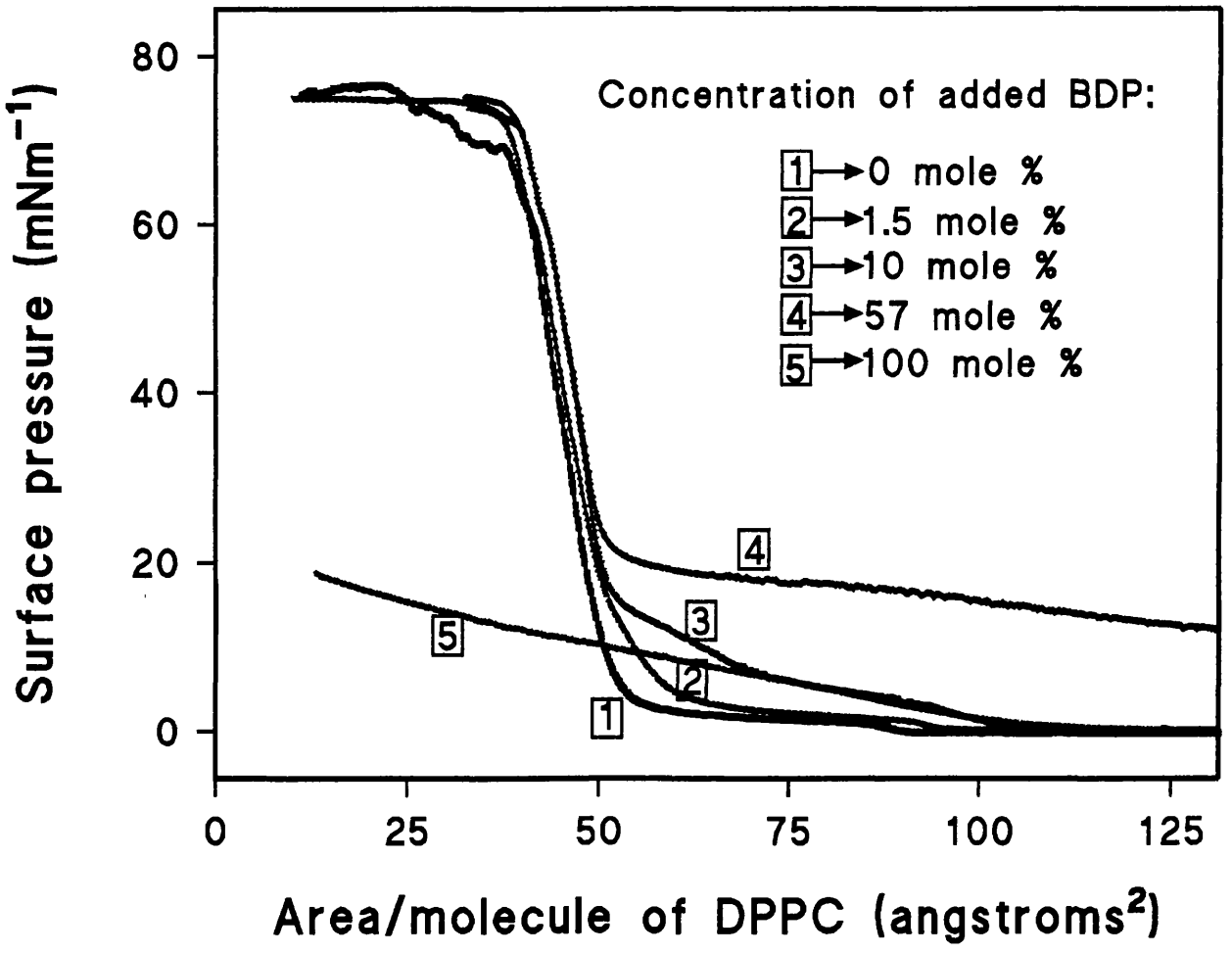


Figure 3.14 Pressure–area isotherms of mixed solutions containing 1mg/ml DPPC and varying amounts of BDP.



BDP, particularly the linear portion of the isotherm after the DPPC phase transition (Figure 3.14.). This presence of this feature, together with the DPPC phase transition, clearly indicates that at these concentrations the two components exist as separate domains at the surface. At 57 mole % BDP the isotherm resembles that of pure BDP (Figure 3.14.). The phase transition of DPPC may be masked by the large number of BDP molecules present. However, further compression leads to a "squeeze out" of BDP molecules, as the condensed phase of the DPPC monolayer begins to form. This is a common feature for all the solutions, but the surface pressure at which the DPPC monolayer begins to condense increases with BDP concentration. This suggests that BDP acts to hinder the formation of this solid phase.

Figures 3.12. and 3.13. are therefore the isotherms obtained by the injection and the premixing methods of BDP incorporation, and can be used to compare the two methods of drug incorporation into monolayers. At identical concentrations of each solution (approximately 25, 58 and 85 mole % BDP) plots may be directly compared. The lack of any phase transition observed in isotherms of the mixed solutions, compared to the transitions with the injection method, indicates that there are differences in the interaction between BDP and DPPC depending on the method of incorporation. The injection method results in BDP molecules penetrating the film unevenly, and this is probably due to the fact that as a solution in chloroform is injected below the subphase surface, the chloroform rises to the surface and evaporates to leave the dissolved BDP deposited at the surface in a domain. In addition, chloroform at the surface may disrupt or dissolve the DPPC monolayer. Therefore the observed "interaction" is probably a result of the method used to introduce BDP into the monolayer. Mixing BDP and DPPC prior to spreading promotes any interaction between the two components, before any effects of compression. Therefore this method is more representative of the liposome manufacturing process (Section 2.3.2.2.). The presence of the DPPC phase transition at certain concentrations of BDP, is a similar finding to that of Lewis and Hadgraft (1990) with other materials. They found that increasing the concentration of azone and oleic acid reduced the phase transition of DPPC in a monolayer until it was abolished. Only 10 mole % of oleic acid was required to decrease the co-operativity of the transition, which indicates a degree of miscibility with the phospholipid. However the same concentration of BDP with DPPC did not result in a decrease in the co-operativity of the DPPC transition, and so

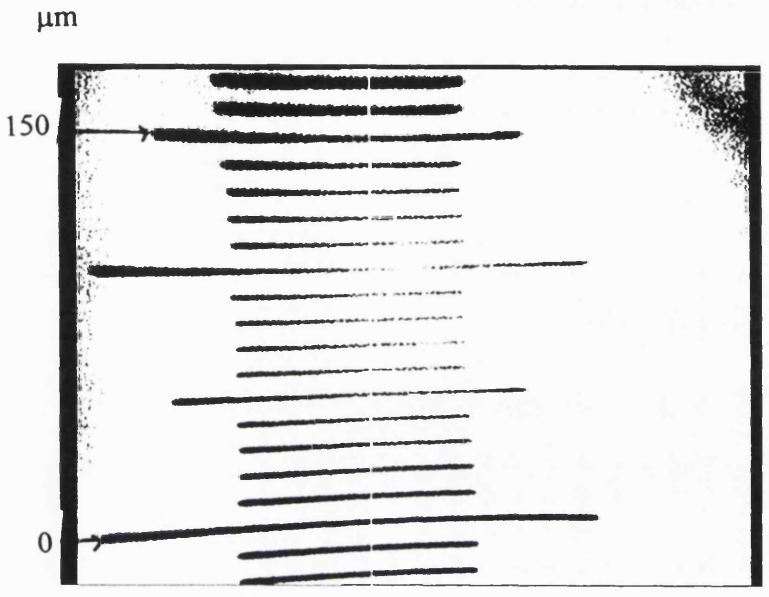
in conclusion, the co-spreading of BDP and DPPC and the subsequent compression of the mixed film has been shown to lead to immiscibility of the two components in the condensed phase, with clear indications of the presence of the domains of each component in the resultant isotherms.

3.4.5. Fluorescence studies of DPPC monolayers containing BDP

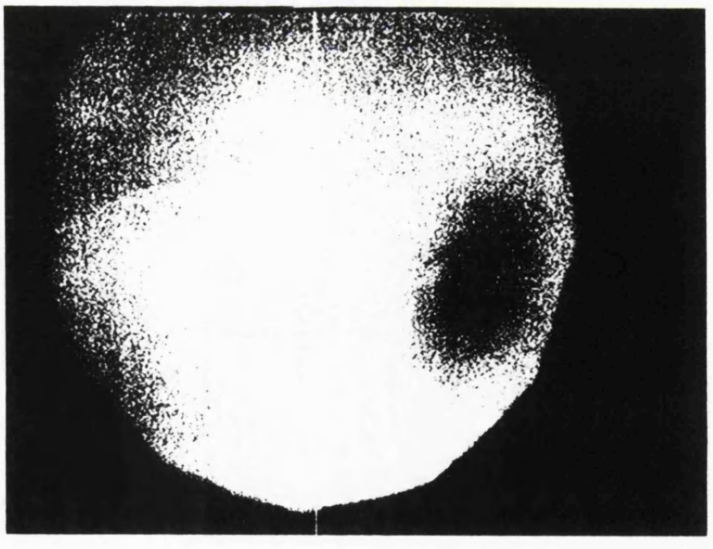
When DPPC alone was spread and compressed, the formation of solid domains was observed using a fluorescence microscope (Figure 3.15.). The domains appeared dark because the fluorescent dye used was only weakly soluble in the solid phase, and so was excluded from the crystalline domains of DPPC. Before compression was commenced, all that could be seen was a white background indicating the presence of dye in the fluid phospholipid (Figure 3.15(a)). Small domains of DPPC appeared at a surface pressure of 5.3 mNm^{-1} (Figure 3.15(b)) and were sparsely distributed in the fluid phospholipid. The onset of the plateau region, which represents the co-existence of fluid phospholipid and solid domains, was coincident with the appearance of these dark domains. On further compression, as surface pressure increased, no new domains formed but the size of the existing domains increased (Figure 3.15(c)). This is because new solid phase was added to existing domains instead of nucleating new domains. The domains had a chiral structure (no superimposable image), since DPPC itself is chiral (commercial preparations are composed of only the L-isomer), and on compression developed thin structures on their "arms" (Figure 3.15(d)). As compression of the monolayer increased, the solid phase domains arranged periodically, were pushed closer together but did not come into contact (Figure 3.15(e)). This indicates a degree of repulsion between them, and Möhwald (1986) suggested that this was due to electrostatic forces arising at the surface, whilst Weis (1991) attributed repulsion to differences in charge and/or dipole densities of the fluid and solid phases. As the domains were forced into contact, the compressibility of the monolayer decreased.

When BDP alone was spread, a white background indicated that the dye was miscible with the steroid molecules, and BDP did not form any solid phase domains on compression (Figure 3.16.).

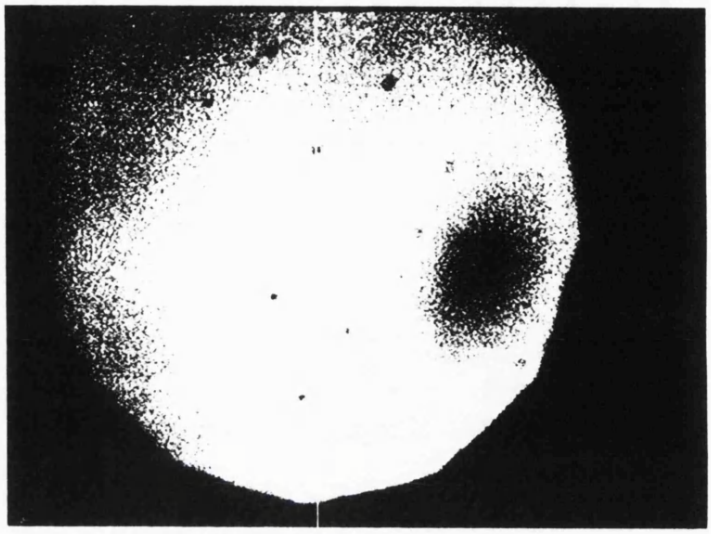
Figure 3.15. Photographs of DPPC monolayer using fluorescence microscopy, showing scale:



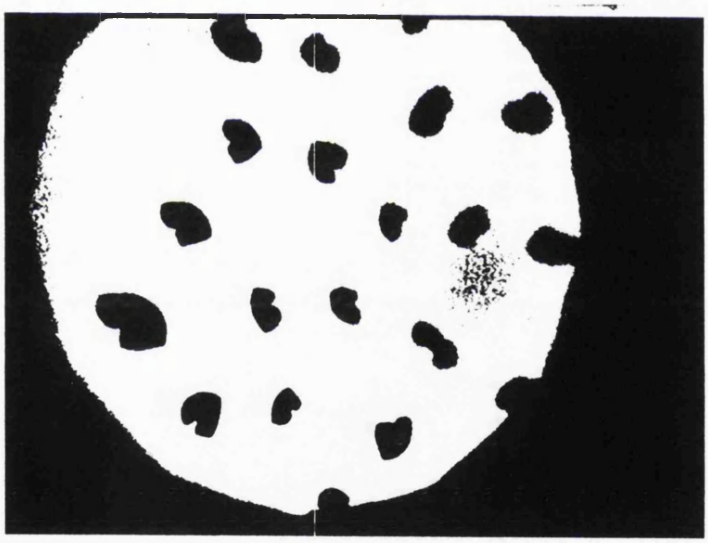
(a) no domains prior to compression:



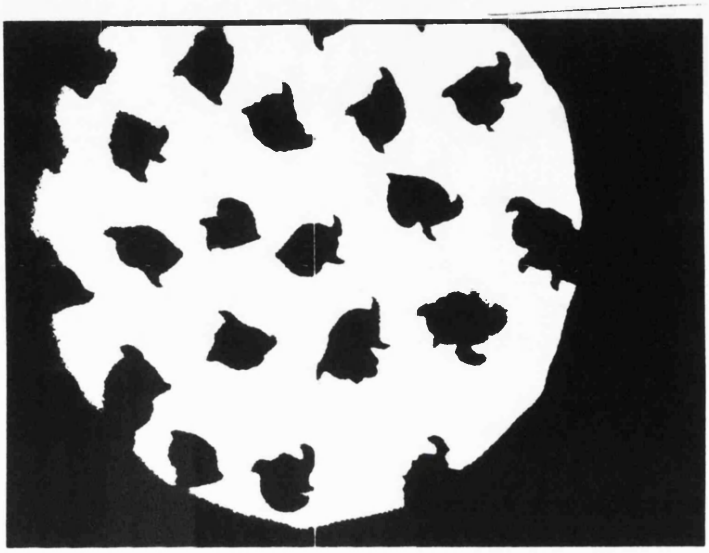
(b) appearance of domains at 5.3 mNm^{-1}



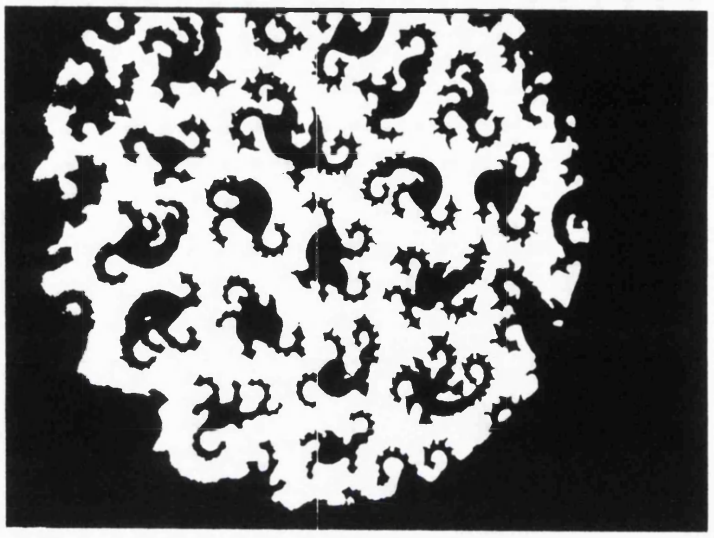
(c) increasing size of domains at 6.5 mNm^{-1}



(d) and at 7.5 mNm^{-1}



(d) development of "arms" on domains at 8.5 mNm^{-1}



(e) repulsion of domains at 10 mNm^{-1}

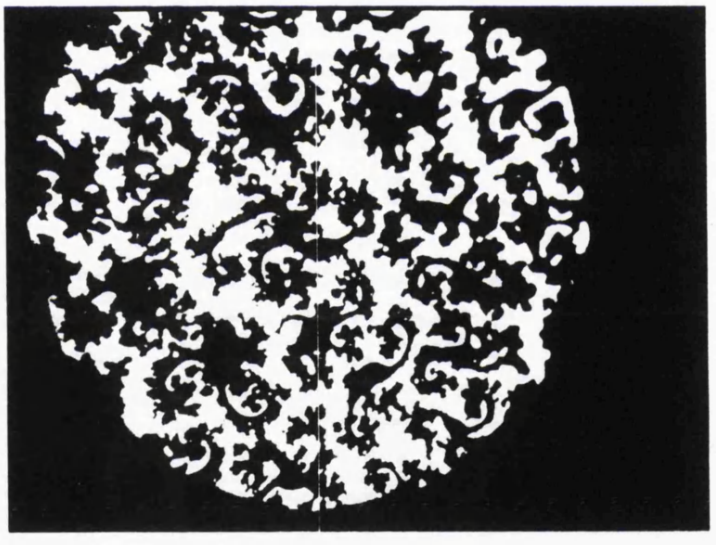
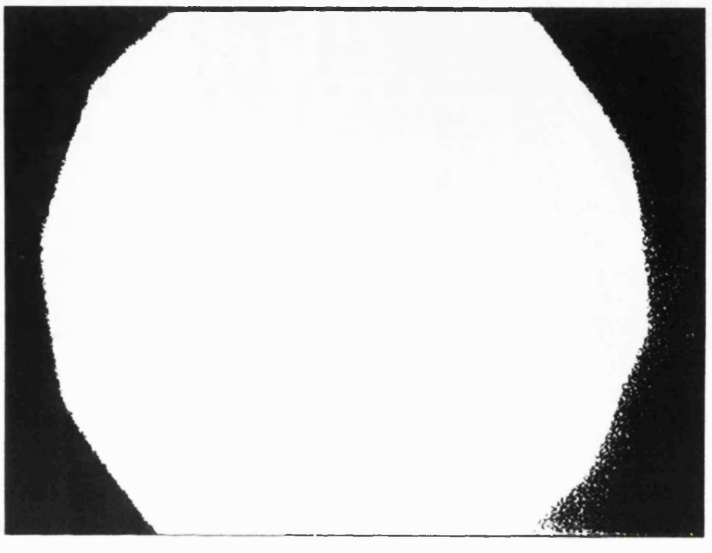


Figure 3.16. Fluorescence of compressed BDP film.



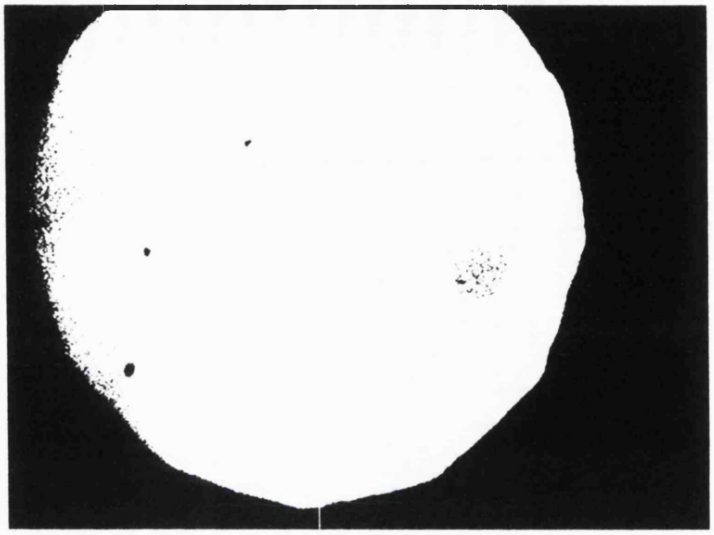
When the mixed solution (containing 2.5 mole % BDP) was co-spread (Figure 3.17.), domains of DPPC formed at approximately 7.5 mNm^{-1} (Figure 3.17(a)). These domains were more irregular in shape than those of pure DPPC (Figure 3.17(b)). Compression of the higher concentration solution (containing 10 mole % BDP) resulted in the formation of DPPC domains at approximately 7 mNm^{-1} (Figure 3.18(a)). On further compression, these domains formed irregular shapes (Figure 3.18(b)) that were different to monolayers containing 2.5 mole % BDP. Figure 3.19. shows that at a given surface pressure of 10 mNm^{-1}, the domains of DPPC in the presence of 2.5 mole % and 10 mole % BDP (Figures 3.19(b) and (c), respectively), were fewer in number than those of the pure DPPC monolayer (Figure 3.19(a)), and domain shapes were very different. Therefore, not only does the presence of BDP increase the surface pressure at which DPPC domains start to form, but the isotherms of mixed monolayers (Figure 3.14.) show that the onset of the DPPC phase transition start at higher surface pressures than that of pure DPPC. This may be explained by a depression in the freezing point of DPPC caused by the mixing of BDP and DPPC at low surface pressures. In addition, at a given surface pressure, the domains of DPPC in the presence of drug are fewer, and of a different shape, than those of the pure monolayer.

The results are similar to the findings of Nag and Keough (1993). They found that the domains of DPPC in the presence of another monolayer component were smaller than those observed for pure DPPC monolayers. In these mixed monolayers the condensed domains were not observed until higher surface pressures had been reached, than those required for DPPC alone. Perez-Gil et al (1992) found that when pulmonary surfactant protein (SP-C) is incorporated into DPPC monolayers, it inhibits lipid condensation, so that the size of individual domains were seen to decrease in size. However SP-C did not prevent high surface pressures being reached, which indicates that SP-C was probably removed from monolayers at high surface pressures, which is also the case for DPPC monolayers that contain BDP (Section 3.4.4.).

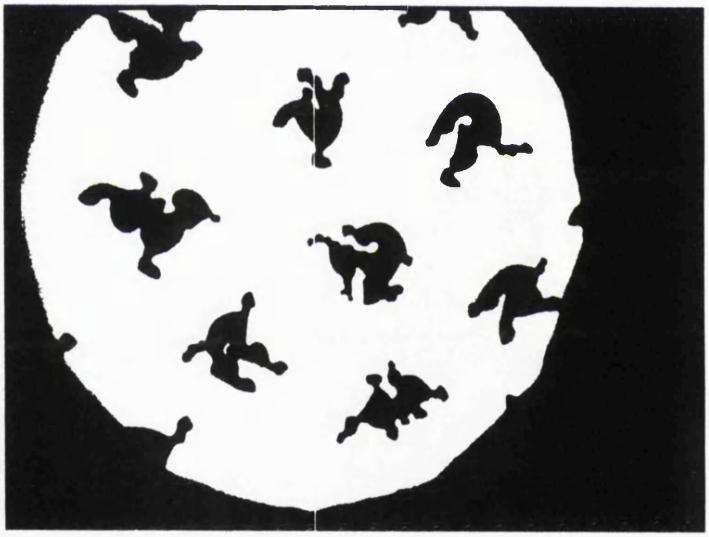
Domain shape can be affected by various factors, one of which is impurities. As mentioned in Section 3.3.5. the fluorescent probe may be considered to be an impurity, but any possible probe effects were minimised by the use of a low concentration. However, other impurities can affect the growth of crystals; increasing the number of domains, by acting as nucleation centres. They may also change the line

Figure 3.17. Photographs of DPPC monolayer containing 2.5 mole % BDP, showing:

(a) appearance of domains at 7.5 mNm^{-1}



(b) shapes of domains at 9.5 mNm^{-1}



(c) and at 10 mNm^{-1}

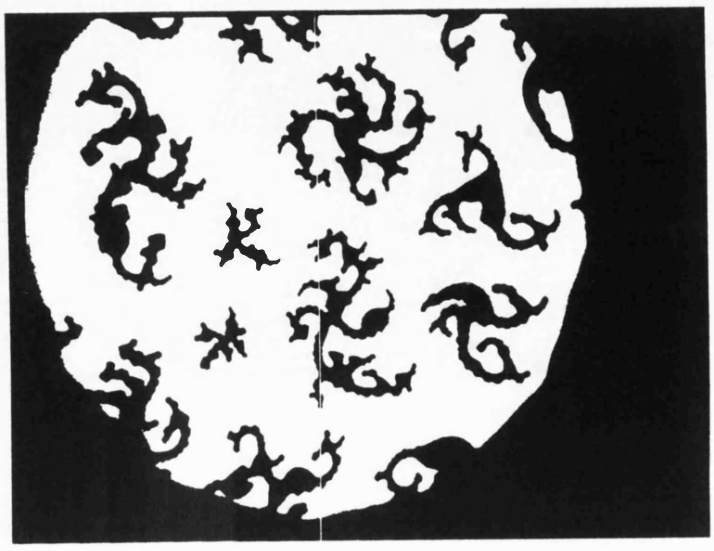
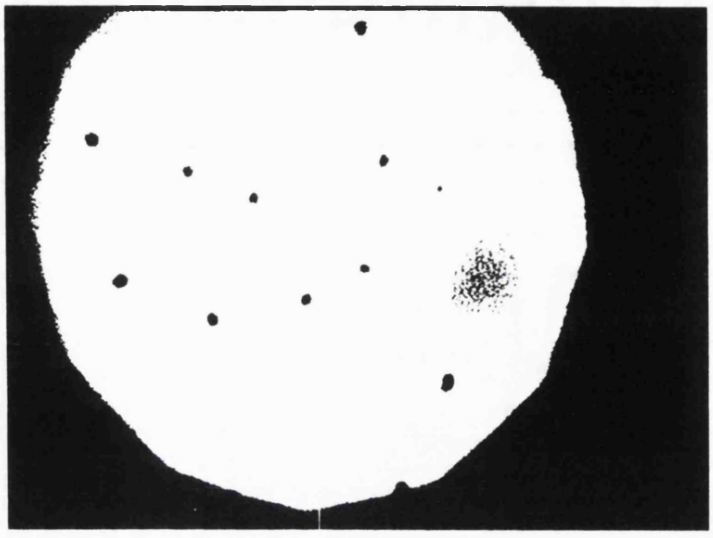
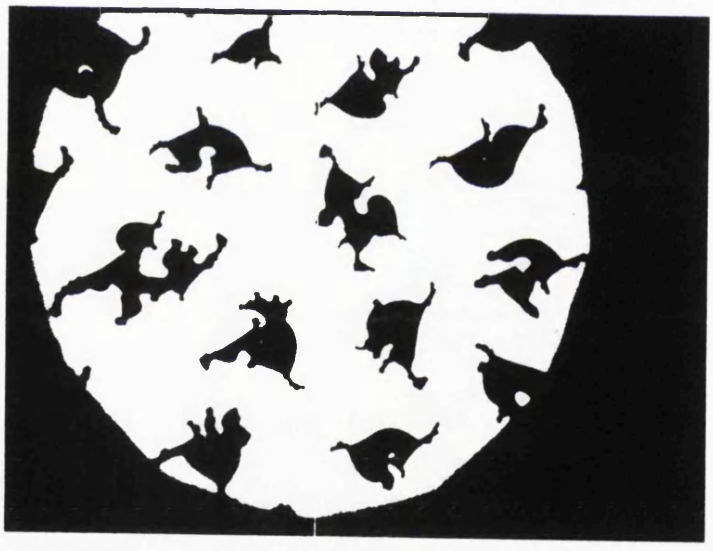


Figure 3.18. Photographs of DPPC monolayer containing 10 mole % BDP, showing:

(a) appearance of domains at 7 mNm^{-1}



(b) shapes of domains at 9.5 mNm^{-1}



(c) and at 12 mNm^{-1}

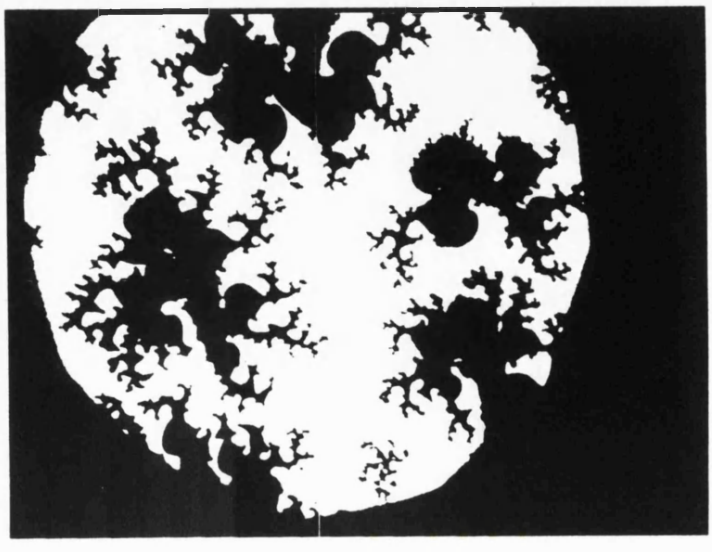
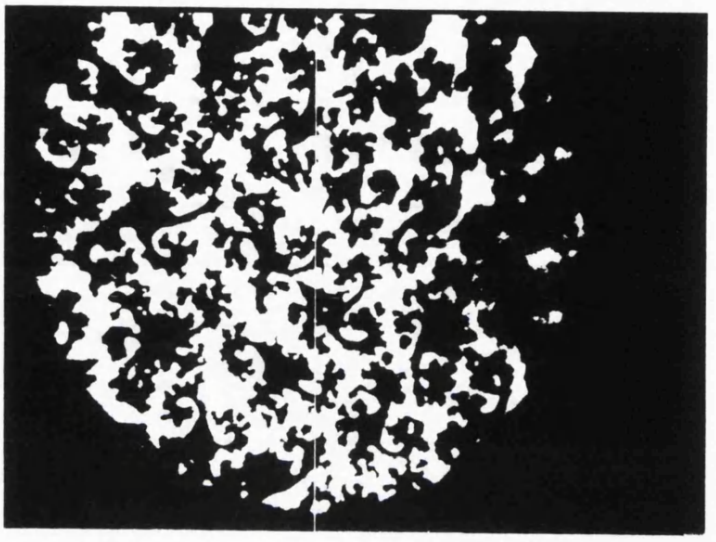
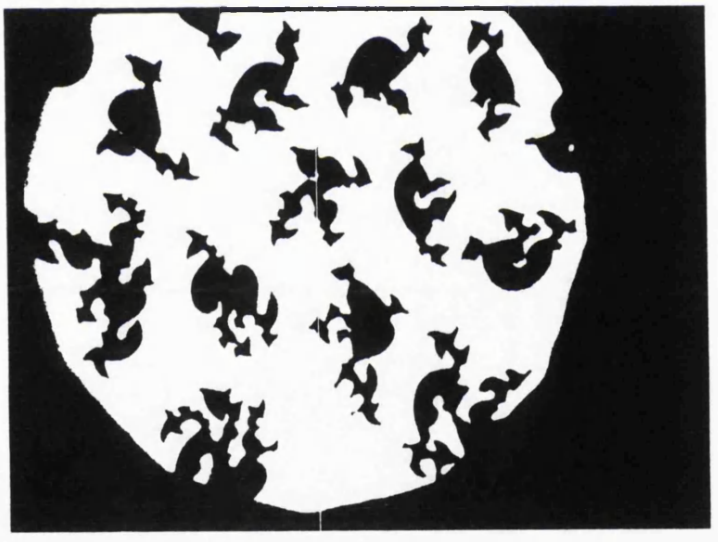


Figure 3.19. DPPC domains, at 10 mNm^{-1}, in the presence of:

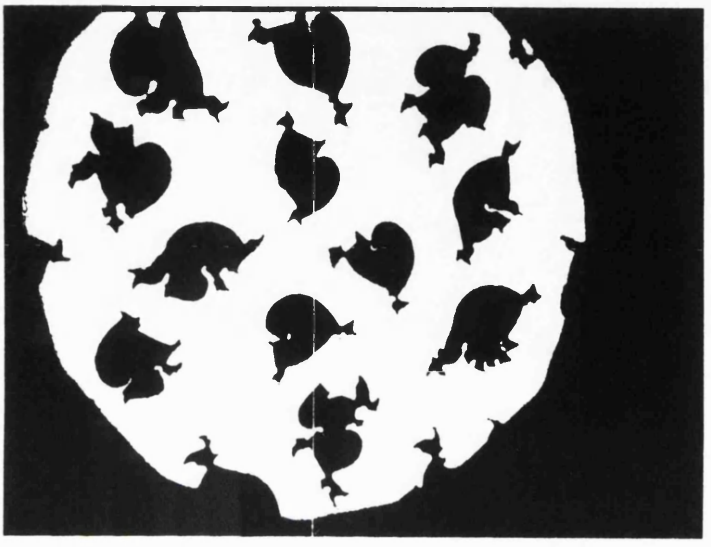
(a) 0 mole % BDP



(b) 2.5 mole % BDP



(c) 10 mole % BDP



tension, and therefore cause peculiar crystal shapes to form (Möhwald, 1986). A line tension acts to minimise the interface between the two phases, and exists at the boundary between the two phases within the monolayer. It has been reported that domain shapes are also affected by electrostatic forces as well as line tension, and that these two forces compete (Weis, 1991). Therefore when line tension dominates, a solid domain forms a circle since this shape minimises the perimeter/area ratio. It was shown that the addition of 2 mole % cholesterol causes the phospholipid hydrocarbon chains to tilt and therefore the crystal symmetry reduces (Weis, 1991). This is due to the fact that cholesterol causes the stabilisation of 1 or 2 crystal faces, thus elongating the crystallite, and turning otherwise-forming circles into spirals (Möhwald, 1986). However these spiral-shaped domains are not observed when DPPC and BDP monolayers are formed. This may be due to the lack of any apparent stabilisation of BDP within the phospholipid film, as demonstrated by its ejection out of the monolayer at high surface pressures. The width of the domains formed in the presence of cholesterol were inversely proportional to the cholesterol concentration in the fluid phase (Weis, 1991). In general when elongated shapes are formed, electrostatic forces have played a more significant role than line tension. Conversely compact shapes are observed when line tension dominates. Another factor affecting the shape of the domain is the compression rate (Weis, 1991). A high rate results in smaller domains being formed (Nag and Keough, 1993) and dendritic shapes may be observed (Weis, 1991). The latter occurs because compression causes an excess of dye to move into the fluid phase, and situate near the solid-fluid phase boundary. This dye inhibits the growth of circular solid domains and so dendritic domains form. If there is a state of non-equilibrium within the monolayer, irregular-shapes may form. This was found when DPPC domains in the presence of BDP were formed. This occurrence has also been noted for DPPC monolayers containing 1 mole % cardiolipin (Weis, 1991), with the growth of non-uniform domains.

To conclude, the presence of BDP in DPPC monolayers has been shown to increase the surface pressure at which domains appear to form, thus confirming earlier findings that BDP increases the onset of the liquid-condensed region. The fewer numbers of domains and irregularity of shapes indicates that BDP is present in the

monolayer. There is a degree of miscibility between the steroid and the phospholipid in the liquid-expanded phase, but the liquid-condensed phase consists purely of DPPC.

3.4.6. Langmuir studies of other phospholipid monolayers

Figure 3.20. shows pressure-area isotherms for different phospholipids, spread at the same concentration (1 mg/ml). It is interesting to note that both DPPG and DPPA compress to smaller molecular areas than DPPC, indicating that the size and nature of the polar head-group is important for condensed monolayers. Both DSPC and DLPC converge to approximately the same molecular area as DPPC on compression, and this suggests that again, chain length is unimportant when examining the behaviour of condensed monolayers.

Figure 3.21. shows that an isotherm of DLPC does not exhibit a phase transition, and the incorporation of low concentrations of BDP (2.5 and 10 mole % BDP) has a small effect on the surface pressures of liquid-expanded DLPC monolayers, but the presence of drug within the monolayer is indicated by the increase in molecular areas. Incorporation of the highest concentration (55 mole % BDP) has a pronounced effect on the surface pressures of liquid-expanded and liquid-condensed monolayers, indicating the presence of BDP in the plane of the monolayer. These results suggest that BDP penetrates DLPC monolayers, and is not "squeezed out" of condensed monolayers to the same extent as with DPPC monolayers. Therefore this indicates that the extent of BDP penetration is related to the phospholipid chain length, with shorter chains able to accommodate BDP molecules such that they are not expelled out on compression of these molecules. Alternatively, this feature may be related to the physical state of the chains in the monolayer, since 20°C is below the main phase transition temperature for DPPC and chains are in the gel state. However, at this temperature DLPC chains are in the liquid-crystalline state, and therefore provide a more fluid environment with respect to entrapped BDP molecules, than DPPC chains. This is reflected in the behaviour of compressed monolayers containing BDP, with DPPC monolayers rapidly expelling out BDP molecules, whilst DLPC monolayers are able to retain drug molecules to a greater extent.

Figure 3.20. Pressure–area isotherms of different phospholipids (1 mg/ml).

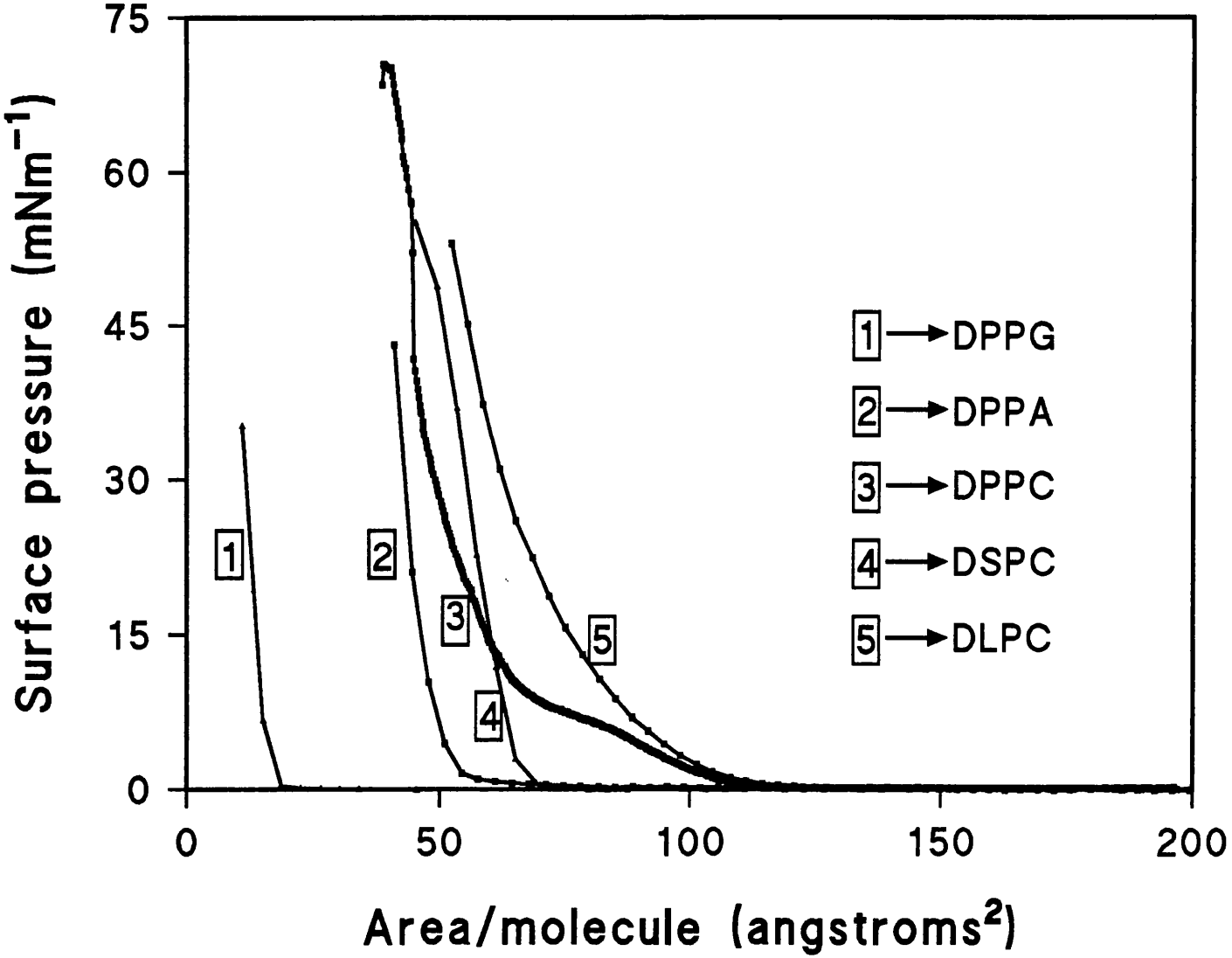


Figure 3.21. Pressure–area isotherms of DLPC monolayers containing BDP.

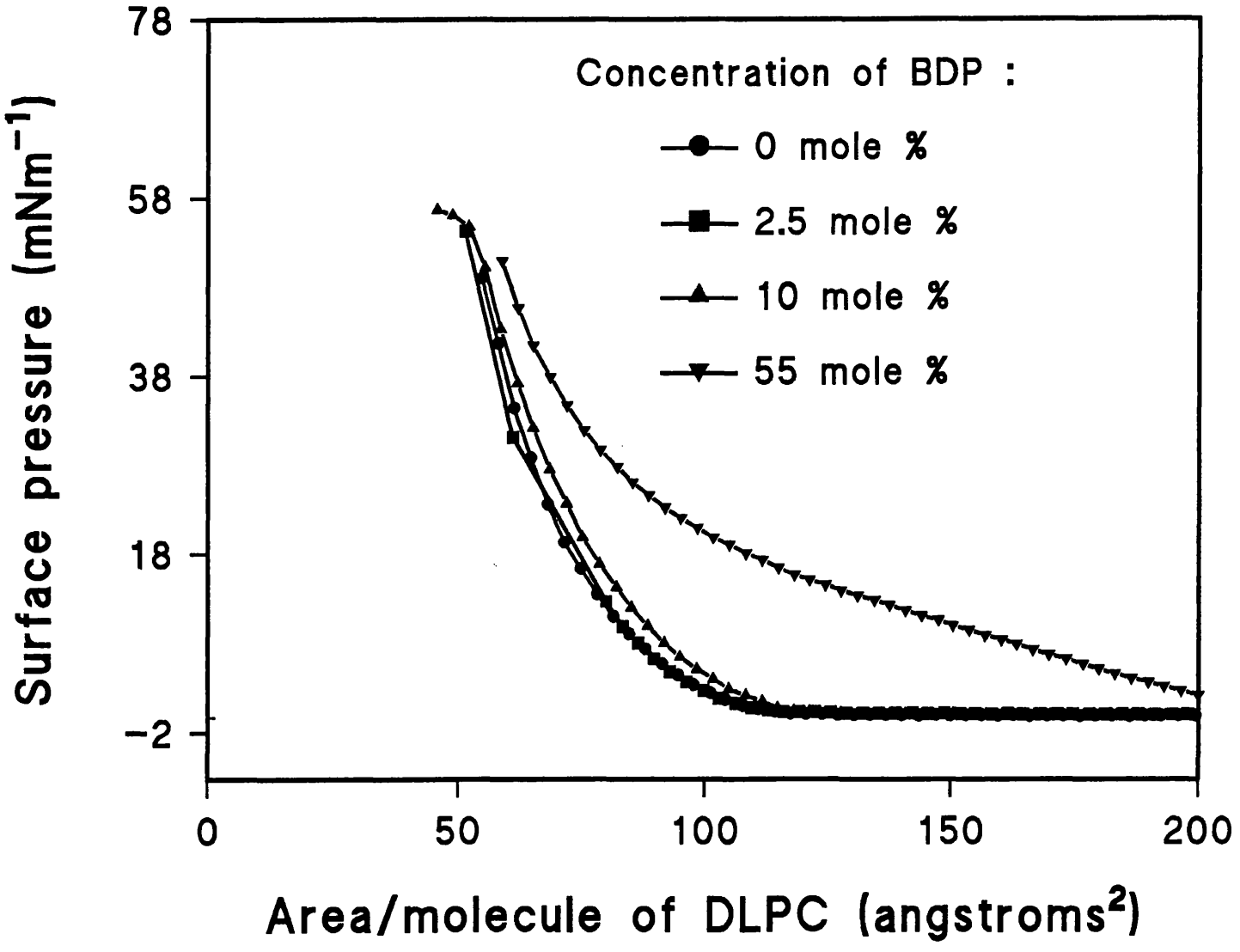


Figure 3.22. shows that DSPC also does not exhibit a characteristic phase transition, and that the incorporation of BDP into DSPC monolayers at all concentrations (2.5, 10 and 55 mole % BDP) has no discernible effect on the surface pressures of mixed monolayers when compared to pure DSPC values. This indicates that BDP is not in the plane of the monolayer, and therefore is probably located in the superphase, above the monolayer. This feature may be related to the chain length of DSPC, which is greater than DPPC or DLPC, suggesting that BDP is not able to penetrate a DSPC monolayer. Alternatively, at 20°C DSPC chains are in a rigid gel state, and may be an unsuitable environment for BDP molecules.

Figure 3.23. shows that surface pressure values of DPPA monolayers in the presence of low concentrations of BDP (2.5 and 10 mole % BDP) are slightly increased. However at the highest concentration of BDP used (55 mole %), the isotherm in the liquid-expanded phase resembles that of pure BDP, similar to isotherms of DPPC containing 57 mole % BDP. This suggests that BDP is present in the plane of the monolayer, but it is not known whether BDP molecules are evenly distributed between those of DPPA, since this phospholipid does not exhibit a phase transition. The isotherm in the condensed phase converges to that of pure DPPA, which indicates that BDP molecules are "squeezed out" of closely packed monolayers of DPPA.

Figure 3.24. shows that whilst low concentrations of BDP (2.5 and 10 mole % BDP) produce a negligible effect on the surface pressures of DPPG monolayers, the highest concentration used (55 mole % BDP) produced an increase in surface pressures with increasing compression. However further compression resulted in the ejection of BDP molecules out of condensed monolayers.

To conclude, the behaviour of phospholipids in the absence of BDP has been shown to be dependent on the nature of the head-group. Finer and Phillips (1973) found that molecular packing in monolayers was influenced by the degree of polar head-group hydration, and concluded that this factor therefore affects the calculated molecular area. It follows that the smaller the molecular area of a condensed monolayer, the more likely that BDP molecules within such a monolayer will be ejected out on compression. The most convincing results to support this hypothesis are the isotherms obtained for DLPC and BDP which showed the presence of drug at the surface at all concentrations, even in condensed monolayers. The smallest

Figure 3.22. Pressure–area isotherms of DSPC monolayers containing BDP.

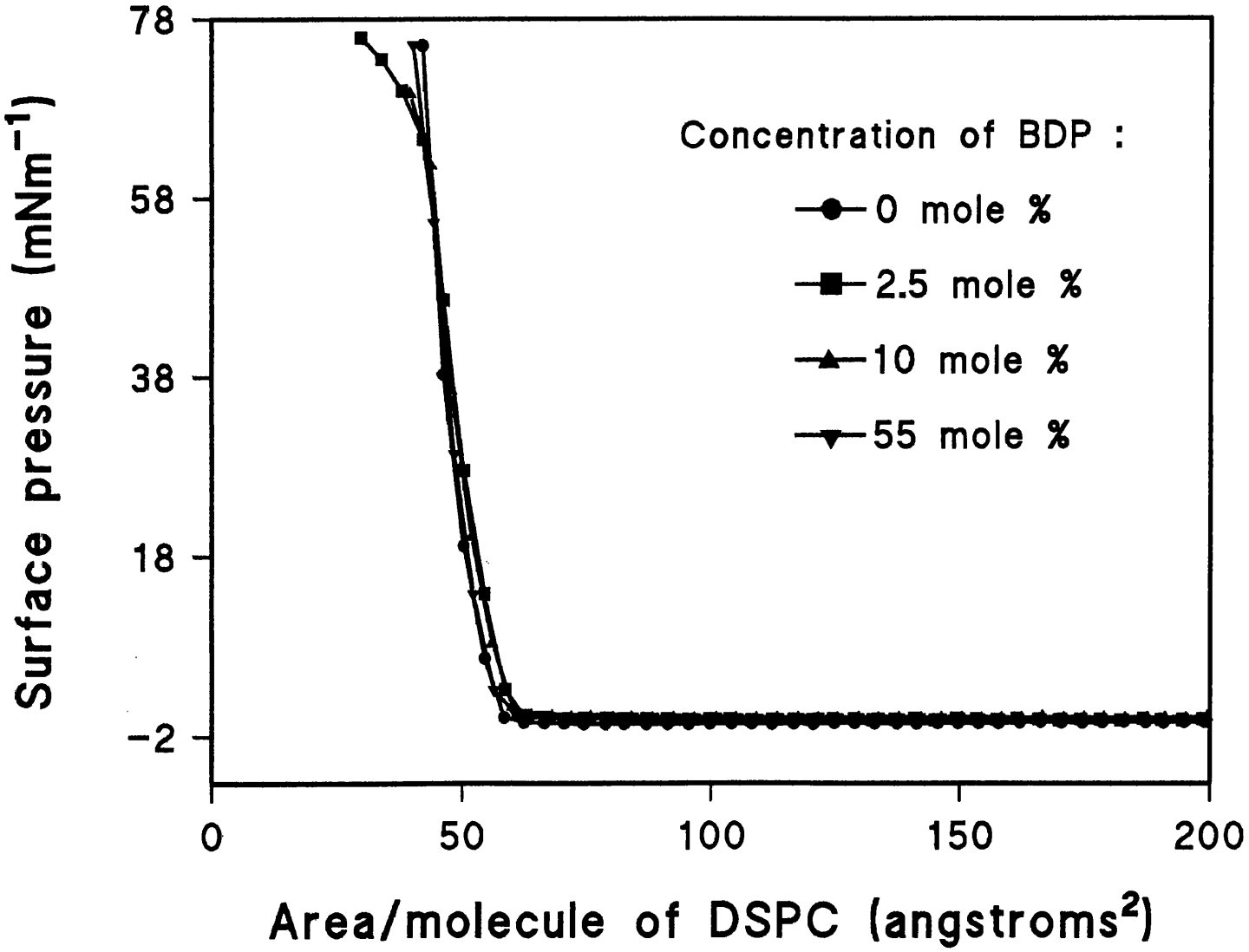


Figure 3.23. Pressure–area isotherms of DPPA monolayers containing BDP.

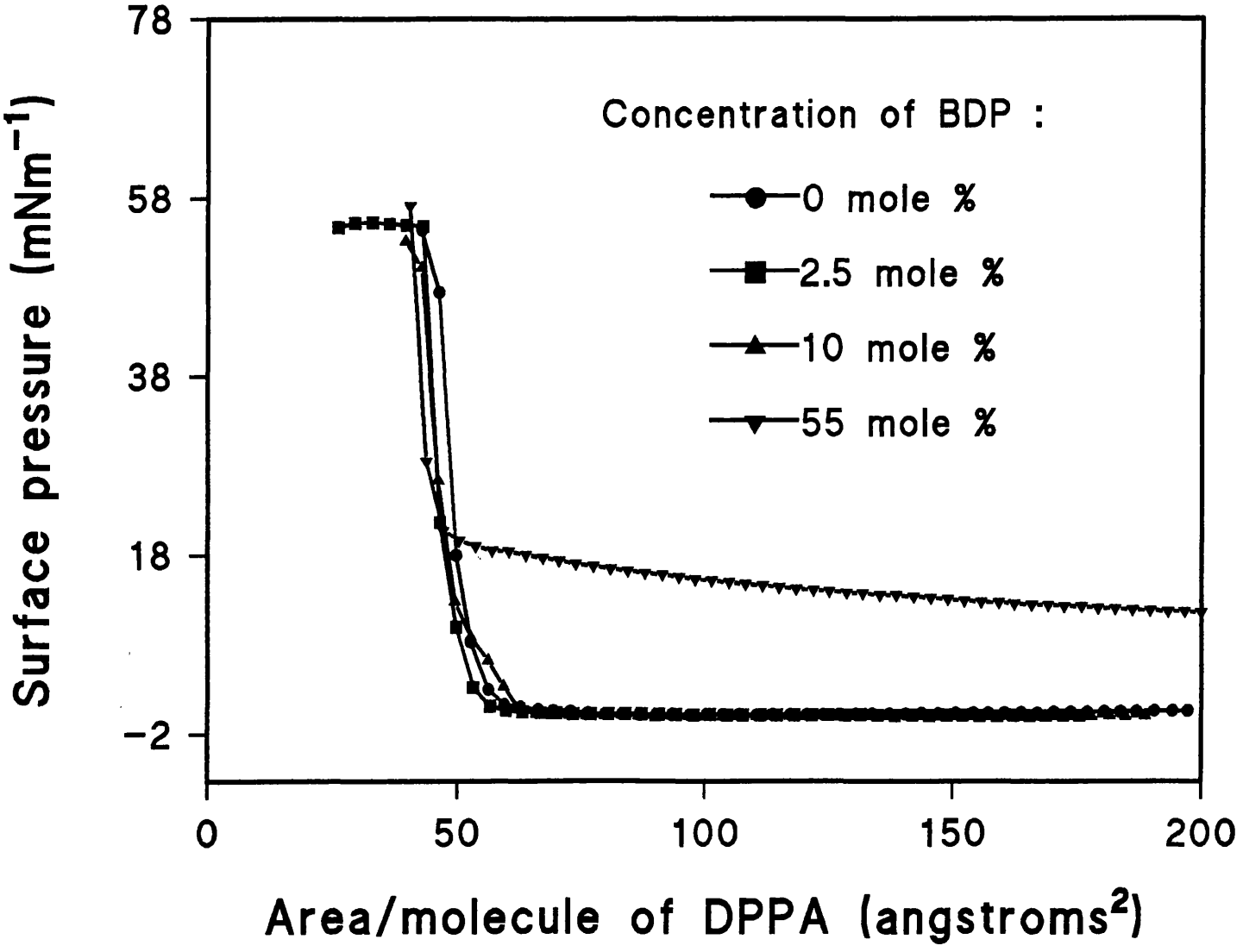
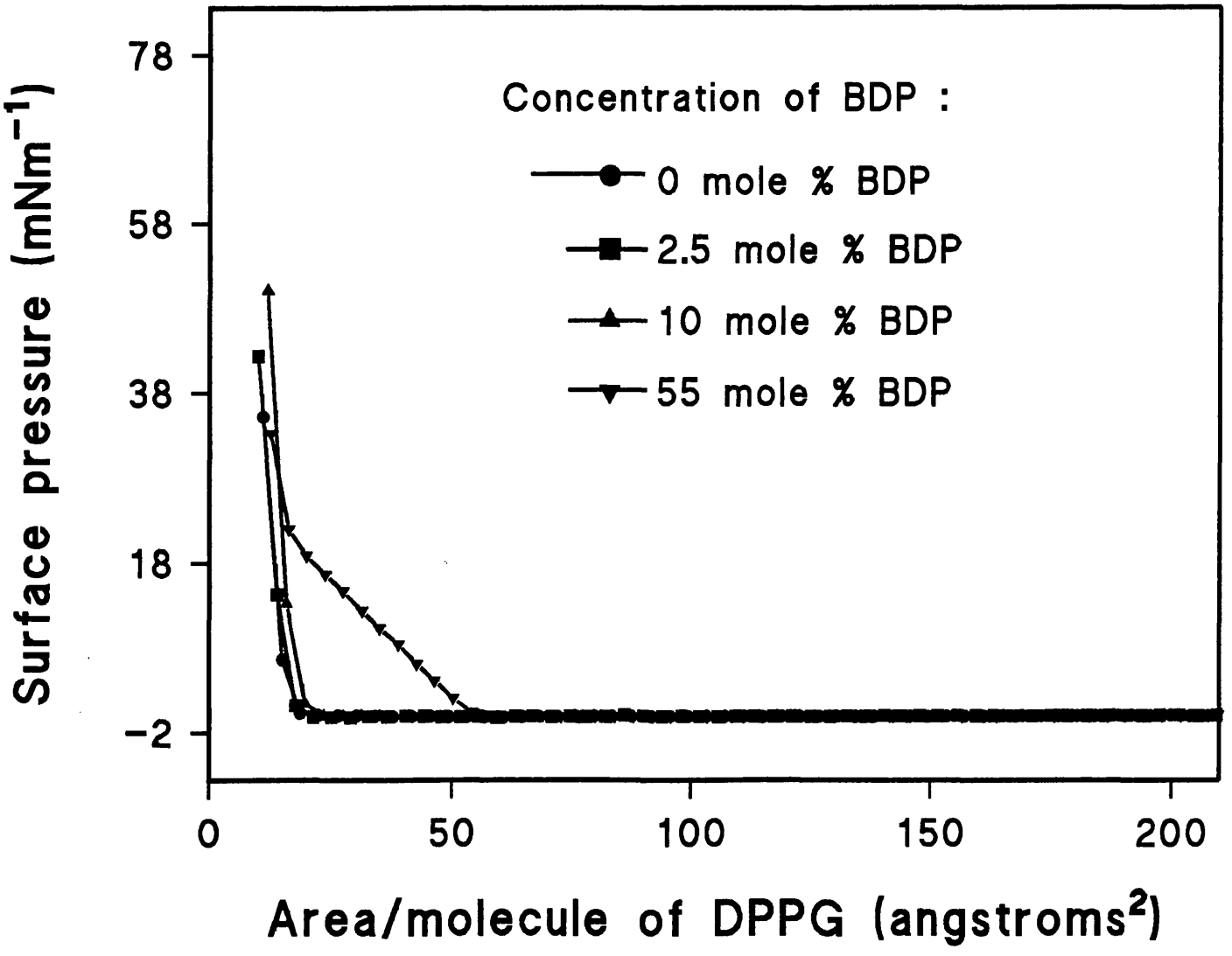


Figure 3.24. Pressure–area isotherms of DPPG monolayers containing BDP.



molecular areas were obtained for solutions of DPPG, and incorporation of BDP into monolayers constructed from this phospholipid, resulted in ejection of drug molecules out of condensed monolayers, at all concentrations. We can therefore assume that the smaller chain length and head-group of DLPC is advantageous when incorporating BDP into highly compressed phospholipid monolayers. In addition, Cleary and Zatz (1973) found that hydrocortisone only interacted with phospholipid monolayers at large areas (being expelled out at smaller ones) and concluded that the interaction between steroid and phospholipid probably involves the polar groups of both substances.

3.5. Conclusions

Results from fluorescence studies of DPPC films containing BDP may be used to conclude that the presence of drug increases the surface pressure at which phospholipid solid domains appear to form, thus confirming earlier findings that BDP increases the onset of the liquid-condensed phase of DPPC monolayers. Whilst there is a degree of miscibility between the steroid and the phospholipid in the liquid-expanded phase, the fewer number of domains and irregularity of domain shapes indicates that BDP and DPPC are not in equilibrium. The liquid-condensed phase meanwhile, consists purely of DPPC, with BDP molecules being ejected out of the closely packed monolayer.

The results gained from studying the effects of incorporating BDP into monolayers composed of various phospholipids, can be used to make predictions regarding equivalent bilayer systems. Blume (1979) concluded on the basis of studying the change in the absolute molecular areas that occurs at monolayer and bilayer phase transitions, that the high pressure region is the most appropriate to examine for behaviour indicative of bulk systems and it is also where phase separation is most likely to occur. So from behaviour in the liquid-condensed region of mixed monolayers, we can predict the behaviour of the components when present in a bilayer. Since BDP is expelled out of closely packed monolayers of DPPC, DSPC, DPPA and DPPG, one can predict that the incorporation of this steroid into liposomes composed of these phospholipids will result in immiscibility of the two components and hence, a low entrapment value. However, BDP may be stabilised in closely packed monolayers of

DLPC as no expulsion was detected by monitoring high surface pressure values. However, it is unclear from these results why the behaviour of BDP in monolayers constructed from phospholipids with the same head-group is different, since the nature and size of polar head-groups are important in dictating the behaviour of drug incorporation into phospholipid monolayers. One possible reason for this fact may be that the temperature at which these investigations were carried out, was above the phase transition of DLPC, and therefore the chains were in the liquid-crystalline state, and hence flexible with respect to the presence of drug. All other phospholipids were in the gel state, and as such were rigid in structure.

Therefore, it must be recognised that these studies cannot determine the effects of BDP on the hydrophobic region of these phospholipids. Whilst the Langmuir technique is a suitable tool with which one can study the compatibility of drugs or other molecules in a monolayer, in order to make predictions regarding liposomal systems, these can be confirmed by performing thermal analysis of liposomes composed of various phospholipids, and containing BDP. The information gained from such investigations can be used to detect the effects of BDP on the packing and conformational states of the phospholipid alkyl chains, thus complementing the information concluded from monolayer studies. In addition, studies on phospholipid monolayers at temperatures above and below their phase transitions may prove to be useful when investigating BDP entrapment into liposomes.

4. THERMAL ANALYSIS OF PHOSPHOLIPID BILAYERS CONTAINING BDP

4.1. Introduction

4.1.1. Thermal analysis of liposomes

Liposomes are a key tool in the study of the physical properties of membrane lipids since they are made up of phospholipids which have characteristic phase transition profiles. When drug molecules are introduced into liposomes, the phase behaviour of these systems is of vital importance in drug delivery, because the altered thermal profile of such a systems determines properties such as the permeability, fusion and aggregation tendencies of these drug-laden liposomes. This in turn affects the clinical characteristics of such a delivery system. For example, the permeability of bilayers to entrapped drug is related to bilayer fluidity and the outcome of phase behaviour studies can help to determine the stability of dosage forms with respect to drug release, both *in vitro* and *in vivo*. Therefore, it is necessary to determine the nature and extent of the interaction between drug molecules and liposomal bilayers.

The effects of drugs on the phase transition profiles of liposomes can be measured using a number of techniques such as DSC (Jain and Wu, 1977; Biltonen and Lichtenberg, 1993), DTA (Ladbrooke and Chapman, 1969), solution calorimetry (Taylor et al, 1990a) and fluorescence depolarisation (Suurkuusk et al, 1976). Information gained in such a manner, such as the maximum amount of drug which may be entrapped (Fildes and Oliver, 1978) and the location of the entrapped material in the bilayer (Jain and Wu, 1977), can help in the design of novel liposomal formulations as drug delivery systems, and also in the optimisation of current formulations.

As described in Section 1.1.3., the transition of hydrated phospholipid bilayers from a relatively well ordered, crystalline state, to a disordered, fluid-like state, can be induced by an increase in temperature. This is the main phase transition of phospholipids, but it is not the only one because during the melting process, phospholipids exist in one or more intermediate forms depending on the amount of water present (Ladbrooke and Chapman, 1969). There are three basic transitions from one form to another, but the main transition is the most prominent one, involving the

largest enthalpy change. All the transitions are detectable by DSC, although some strict criteria must be observed with regard to storage conditions to detect smaller transitions. These other transitions are described in detail in Section 1.1.4.

Historically, DSC has been used primarily to study the thermally induced transitions of phospholipid bilayers and biological membranes (Ladbrooke and Chapman, 1969). However it is not the only technique that is capable of detecting changes in the state of the bilayer. This main transition results in an increase in the fluidity of the bilayer, and is associated with changes in molecular motion of the hydrocarbon chains. Therefore other techniques may be employed to detect phospholipid transitions by the measurement of molecular motion, such as nuclear magnetic resonance (NMR) (Lewis et al, 1984) and electron spin resonance (ESR) spectroscopy (Cater et al, 1974), which both utilise probes. However DSC is suitable for these current studies, since it does not require the introduction of foreign probes into liposomes, therefore ensuring that the transitions of interest are left undisturbed. Also, modern instruments are capable of greater accuracy and sensitivity than their previous counterparts.

DSC can serve as a physical tool for the quality control of liposomes as not only is it sensitive to their chemical composition, but also to the physical state of liposomal bilayers. From these types of investigations further studies may be performed on liposomes containing drug, if the drug is sufficiently hydrophobic to create a quantifiable change in the phospholipid thermogram (Arrowsmith et al, 1983b). Drugs that are hydrophilic will be incorporated in the aqueous core and compartments within a liposome, and will cause minimal, if any, disruption of the bilayer chain packing, and therefore DSC will not detect any effects due to the presence of such drugs.

It is evident from previous studies (see Chapter 2) that when a hydrophobic drug such as beclomethasone dipropionate (BDP) is incorporated into liposomes, a given amount of phospholipid will only have a limited affinity for the steroid. In excess of this limit, steroid molecules might separate as a discrete phase. Incorporation of BDP into bilayers is expected to disturb chain packing, and therefore any effects will be detectable by DSC. However, when investigating bilayer phase transitions, the behaviour of excess steroid (not associated in any way with the phospholipid) may remain undetected by DSC. Solution calorimetry has suggested that the addition of

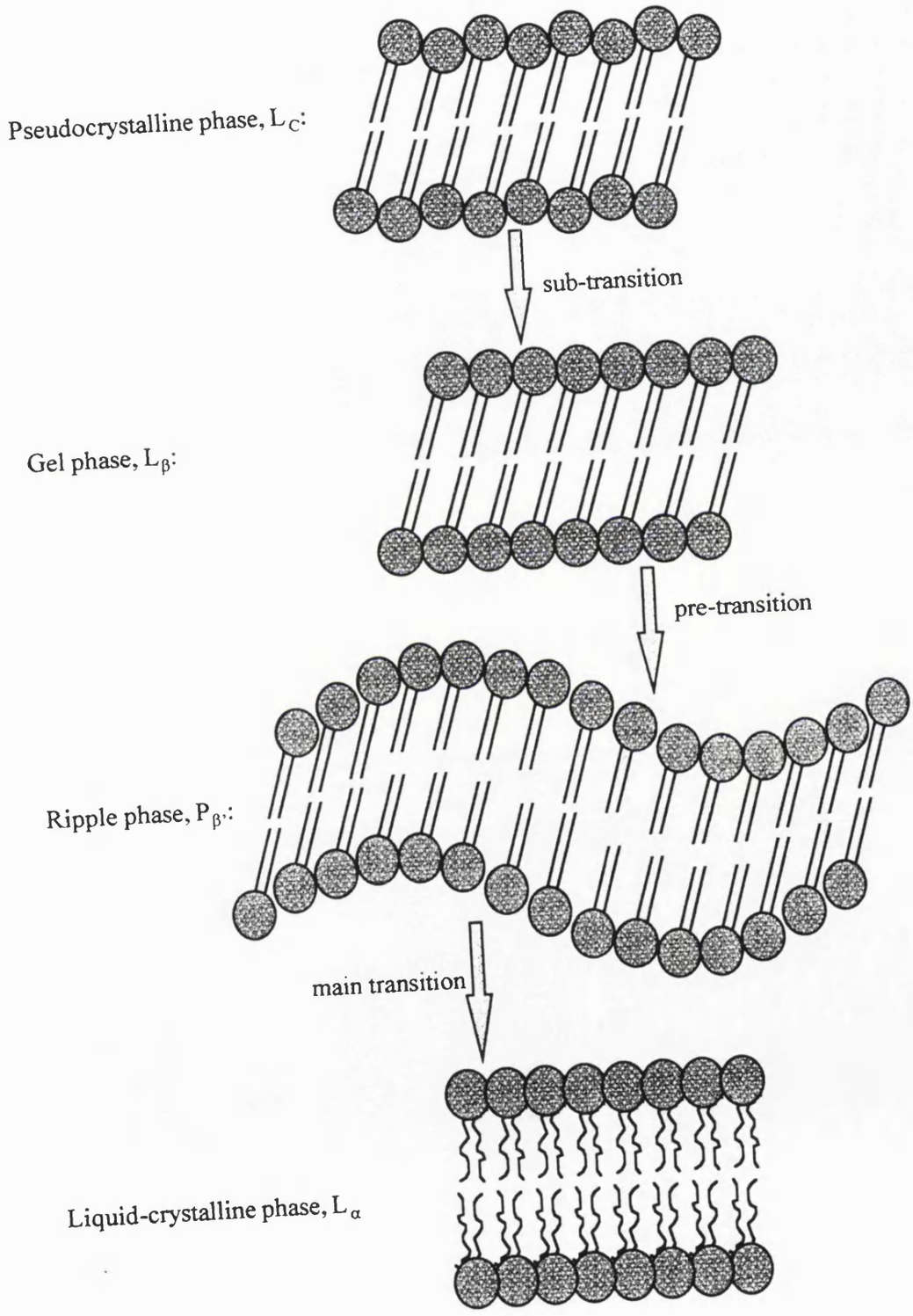
larger proportions of BDP results in a solid drug phase co-existing with a more liquid-crystalline phospholipid phase (Taylor et al, 1990a). This was detected by the effect of different amounts of BDP on the enthalpy of liposome formation from anhydrous phospholipid films.

4.1.2. Thermal analysis of DPPC liposomes

When the temperature of a liposomal sample is increased, the system changes energy states, and undergoes a thermotropic phase transition in a co-operative manner (Hinz and Sturtevant, 1972; McElhaney, 1982). In their pure forms, synthetic long-chained phospholipids can undergo a number of transitions at defined temperatures, including the sub-, pre- and main transitions. The nomenclature used in the following descriptions of the transitional states of phospholipids, is adapted from that used by Tardieu et al (1973). Figure 4.1. is a diagrammatic representation of the various structures and transitions of DPPC bilayers.

When samples are incubated for prolonged periods at low temperatures they exist in the subgel (L_c) state, where hydrocarbon chains are all fully extended and the polar head-groups are relatively immobile (Fuldner, 1981). The relatively large head-group cross-section of DPPC compared to that of the alkyl chains results in the chains adopting a tilt of 58 ° C relative to the plane of the bilayer in the gel state (Hauser et al, 1981). Adjacent hydrocarbon chains are aligned parallel to each other, and stabilise the gel phase through van der Waals interactions. A small gap between the terminal methyl groups exists due to the tilted alignment of the chains. On heating, L_c state synthetic phospholipids undergo the sub-transition to the $L_{b'}$ state. This transition usually occurs approximately 30 ° C below the temperature of the main transition (Chen et al, 1980), but DPPC has a sub-transition temperature reported to be 21°C (Biltonen and Lichtenberg, 1993), approximately 20°C below the main transition temperature. In the $L_{b'}$ state, head-groups have greater mobility and there is increased penetration of water molecules into the interfacial region (Lewis et al, 1984). A feature of the sub-transition is that it can only be observed if the sample is incubated below the sub-transition temperature for several days (for example 4°C for 5 days) (Chen et al, 1980; Fuldner, 1981). There are two types of sub-transitions. Type I sub-transition is a

Figure 4.1. Diagrammatic representation of structures of different transitional states of DPPC bilayers.



solid-solid transition between the subgel and gel states that occurs in saturated phosphatidylcholines (Finegold and Singer, 1984) and also in DMPG bilayers (Blaurock and McIntosh, 1986). It is characterised by a small change in rotameric ordering. Type II sub-transition involves more rotameric disordering and more melting of the subgel phase into the liquid-crystalline phase. Head-group interactions play a particularly important role during this transition since the addition of the opposite stereoisomer prevents the sub-transition from occurring (Slater and Huang, 1987). A sub-sub-transition has been described for DPPC, with a T_m of approximately 6.8°C (Slater and Huang, 1987), which does not require a prolonged incubation time in order to be detected.

On heating the $L_{b'}$ state, phospholipids undergo the pre-transition to the $P_{b'}$ state. The pre-transition is an endothermic event which usually occurs around 5-10°C below the main transition, and with a lower enthalpy (Hinz and Sturtevant, 1972). The pre-transition has been attributed to a number of structural features, including rotations of the polar head-groups (Ladbrooke and Chapman, 1969), or a co-operative movement of the hydrocarbon chains prior to their melting temperature (Hinz and Sturtevant, 1972). A third possibility is that the pre-transition is associated with tilting of the chains prior to melting (Chapman et al, 1974). The lack of any definitive explanation for the pre-transition results in vague interpretations of the effects of other molecules, such as drugs, on the liposome bilayer. The pre-transition for DPPC liposomes occurs at approximately 36°C (Biltonen and Lichtenberg, 1993), and has been suggested to be due to structural changes in the lamellar lattice (Janiak et al, 1976) with the bilayer reorganising from a one-dimensional lamellar structure into a two-dimensional lattice distorted by periodical ripples. Within this structure the surface area occupied by the head-groups remains constant, whilst the differences in area resulting from the change in chain conformation is accommodated. The pre-transition is only thought to be seen for phospholipids that are sufficiently hydrated (Janiak et al, 1976), and is very sensitive to small amounts of contaminants. Also the temperature at which this transition occurs decreases with decreasing scan rate (Biltonen and Lichtenberg, 1993).

At the main transition, where the P_β state hydrocarbon chains "melt" to produce the L_α state, rotation of the carbon-carbon single bonds of the chains is altered

such that their configuration changes from a situation where the chains are predominantly trans to one where a number of gauche conformations are present. The hydrocarbon chains of the phospholipid molecules assume a liquid-like conformation and the lattice once more reverts to a one-dimensional structure (Janiak et al, 1976). The polar head-groups are anchored at the aqueous interface which imposes an anisotropic constraint on the molecule. The lamellar bilayer conformation is preserved throughout the transition (Engelman, 1971), but the tilted structure characteristic of the gel-phase bilayer reverts to a perpendicular orientation in the liquid-crystalline phase. Since the intermolecular distance between molecules is only around 2 nm, the rotation in one molecule affects adjacent perpendicular molecules making this transition a co-operative event (Nagle, 1980). A decrease in the thickness of the bilayer is also observed (Juliano and Layton, 1980). The main transition of DPPC occurs at approximately 41.3 °C, and the enthalpy change associated with this transition has been reported to range from 21 J/g (Miyajima et al, 1993) to 50 J/g (Ladbrooke and Chapman, 1969; Biltonen and Lichtenberg, 1993) and 57 J/g (Hinz and Sturtevant, 1972).

4.1.3. Thermal analysis of liposomes other than MLVs

The behaviour of phospholipid bilayers so far described applies to large MLVs or LUVs, but SUVs show a characteristic profile of their own. High-sensitivity DSC studies showed that freshly sonicated vesicles of disaturated phosphatidylcholines had no pre-transition and a decreased enthalpy of the main transition (Suurkuusk et al, 1976). SUVs of DPPC exhibit a T_m approximately 4°C less than equivalent MLVs, and also broader with a smaller enthalpy (Melchior and Stein, 1976). The anchoring of the polar head-groups at the aqueous interface imposes a restraint on the motion of the molecules; however as liposome size decreases so the curvature of the membrane increases, and a degree of asymmetry develops. The small radius of curvature of SUVs results in thermodynamically unfavourable packing constraints on the hydrocarbon chains which may be relieved by intermembrane fusion, seen particularly at low temperatures. Therefore, below the T_m chains are in a more highly disordered

state than in planar membranes, and their tight packing prevents SUVs undergoing a pre-transition.

SUVs of DPPC undergo the main phase transition at 37 ° C, compared to 41°C for MLVs (Biltonen and Lichtenberg, 1993), indicating that vesicle size influences the thermal behaviour of phospholipids. LUVs are likely to have a broader transition than MLVs, due to a slight alteration of the lipid-lipid interactions. This may be explained as follows: the transformation of a bilayer from liquid-crystalline to gel phase is an exothermic process, and may be attributed to the formation of van der Waals contacts. The effect of curvature is elevated for smaller vesicles, and packing of phospholipid chains is altered such that fewer van der Waals contacts are formed in SUVs than in LUVs. Therefore in large vesicles, the effect of curvature is unlikely to have a pronounced effect on the packing of the lipids in the gel phase bilayer, and the transition temperature does not change significantly upon increasing the vesicle size. This also results in the larger enthalpy difference associated with the gel to liquid-crystalline transition of the LUVs in comparison to the SUVs.

4.1.4. Thermal analysis of liposomes composed of phospholipids other than DPPC

The structures of several lipids, differing in chain length and/or head-group may be seen in Section 1.1.5. The main gel to liquid-crystalline phase transition of phospholipids is not only dependent on chain length and the chemical structure of the phospholipid hydrocarbon chains, but also on the nature of the head-group, the interactions of which are in turn affected by the pH and ionic composition of the aqueous phase (Melchior and Stein, 1976). Therefore thermal profiles of liposomes composed of different phospholipids vary according to the chain length and head-group.

Firstly, transition temperatures and enthalpies are dependent on the lengths of the two acyl chains of phospholipid molecules. For example, in symmetrical saturated 1,2-diacyl phosphatidylcholines, increasing the chain length results in an increase in the transition temperature by 7-14 ° C per additional carbon atom, and the enthalpy increases with the number of methyl groups by 4.18-8.37 kJ/mole per methyl group

(Biltonen and Lichtenberg, 1993). DSPC and DLPC have the same head-group as DPPC but chain lengths of C_{18} and C_{12}, respectively. In general, increasing the chain length increases the enthalpy of the T_m (Chapman et al, 1967). Therefore, DLPC has a reported enthalpy of only 19.5 J/g compared to 53.5 J/g for DSPC (Biltonen and Lichtenberg, 1993). Phospholipids with longer chains have a higher T_m than those with the same head group but shorter chains (Chapman et al, 1967). Accordingly DSPC has a T_m of 58°C whilst DLPC has a T_m of 0°C (Ladbrooke and Chapman, 1969). In addition, the interval between the pre- and main transition increases as the chain length decreases (McMullen et al, 1993).

As well as chain length, saturation is another important factor which determines transition temperatures and enthalpies. In general trans double bonds only have a slight effect on phospholipid packing, and consequently on transition temperature. But the introduction of a cis bond can dramatically decrease the T_m, because the free volume and rotational degree of freedom of chains is increased. This leads to decreased order and therefore increased entropy of the phospholipid. Therefore increasing the saturation of the chains increases the enthalpy of the T_m (Chapman et al, 1967), and for phospholipids with the same head-group and degree of hydration, increasing saturation in the hydrocarbon chains also increases the T_m (Ladbrooke et al, 1968).

Because T_m is highly dependent on chain length and degree of unsaturation, this suggests that the interactions between the non-polar chains is important. However the head-group also affects phospholipid transitions, depending mainly on the ionisation of the phospholipid and the pH of the surrounding solution (Biltonen and Lichtenberg, 1993). For instance, DSC studies indicate that the choline group of phospholipids is essential for the pre-transition. For example, DPPC and DPPE transition profiles are similar but the latter does not show a pre-transition (Ladbrooke and Chapman, 1969) because of the lack of choline groups in its structure. This suggests that the interaction between the choline groups and surrounding water molecules is responsible for the structural transformation involved in the pre-transition (Janiak et al, 1976). However, it has also been reported that phospholipids with bulky head-groups, such as DPPC and DPPG, do exhibit a pre-transition due to the fact that their large polar head-groups are unable to fit into the same plane and segments of the bilayer extrude to form the ripple phase (Lo and Rahman, 1995).

In negatively-charged phospholipids, such as dipalmitoylphosphatidic acid (DPPA) and dipalmitoylphosphatidylglycerol (DPPG), steric hindrance, hydrogen bonding and electrostatic charges can affect head-group interactions, and therefore phase transition properties. When dispersed at neutral pH, DPPG has an enthalpy and T_m almost identical to DPPC (approximately 41°C) according to McElhaney (1982). However, New (1990f) claims that the bulky glycerol group in conjunction with electrostatic repulsion of the unprotonated phosphate at pH 7, gives DPPG a main transition of almost 10°C below that of DPPC. In contrast, DPPA has a small head-group and a high T_m (67°C) which may be explained by the intermolecular interactions (in this case hydrogen bonding) between adjacent molecules, since at neutral pH one dissociable proton is still present. At high and low pH, the T_m is reduced, particularly at high pH where electrostatic repulsion can push the head-groups apart.

4.1.5. Principles of DSC

When the temperature of any material is increased the system will change energy states. This may occur in a co-operative manner as is the case for the thermotropic phase transition of phospholipids (Hinz and Sturtevant, 1972; McElhaney, 1982) where rotational isomerism in alkyl chains gives rise to trans and gauche conformers. DSC is a suitable technique with which to study these phase transitions.

DSC involves the indirect measurement of differential power during a thermal event. The principles behind DSC involve the simultaneous heating of a sample and a reference in two small aluminium pans at the same rate. The reference pan may be empty or may contain material that does not undergo any transition within the temperature range of interest. The temperatures of both pans are expected to initially increase linearly with time and the temperature difference between them is maintained at zero. If the sample under investigation undergoes a thermally-induced event, then a temperature differential between the two pans will result, and the control system detects this. These events can be either endothermic (such as melting or dehydration) or exothermic (such as crystallisation), and the magnitude of deflection depends on the magnitude of the heating rate. Alternatively the event may involve a change in the heat capacity of a sample (known as glass transition phenomena). The recorded DSC

parameter is excess specific heat or differential heat, as a function of temperature (heat flow), usually expressed as mW.

There are two designs of DSC, the first being the power compensation DSC which involves individual sample and reference pans being heated separately by microfurnaces. These furnaces, which are made of inert platinum-iridium alloy, are mounted in a heat "sink" made of aluminium. A power compensation DSC works on a "null balance" principal. In other words two separate control loops are used to precisely control the temperature of the furnaces. The average temperature control loop ensures that both furnaces are provided with the same amount of heat at the selected rate, whilst the differential temperature control loop is used to measure the differences in heat that occurs between the sample and reference. It is the latter loop that acts to adjust the heat input of the sample pan to bring the two furnaces back to the same temperature. Thus the system is always kept at a thermal null. The amount of energy provided by the differential control loop is directly proportional to the energy change of the system, and hence power compensation DSC is capable of direct energy measurements. For example, for a liposomal sample at the phase transition, the system requires extra heat to be supplied to the sample pan in order for the temperatures of the two pans to remain equal.

The second DSC design is known as heat flux DSC. This consists of a sole furnace containing both the sample and reference pans which are heated from a single heat source. This system operates on a "heat leak" principle, whereby the temperature of the heat sink containing the sample and reference pans, is raised at a constant heating rate. The temperature difference that arises between the two pans when the sample undergoes a thermal event is measured, and the resultant signal is converted to heat flow which is then used to calculate heat capacity.

DSC has many advantages over other techniques used to measure thermal transitions. It is relatively inexpensive, measurements and data interpretation is fairly straightforward and DSC accurately reports on the entire course of broad phase transitions (such as the onset, maximum and the shape of the transition peak). More importantly DSC does not require the introduction of foreign probe molecules, which may localise at phase boundaries and cause microenvironmental perturbations. But, whilst DSC can provide useful information regarding the changes associated with the

phospholipid bilayer transformation, it does not provide direct information about the structural state of the phospholipid under a given set of conditions.

4.1.6. Information derived from thermoanalytical data

DSC data are presented in the form of a thermoanalytical curve (sometimes referred to as a thermogram), with differential power usually plotted against temperature. Heat capacity is a value that is directly obtainable by DSC, and the specific heat capacity for a unit mass of material is defined as the energy required to raise the temperature of the material by 1 K. Endothermic and exothermic events are registered as peaks and troughs respectively. The integral under the curve is equal to the enthalpy of the transition, which is directly proportional to the heat evolved or absorbed during a thermal event. The area is constructed from the departure of the program line from the base line, to the point at which the endotherm or exotherm rejoins the base line. This method involves a high degree of operator bias, and whilst sharp transitions can be accurately measured, for broader transitions the departure temperatures are less easily defined, potentially giving false estimates of the transition energy.

Heat capacity curves are those constructed using heat capacity and temperature as the y and x axes, respectively. The information derived from these curves has been limited to the determination of standard enthalpy changes (ΔH) of the transition, the onset and peak transition temperatures (the maximum heat capacity) and the entropy change (from the transition temperature and enthalpy values) (Biltonen and Lichtenberg, 1993). However the shape of the curve may be indicative of the nature of the interaction between the drug and the bilayer (Jain and Wu, 1977). For the main transition of phospholipids, T_o represents the onset of the transition, whilst T_m represents the temperature at which the transition is half complete. However for asymmetric traces (characteristic of certain phospholipids), the T_m does not represent the midpoint of the transition and instead $T_{½}$ may be reported. $T_{½}$ is the width of the peak at half its height (sometimes described as the half-height width or HHW), and has been advocated as a measure of the interaction of compounds with the phospholipid bilayer (Jain and Wu, 1977).

4.1.7. Effects of drugs on the transition behaviour of phospholipids

Since the bilayers of liposomes are made up of co-operative units, conformational changes within this region, such as chain motion, can be transmitted to other surrounding chains. Small phospholipid-soluble molecules, such as hydrophobic drugs, can be incorporated in between these hydrocarbon chains, and they exert a disruptive effect on bilayers by interfering with chain packing. They also act as a block, hindering co-operative transmission. The result of this is a local effect rather than one involving the whole co-operative unit. Therefore the presence of small additives can have a very pronounced effect on the transition profile of DPPC bilayers (Jain and Wu, 1977). Whilst the type of effect is dependent on the nature of the entrapped molecule and its location in the bilayer, the extent of the effect is dependent on the concentration of the entrapped material.

DSC is very sensitive to alterations in phospholipid packing, and if packing is interrupted, then the order parameter is reduced and the entropy of the phospholipid increases. Therefore the phase transition occurs at lower temperatures. In other words the presence of any impurity that interferes with chain packing, reduces the temperature of the main transition. However, in most cases the effect of impurities in a liposome sample will have a more pronounced effect on the pre-transition than on the main transition (Fildes and Oliver, 1978; Arrowsmith et al, 1983b).

DSC can be used to measure the entrapment of hydrophobic drugs within liposomes. Thermograms of liposomes, in the presence of drugs, generally show broadening of the curve of the main transition, measured using the HHW which indicates that the drug is affecting the co-operativity of this transition (Biltonen and Lichtenberg, 1993). The HHW has been used in the past to describe the effects of concentration when studying the entrapment of small molecules (Jain and Wu, 1977). This value has been further used to quantify accurately the maximum entrapment of drugs into liposomes (Fildes and Oliver, 1978; Arrowsmith et al, 1983b), with the maximum value of HHW indicating saturation of the bilayer with the drug under investigation.

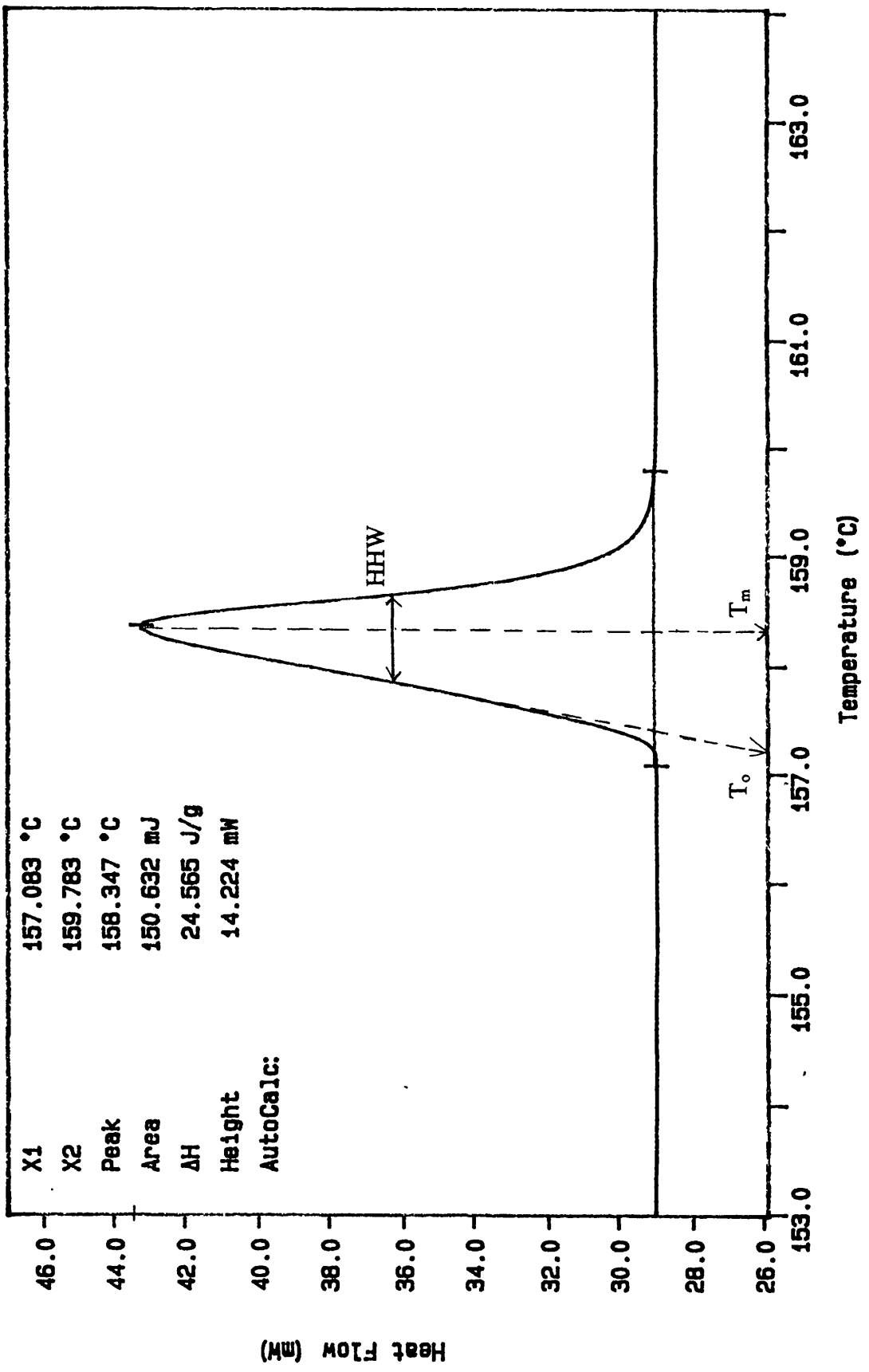
Entrapment has also been measured by assessing the decrease in the temperature of the onset of the transition (T_o) (Shaw et al, 1976). However a decrease

in T_o follows the same pattern as an increase in HHW. Since the quoted transition temperatures of phospholipids are the measured peak temperatures, these have been measured when comparing phospholipid transition temperatures in the absence and presence of BDP. The measurement of the peak temperature (T_m) has been reported to be better than T_o, when quantifying shifts in the transition temperature (Arrowsmith et al, 1983b), and therefore was measured in preference to T_o. In addition, HHW values were calculated in order to examine the effects of BDP concentrations on the widths of transition peaks. T_o, T_m and HHW measurements are outlined in Figure 4.2.; a DSC trace for a sample of indium (for calibration purposes), with heat flow (mW) plotted against temperature (°C).

4.1.8. Steroid-phospholipid interactions

If the effects of incorporating steroid molecules into DPPC liposomes are to be considered, then it is first necessary to understand the structure of DPPC bilayers. The head-group cross-section area of DPPC is 0.42 nm^2, compared to that of its alkyl chains, which is 0.39 nm^2 (Street, 1993). This difference results in DPPC molecules adopting a tilted orientation with respect to the bilayer normal (Lo and Rahman, 1995). Orienting these chains perpendicular to the interface without the introduction of "spacer" molecules would introduce considerable free space volume into the bilayer, that may be occupied by any entrapped molecules. Cholesterol is often incorporated into liposomes to modify the fluidity of bilayers, and its action in condensing phospholipid monolayers has been partially ascribed to a such a space-filling role (Zatz and Cleary, 1975), where cholesterol molecules remove the hydrocarbon chain tilt from the gel state. On inspection of thermograms of DPPC liposomes containing cholesterol, it is evident that as the concentration of cholesterol increases (between 5 and 10 mole %) the pre-transition peak is eliminated, and as the concentration of cholesterol is further increased (when the molar concentrations of cholesterol and DPPC are equal), the main transition peak is also eliminated (McMullen et al, 1993). The elimination of the pre-transition peak is attributable to cholesterol molecules removing the bilayer tilt. McMullen et al (1993) found that the concentration of cholesterol required to abolish the pre-transition of DPPC liposomes was identical to

Figure 4.2. DSC thermogram of a sample of indium, showing T_o, T_m and HHW measurements.



that required to remove the pre-transition in liposomes composed of phospholipids with differing chain lengths (C_{14}-C_{20}), thus suggesting that the effect of cholesterol on the pre-transition is not dependent on the hydrocarbon chain length of the liposomal phospholipid. Since the molar concentrations of cholesterol and DPPC are equal when the main phase transition is abolished we may assume that cholesterol affects the mobility of individual chains of phospholipids by intercalating between individual DPPC molecules.

DSC has been used in the past to create thermal profiles of DPPC liposomes containing steroidal drugs. For example, DSC has been performed on liposomes in the absence and presence of the esterified form of hydrocortisone (hydrocortisone-21-palmitate), which was found to have a greater percentage entrapment than hydrocortisone itself (Fildes and Oliver, 1978), due to anchoring of the ester chain within the bilayer. Fildes and Oliver (1978) found that on increasing the concentration of hydrocortisone-21-palmitate in DPPC liposomes, the HHW increased to a maximum (at 13.2 mole % steroid) and then showed a decrease. This was interpreted as saturation of the bilayer with drug at this concentration, with a subsequent separation of a new steroid-rich phase on further increasing the drug concentration. In addition, Arrowsmith et al (1983b) found that the pre-transition of DPPC liposomes was abolished on the addition of 2.5 mole % hydrocortisone palmitate, and HHW values indicated bilayer saturation at 11.25 mole % steroid.

4.1.9. Aim of DSC studies

The use of liposomes as a vehicle with which to deliver BDP to its target site requires that the dose of BDP within this system is completely liposome-associated and that the dose can be accurately determined. Whilst other work has focused on the identification of excess steroid in liposomal samples, their subsequent removal, and determining the entrapped concentration, there is still a need to characterise the behaviour of BDP within phospholipid bilayers. DSC is a technique that is clearly suited to measuring phospholipid phase transitions (Ladbrooke and Chapman, 1969), which are sensitive to the presence of hydrophobic drugs in the bilayer. Therefore the aim of these studies was to utilise this technique in order to determine the effects of BDP on bilayers composed of different phospholipids, and containing various amounts

of the steroid. By doing so it was hoped that a thermal profile of the effects of BDP on different bilayers could be established. This profile could then be used to establish the events that occur at the saturation concentration, after which excess BDP crystallises out into domains. Similar profiles may be useful in the future when determining the compatibility of steroidal drugs with different phospholipids.

4.2. Materials

The phospholipids used throughout these investigations were dipalmitoylphosphatidylcholine (DPPC) (approximately 99 % pure), dilauryl-phosphatidylcholine (DLPC) (approximately 99 % pure), distearoylphosphatidylcholine (DSPC) (approximately 99 % pure), dipalmitoylphosphatidylglycerol (DPPG) (approximately 98 % pure) and dipalmitoylphosphatidic acid (DPPA) (approximately 98 % pure), the structures of which are depicted in Section 1.1.5. All phospholipids were purchased from Sigma Chemicals (Poole, Dorset), and stored below 0°C. Beclomethasone dipropionate was kindly supplied by Glaxo Wellcome (Ware, Herts.) as a micronised powder of the monohydrate (MW = 539.1). Distilled water used to make liposomes was further purified by passing through an Elgastat UHQ-PS unit. Chloroform used in the manufacture of liposomes was purchased from BDP (Poole, Dorset) and was AnanlaR Grade. Indium used in DSC calibration was supplied by Perkin-Elmer (Beaconsfield, UK).

4.3. Methodology

Perkin Elmer was the first company to design the power-compensated DSC, and the most recent model, the DSC7, has incorporated the PE 3700 computer. This model is capable of heating samples at rates of 0.1 to 200°C/minute, in 0.1°C increments, over a temperature range of -170 to 725°C. Once the sample and reference pans are prepared, they are placed in their respective furnaces by means of forceps. In addition, an inert gas atmospheres is created around the immediate vicinity of the sample and reference pans, by the use of nitrogen as a purge gas, at a flow rate of 30 ml/minute (and flow-through covers). Purges are necessary when samples are in

open pans, as the inert gas sweeps away any volatile products. In these studies however, purges were essential to decrease baseline noise and prevent ice formation on furnaces, as a result of refrigeration. A refrigeration system was in operation continuously throughout this study, which required a further nitrogen purge in operation. The run conditions were stored in the PE 3700 computer, and all runs were carried out four times. The PE 3700 computer is capable of simultaneous data analysis and operation, and hard copies were made using a multiple-pen printer plotter.

4.3.1. DSC calibration

Quantitative analysis requires temperature and enthalpy calibration, and the substance most commonly used for this purpose is indium, which melts at 156.6°C, with an enthalpy of fusion of 28.71 J/g. This material is widely available as a very pure (greater than 99.9999 %) solid that can be reused frequently.

A sample of indium was prepared for calibration by weighing a small amount of the calibrant into an aluminium DSC pan, and then hermetically sealing this pan. DSC was performed against another sealed, but empty, reference pan, over a temperature range of 100-200°C, at scan rates of either 5°C or 10°C/minute, depending on the parameter under investigation. The maximum (peak) temperature achieved was noted and corrections were made based on the true melting temperature of indium.

Variables which may affect calibration include heating rates, instrument warm-up (which influences the temperature of the block) and the purge gas used. For these reasons, the instrument was allowed an equal warm-up time every day (1 hour), after which calibration was performed, at the same heating rate as the experiment. In addition, calibration was carried out every time the encapsulation procedure was altered, at the new settings. Every month, the calibration values for the melting temperature and enthalpy of indium were entered into the calibration menu of the DSC7 instrument.

4.3.2. Sample preparation

4.3.2.1. Preparation of liposomes

Liposomes were prepared according to the procedure outlined in Section 2.3.2.2. The temperature of the water bath was held at approximately 15°C higher than the main phase transition temperature of the respective phospholipid.

4.3.2.2. Sample encapsulation in DSC pans

Due to the liquid nature of the liposomal suspensions, samples require encapsulation in sealed pans in order to prevent any of the sample leaking out during the experiment. Proper encapsulation is an important factor in achieving thermal contact, and thus reducing thermal lag and obtaining accurate results. This was achieved by depositing a small predetermined amount of the liposomal sample into an aluminium pan drop-wise, by means of a microsyringe (Hamilton, Nevada, USA), to ensure uniform sample contact with the pan base. The weight of the sample was determined by taring the empty pan and reweighing it after deposition of the sample. Then an aluminium lid was immediately placed on top using forceps, to avoid any sample loss. The whole ensemble was then transported to a sealing device, which works by pressing the pan and lid together to achieve a seal, thus the sample is fully encapsulated in a hermetically sealed closed pan. This achieves optimum thermal conductivity, and the airtight seal ensures that leakage of the liquid sample does not occur. References were also hermetically sealed pans, which were either empty or contained water, depending on the parameter under investigation.

4.3.3. DSC parameter investigations

The baseline deflection seen at the start of a scan, is associated with the system changing from isothermal to dynamic status. The extent of this initial deflection depends on the sample mass, specific heat, heating rate employed and the sensitivity of the instrument (Ford and Timmins, 1989).

Ford and Timmins (1989) have stated that in the determination of transition temperatures, "the greatest accuracy will be achieved with a small sample size, proper sample encapsulation, slow scanning speeds and correct instrumental calibration".

4.3.3.1. Investigations into different sample concentrations

The sample size (and particle size of that material) can introduce procedural variables, which must be eliminated prior to investigations. Whilst larger sample sizes are preferable for increasing sensitivity (thus aiding the detection of small events) and accuracy (in terms of enthalpy determinations), smaller sample sizes have the advantages of optimum pan contact (thus decreasing thermal lags), better resolution and increased accuracy (in terms of temperature determinations). Usual sample sizes range from 5 to 10 mg.

Various concentrations of DPPC liposomes were prepared in order to determine differences, if any, arising from using different concentrations. It may be that the observation of some thermal events are more clearly outlined when the concentration of phospholipid is higher. For example, a small event such as the pre-transition has a larger enthalpy for higher concentrations, and therefore produces a larger peak. Whilst this may be beneficial when making comparisons with liposomes containing drug, it may also be costly and wasteful to manufacture liposomes containing such a high concentration of phospholipid. Therefore it is important to determine whether lower concentrations of phospholipid can also produce peaks that are consistent and reproducible, before higher concentrations are utilised. In the past, transitions of phospholipids have been studied using liposomal suspensions that have ranged from very dilute concentrations of 0.04 -0.66 % w/w (Hinz and Sturtevant, 1972), to concentrations of up to 50 % w/w (Fildes and Oliver, 1978). The higher concentrations have been used when drug-bilayer interactions are under investigation. Accurate weighing is dependent on the quality of the analytical balance. In these studies a Perkin Elmer Autobalance (Model AD-4, Perkin Elmer, Beaconsfield) was calibrated daily prior to use.

DPPC liposomes were made, according to the procedure outlined in Section 2.3.2.2., to achieve final concentrations of 25, 50 and 100 mg/ml. A scan rate of 10°C

was chosen for this study since we were not interested in any rate effects, and for similar reasons a reference of air was chosen.

4.3.3.2. Investigations into different scan rates

It has been reported by van Dooren (1982) that an increase in the heating rate of the experiment resulted in a decrease in the observed transition peak temperature of adipidic acid. Another feature related to increasing the scan speed is the peak area, which will also decrease (and hence resolution will decrease). A high scan rate may cause the merger of two peaks, due to instrumental thermal lag. Therefore to separate events that occur close to one another, and to obtain accurate transition temperatures, a slower scan speed is recommended (Ford and Timmins, 1989). Heating rates are usually between 10 and 20°C/minute. However, whilst lower rates generally result in higher resolution, low sensitivity is also experienced, and therefore smaller events may not be detected. In addition, longer scan times may result in sample degradation (Coleman and Craig, 1996). Therefore experiments were performed at scan rates of 5 and 10°C/minute to determine which rate should be used in further investigations.

4.3.3.3. Investigations into different references

Reference samples usually consist of an empty closed pan, but recently they have included a quantity of the vehicle of the sample system (Coleman and Craig, 1996). A suitable inert material should be used to balance the reference and sample pans with respect to weight. Therefore these studies have investigated the possibility of reference pans containing distilled water from an Elgastat UHQ-PS system, and also empty (air) reference pans.

DPPC liposomes having a concentration of 100 mg/ml were tested against references of either air or water. The latter were prepared by injecting an appropriate amount of Elgastat UHQ-PS water (approximately equal in weight to the corresponding liposome sample - 5 to 7 mg) into pans which were subsequently sealed.

4.3.4. Studies on liposomes containing BDP

Liposomes composed of various phospholipids were prepared in the absence and presence of BDP. DSC thermograms were obtained for each sample scanned to compare the effects of BDP in different bilayers.

Liposomes containing 100 mg/ml DPPC with BDP (0, 0.5, 1, 2.5, 5 and 6 mole %) were prepared by the technique described in 2.3.2.2. The steroidal drug was added during the initial stage of manufacture when all the components were dissolved in chloroform. Liposomes containing 100 mg/ml DSPC, DLPC, DPPG, DPPA with BDP (0, 1, 2.5 and 5 mole %) were also prepared and tested.

Since the measurement of the main phase transition of DLPC liposomes coincides with the freezing of water in the sample, only the pre-transition (which occurs at approximately -8°C) was measured.

4.4. Results and Discussion

For all samples, the temperatures noted for the pre- and main transitions were the maximum peak temperatures obtained, T_p and T_m (Figure 4.2.). Enthalpy values, ΔH, were recorded as J/g and automatically calculated using computer software, but later corrected per g of phospholipid in the sample. The half-height widths of peaks, $T_{½}$ (or HHW) were measured manually and converted to °C (Figure 4.2.). Whilst the DSC thermograms represented are typical of the sample under investigation, it must be noted that the temperature values summarised in the tables have been corrected for calibration with indium.

4.4.1. Experimental parameter investigations

Procedural variables are likely to affect the observed transition temperatures and enthalpies (Section 4.3.3.). In order to improve the accuracy of calculated values, parameters such as sample concentration, scan rates and references were investigated to determine their suitability.

4.4.1.1. Investigations into different sample concentrations

Table 4.1. shows the values obtained from thermograms of liposomes containing different amounts of DPPC, whilst Figure 4.3. is a typical DSC trace for a sample containing 100 mg/ml DPPC.

Table 4.1. Mean transition temperatures and enthalpies (± standard deviation), and coefficient of variation values obtained for different concentrations of DPPC liposomes.

Liposome sample	Pre-transition		Main transition	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
25 mg/ml	35.25 ± 1.02	3.40 ± 0.44	40.66 ± 0.05	37.92 ± 7.36
Coefficient of variation	2.9 %	12.9 %	0.1 %	19.4 %
50 mg/ml	32.14 ± 1.53	7.10 ± 3.60	39.53 ± 0.16	49.18 ± 26.40
Coefficient of variation	4.8 %	50.7 %	0.4 %	53.7 %
100 mg/ml	36.21 ± 0.79	5.50 ± 0.66	40.83 ± 0.21	44.34 ± 0.94
Coefficient of variation	2.2. %	12.0 %	0.5 %	2.0 %

The mean temperatures obtained for both transitions correlated well with literature values: Suurkuusk et al (1976) reported peak temperatures for the pre- and main transitions as 35.4°C and 41.2°C, respectively, whilst Hinz and Sturtevant (1972) found these values to be 34.0 ± 0.2°C and 41.75 ± 0.05°C, respectively. The consistency of results were reflected by coefficient of variation values. In terms of pre-transition temperatures and enthalpies, the smallest variation was seen with liposomes composed of 100 mg/ml DPPC. For main transition temperatures the coefficient of variation values were similar, but for transition enthalpies, 100 mg/ml DPPC liposomes produced the smallest variation. In addition, liposomes containing 100 mg/ml DPPC

produced larger events (in terms of peak enthalpies) than lower concentrations, and are therefore probably more useful when making comparisons with other samples. Therefore all future studies employed samples containing 100 mg/ml phospholipid.

4.4.1.2. Investigations into different scan rates

Table 4.2. shows the values obtained from thermograms of liposomes containing 100 mg/ml of DPPC, when run against empty reference pans, at scan rates of 5 and 10 °C/minute. Similarly Table 4.3. shows the values obtained when reference pans contained water.

Temperature values for the pre- and main transitions were similar to those quoted in the literature; Biltonen and Lichtenberg (1993) found the main phase transition peak temperature to be 41.3°C, whereas the temperature of the pre-transition decreased with decreasing scan rate (as seen in Tables 4.2. and 4.3.). Main transition enthalpy values were also found to be similar to those quoted in the literature; Suurkuusk et al (1976) reported main transition enthalpies of DPPC liposomes as 46.74 J/g, which is comparable to the results obtained in Tables 4.1., 4.2. and 4.3., but Hinz and Sturtevant (1972) found this value to be 55.29 J/g. Pre-transition enthalpies were smaller than those quoted in the literature; 13.11 J/g (Hinz and Sturtevant, 1972) and 9.12 J/g (Suurkuusk et al, 1976). Suitable references and scan rates were chosen based on the consistency of the observed results. From Table 4.2., the lower scan rate produces more consistent pre-transition temperatures and enthalpies, whilst the higher scan rate gives more consistent main transition temperatures and enthalpies.

Table 4.2. Mean transition temperatures and enthalpies (± standard deviation), and coefficient of variation values obtained for DPPC liposomes (100 mg/ml), analysed against empty reference pans.

Scan rate	Pre-transition		Main transition	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
5°C/minute	35.78 ± 0.36	6.46 ± 0.58	40.24 ± 0.47	52.75 ± 11.06
Coefficient of variation	1.0 %	9.0 %	1.2 %	21.0 %
10°C/minute	36.21 ± 0.79	5.50 ± 0.66	40.84 ± 0.21	44.34 ± 0.94
Coefficient of variation	2.2 %	12.0 %	0.5 %	2.1 %

Table 4.3. Mean transition temperatures and enthalpies (± standard deviation) and coefficient of variation values obtained for DPPC liposomes (100 mg/ml), analysed against reference pans containing distilled water.

Scan rate	Pre-transition		Main transition	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
5°C/minute	35.41 ± 0.19	6.45 ± 0.34	40.09 ± 0.20	44.97 ± 2.00
Coefficient of variation	0.5 %	5.3 %	0.5 %	4.4 %
10°C/minute	37.13 ± 0.23	5.27 ± 0.71	41.45 ± 0.20	44.59 ± 6.07
Coefficient of variation	0.6 %	13.5 %	0.5 %	13.6 %

However, the transition temperatures in Table 4.3. show the least variation when a lower scan rate was chosen. Since the pre-transition is especially important

when studying the effects of drug molecules on phospholipid bilayers (Section 4.1.7.), the lower scan rate of 5°C/minute was selected for future DSC studies.

4.4.1.3. Investigations into different references

Tables 4.2. and 4.3. show the values obtained for the pre- and main transition temperatures and enthalpies of liposomes containing 100 mg/ml of DPPC, against empty reference pans (Table 4.2.) and those that contain water (Table 4.3.). As with choosing a suitable scan rate, an appropriate reference was determined from the coefficient of variation values. At the lower scan rate, both the pre- and the main transition temperatures and enthalpies were more consistent when water was used in the reference pan. At the higher scan rate, the pre-transition temperatures and enthalpies were also more consistent with a water reference, whilst the main transition temperatures and enthalpies were more consistent with empty reference pans. Since a lower scan rate was selected from the results in the Section 4.4.1.2., water was chosen as a suitable reference material.

4.4.2. Studies on DPPC liposomes containing BDP

The addition of varying molar concentrations of BDP to DPPC liposomes caused differences to appear in the thermograms of the drug-liposomal systems. These features were apparent when measuring the maximum peak temperatures and peak enthalpies of the pre- and main transitions. When these values were compared to those of non-drug-containing liposomes, direct comparisons were made to elucidate the effects of BDP on these bilayers.

Table 4.4. displays the values obtained for the pre- and main transitions of DPPC liposomes containing varying amounts of BDP, which have been corrected for calibration. Figures 4.3, 4.4(a), (b) and (c) are typical DSC traces of DPPC liposomes containing 0, 1, 2.5 and 5 mole % BDP, respectively.

The relationship between transition temperature and BDP concentration can be seen more clearly in Figure 4.5., where the addition of 0.5 mole % BDP resulted in a reduction in the temperature and enthalpy of the pre-transition, and the addition of

Figure 4.3. DSC thermogram of DPPC liposomes (100 mg/ml).

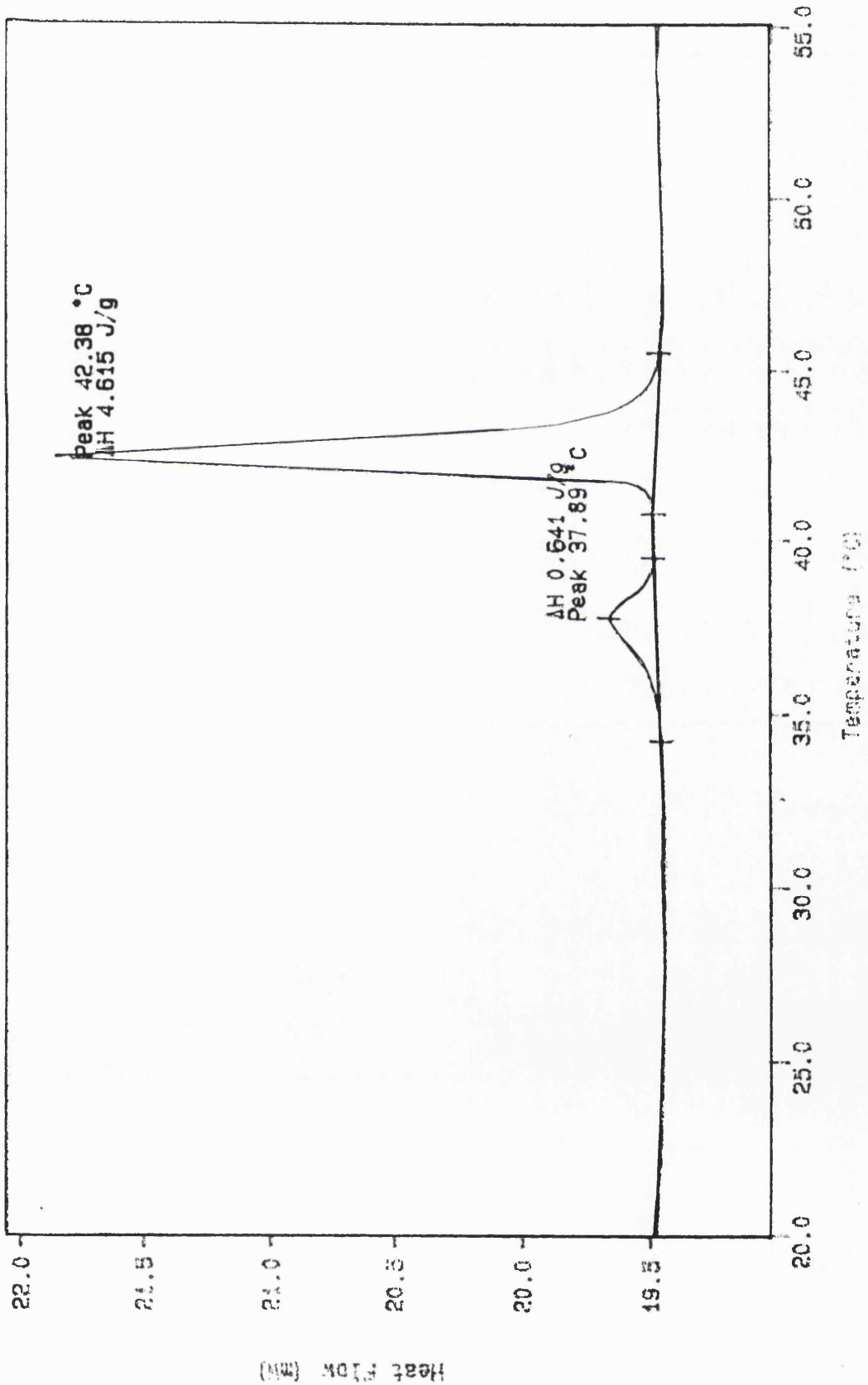


Figure 4.4(a) DSC thermogram of DPPC liposomes containing 1 mole % BDP.

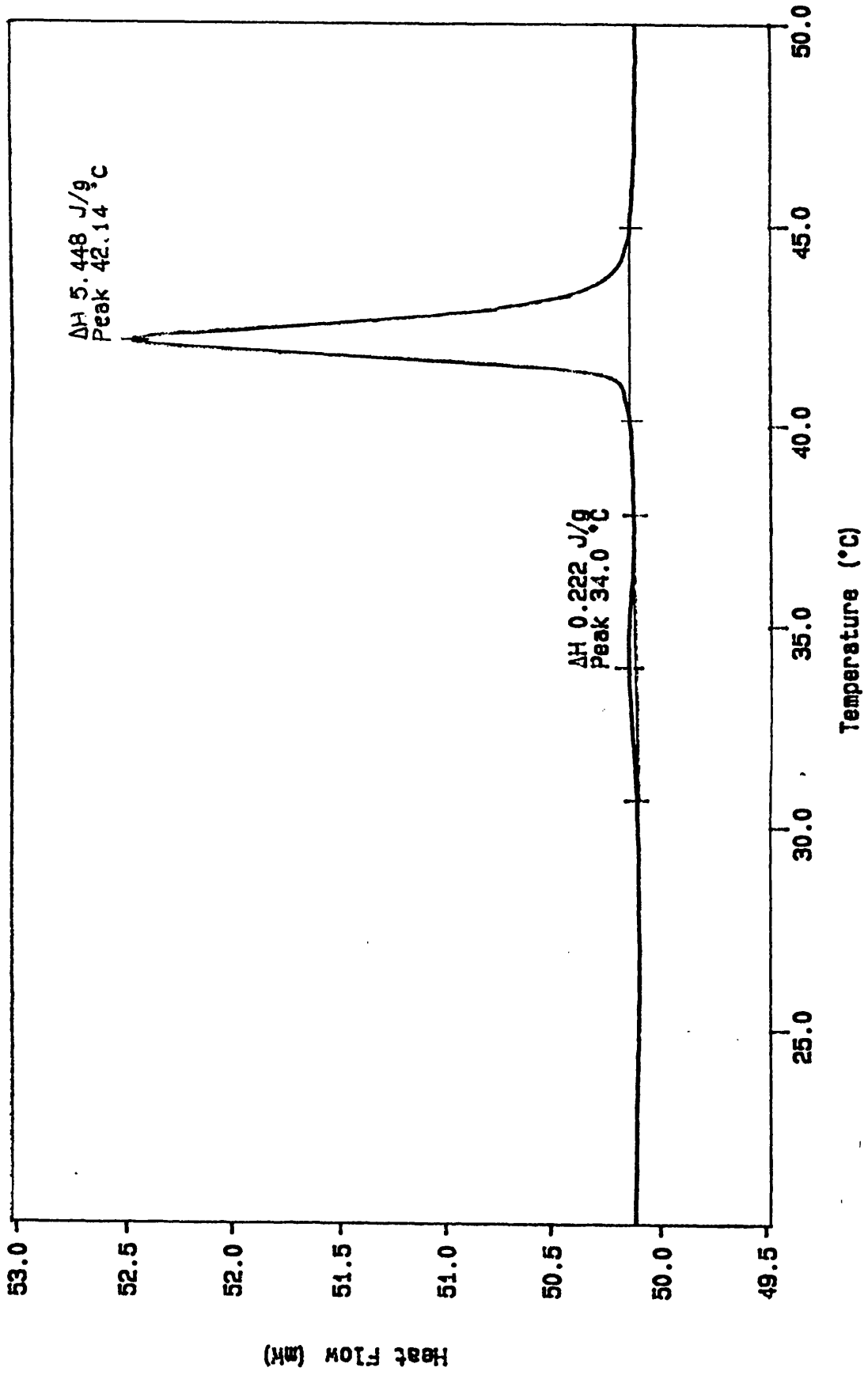


Figure 4.4(b) DSC thermogram of DPPC liposomes containing 2.5 mole % BDP.

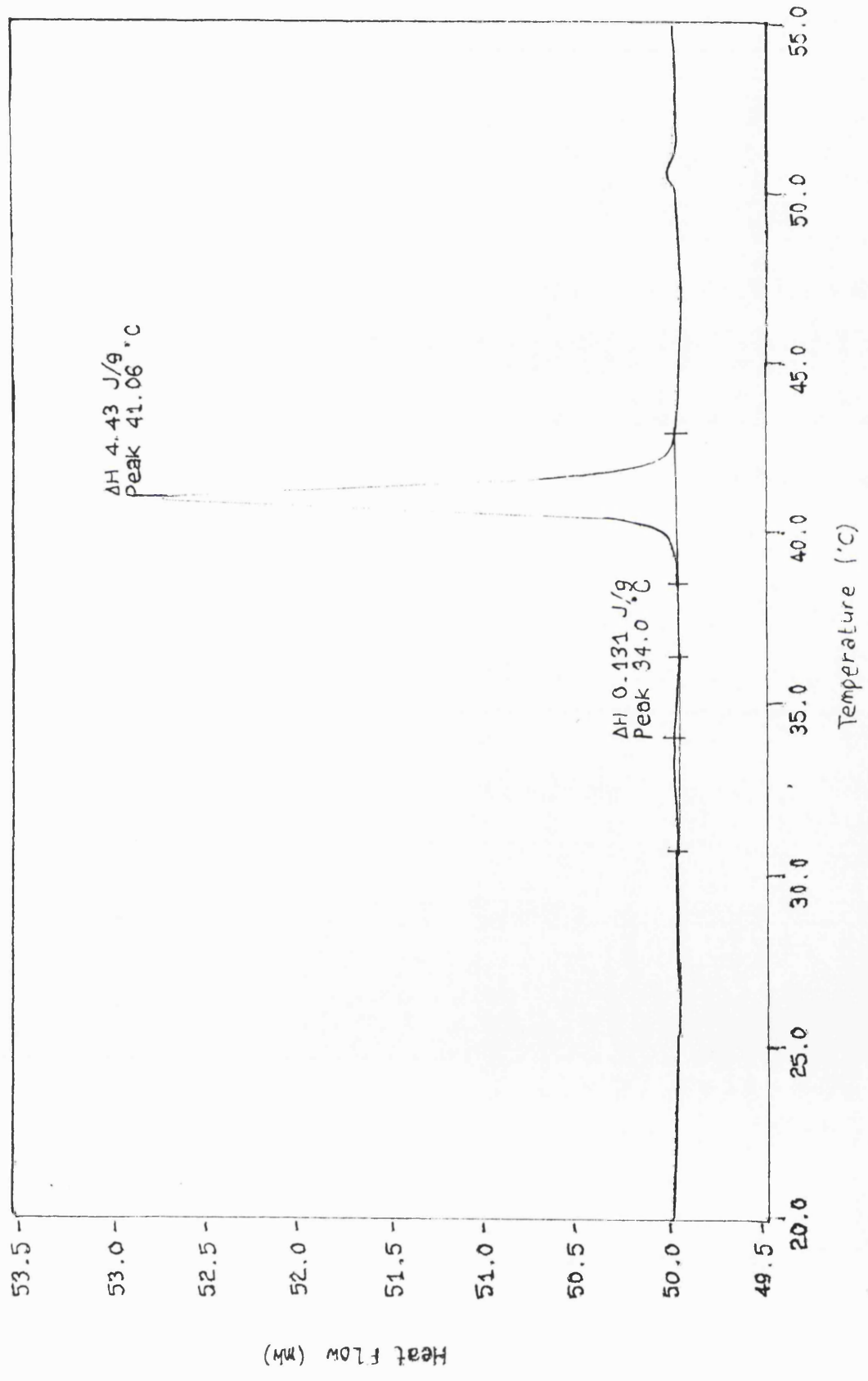


Figure 4.4(c) DSC thermogram of DPPC liposomes containing 5 mole % BDP.

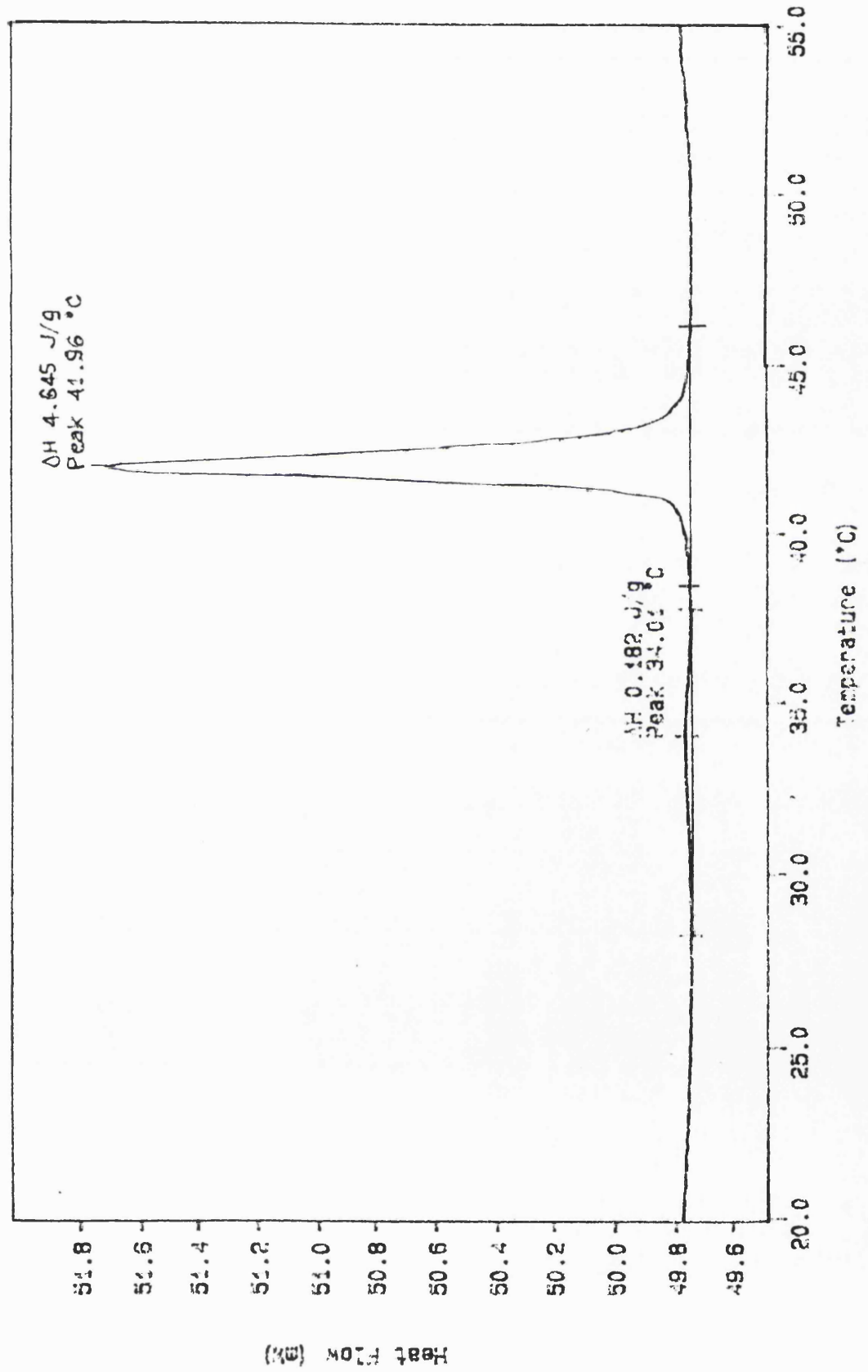
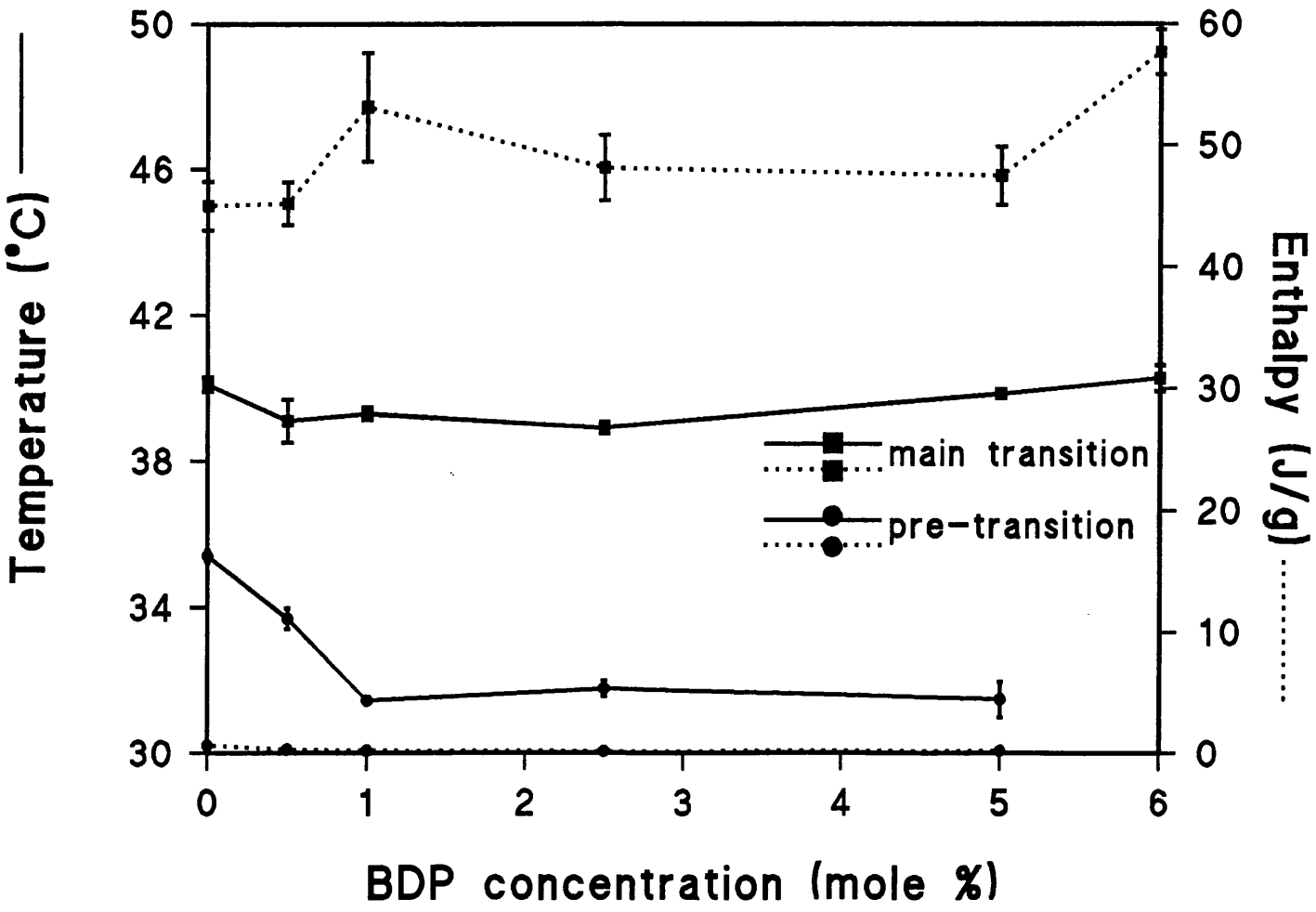


Figure 4.5. Pre- and main transition temperatures and enthalpies of DPPC liposomes containing BDP.



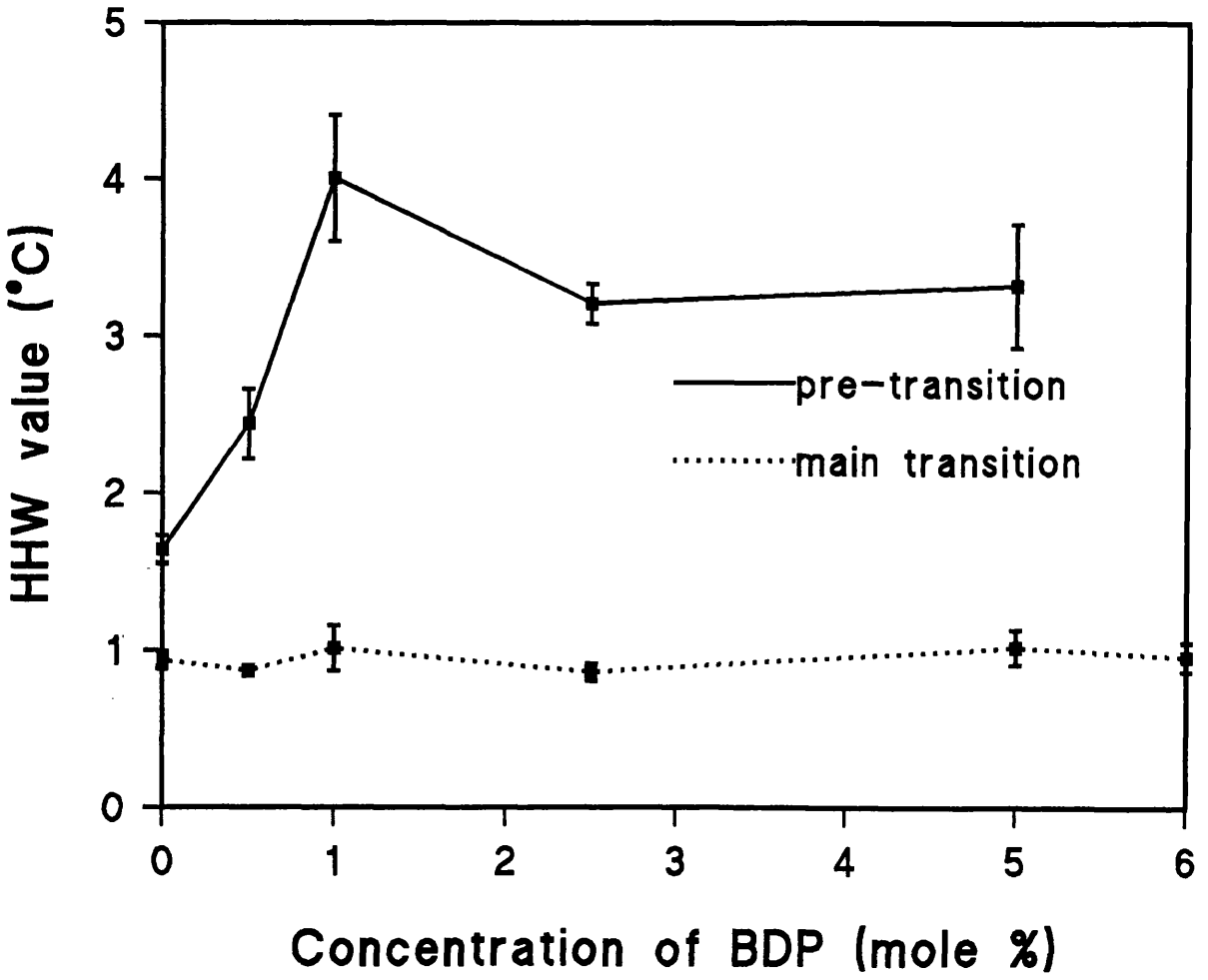
higher concentrations of BDP (above 2.5 mole %) had little effect on either parameter. At a concentration between 5 and 6 mole % BDP the pre-transition was abolished altogether. The effect of BDP on the main transition (Figure 4.5.) was not quite so apparent, with transition temperatures increasing after 2.5 mole %, and enthalpy values increasing after the addition of 5 mole % BDP. Figure 4.6. shows the effect of BDP concentration on the HHW values of both transitions. The width of the pre-transition peak increased to a maximum at 1 mole % BDP and then levelled off, whilst no real differences in the peak widths of the main transition were observed.

Table 4.4. Mean transition temperature and enthalpy values (± standard deviation) of DPPC liposomes (100 mg/ml) containing BDP.

BDP concentration	Pre-transition		Main transition	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
0 mole %	35.41 ± 0.19	6.45 ± 0.34	40.09 ± 0.20	44.97 ± 2.00
0.5 mole %	33.70 ± 0.32	3.01 ± 0.57	39.35 ± 0.20	45.17 ± 1.75
1 mole %	31.45 ± 0.08	2.05 ± 0.45	39.29 ± 0.17	53.14 ± 4.50
2.5 mole %	31.77 ± 0.24	1.40 ± 0.21	38.92 ± 0.16	48.11 ± 2.71
5 mole %	31.48 ± 0.56	1.81 ± 0.31	39.85 ± 0.13	47.48 ± 2.41
6 mole %	-	-	40.28 ± 0.36	57.66 ± 1.85

The modified thermal profile of DPPC liposomes in the presence of BDP indicated the perturbation of the bilayer by drug molecules. The results may be interpreted firstly in terms of the effects of BDP on the pre-transition, which is known to be so sensitive to impurities that it is eliminated in the presence of only 0.005 mole % gramicidin A (Chapman et al, 1974). In this case, the pre-transition was maintained up to 5 mole % BDP, but the addition of 0.5 mole % BDP reduced the pre-transition temperature (Figure 4.5.). Student t-test calculations showed that the t value obtained (7.94) was greater than tabulated values, at a probability level of 95 %, indicating that 0.5 mole % BDP significantly reduced the pre-transition temperature. Hydrocortisone-21-palmitate abolishes the pre-transition of DPPC liposomes at a concentration of 3.8

Figure 4.6. HHW values for pre- and main transition of DPPC liposomes containing varying amounts of BDP.



mole % steroid (Fildes and Oliver, 1978), whilst Arrowsmith et al (1983b) found this value to be 2.5 mole % for the same steroid.

The enthalpy of the pre-transition also decreased significantly ($p < 0.05$) on the addition of 0.5 mole % BDP and reached a minimum at 2.5 mole % BDP (Figure 4.5.). This feature is similar to that found by Taylor et al (1990a), when BDP was incorporated into DMPC films. At 5 mole % BDP the enthalpy associated with liposome formation decreased, and after this concentration the addition of more drug had a less pronounced effect. This, the authors suggested, indicated that ideal mixing was not occurring, and that a solid drug phase had to begun to co-exit with a fluid phospholipid phase. This therefore suggests that BDP domains form between 2.5 and 5 mole % BDP.

HHW values of the pre-transition reached a maximum at 1 mole % BDP (Figure 4.6.). This value has been suggested by some authors to be an indication of bilayer saturation (Fildes and Oliver, 1978; Arrowsmith et al, 1983b), whilst others have interpreted HHW values as simply a measure of the interaction between compounds and phospholipids (Jain and Wu, 1977). This then suggests that the maximum amount of BDP that can be entrapped into DPPC liposomes is 1 mole %, after which excess drug forms solid domains. However, pre-transition enthalpy values suggest that domains do not form until after 2.5 mole % BDP. Therefore, an assumption can be made that optimal accommodation of the drug in the bilayer occurs at 1 mole % BDP (or between 1 and 2.5 mole % BDP), and the formation of BDP domains affect thermal profiles at 2.5 mole % BDP.

The effect of BDP on the main transition of DPPC liposomes was a significant increase in the transition temperature between 2.5 and 6 mole % BDP ($p < 0.05$), and enthalpy between 2.5 and 6 mole % BDP ($p < 0.05$). It was thought that the enthalpy values of samples containing 5 mole % BDP may have been prone to error, such as sample preparation and handling. Lack of reproducibility in calculated enthalpies has been reported in the past by Chen & Sturtevant (1981), whilst others claimed inconsistencies to be a significant cause of discrepancy (O'Leary et al, 1984). HHW values of the main transition peaks were not altered significantly on the addition of incremental amounts of BDP up to 6 mole % BDP (all t values were less than 2.447 at a probability level of 95 %).

The addition of 0.5 mole % BDP caused the main transition temperature to decrease by 0.7°C, but further drug had little effect on temperatures up to 2.5 mole % BDP (Figure 4.5.). This may indicate that at very low concentrations the presence of BDP affected chain mobility, but the consistency of temperatures up to 2.5 mole % BDP suggests that there was little or no effect on the actual mobility of individual chains at these concentrations, since inhibition of chain motion manifests as a change in transition temperatures (Jain and Wu, 1977). When cholesterol, for example, is incorporated into bilayers at low concentrations, both the pre- and main transition temperatures are altered (McMullen et al, 1993), suggesting cholesterol produces a change in the degree of bilayer fluidity. In addition, HHW values were almost constant from 0 to 6 mole % BDP, and therefore these initial findings suggested that the steroid nucleus was probably positioned within the head-group region of bilayers, as proposed by Fildes and Oliver (1978). However, a hydrophobic molecule is unlikely to associate solely with the polar region, and the increase on transition temperature and enthalpy at higher concentrations of BDP indicates that the behaviour and location of this steroid within bilayers alters at higher concentrations. Whilst Jain and Wu (1977) categorised interactions between molecules and bilayers under four divisions, they also recognised the ability of some materials to behave ambiguously under different concentrations. In other words, whilst the extent of the interaction between phospholipid and steroid is solely dependent on the concentration of the steroid molecule, the type of interaction may be dependent on both the nature and concentration of the steroid molecule. Therefore, these results are consistent with the location of BDP molecules in the centre of the bilayer acting as transmitter blocks so that any effects on chain motion remain relatively local and an alteration in the temperature of the main transition is not observed. The incidence of gauche conformers above and below the main phase transition is greater nearer the methyl-end of the phospholipid alkyl chain, than at the head-group end (Jain and Wu, 1977). Chain ends are therefore in a relative state of disorder and this region is more fluid than elsewhere in the hydrophobic region (Phillips et al, 1969). This may explain the ease with which bulky steroid molecules are taken up and rapidly released by bilayers. Since molecules that are located within this fluid region do not interact with phospholipid molecules and have little effect on thermal profiles, HHW values remain constant. Fildes and Oliver (1978) claimed that a

constant transition temperature together with an increase in HHW values is indicative of the formation of a separate phospholipid-steroid mesophase, but results from these studies imply the formation of steroid domains is possible whilst HHW values remain constant. A broadening of the main transition peak may be observed for liposomal systems in which ideal solutions (of phospholipid and drug) are formed in both the gel and the liquid-crystalline phases (Sturtevant, 1982), and such behaviour is also compatible with the segregation of the two components into pure phospholipid and phospholipid-steroid regions. Therefore results that display a broadening of the main transition peak cannot discriminate between these two possibilities, and the possibility of domain formation can only be determined from additional techniques.

Since the pre-transition has been attributed to a number of different structural features (Section 4.1.2.), the abolition of this transition may also be ascribed to various causes. Therefore, accurate identification of the nature of the interaction between the steroid and phospholipid molecules at the pre-transition is not always possible. However, the results above suggest that BDP is located in the centre of the bilayer and is unlikely to affect the head-group region. Therefore, the pre-transition probably involves changes in the chain region, as proposed by Janiak et al (1976), who found that the pre-transition of DPPC liposomes is associated with changes in the lamellar lattice. Therefore small amounts of BDP in the terminal regions of alkyl chains are likely to produce effects on this transition.

4.4.3. Studies on liposomes composed of phospholipids other than DPPC

Figure 4.7. is a typical trace for a liposomal sample containing 100 mg/ml DSPC, whilst the mean temperature and enthalpy values obtained for the pre- and main transitions in the presence of different BDP concentrations, are displayed in Table 4.5.

Figure 4.7. DSC thermogram of DSPC liposomes (100 mg/ml).

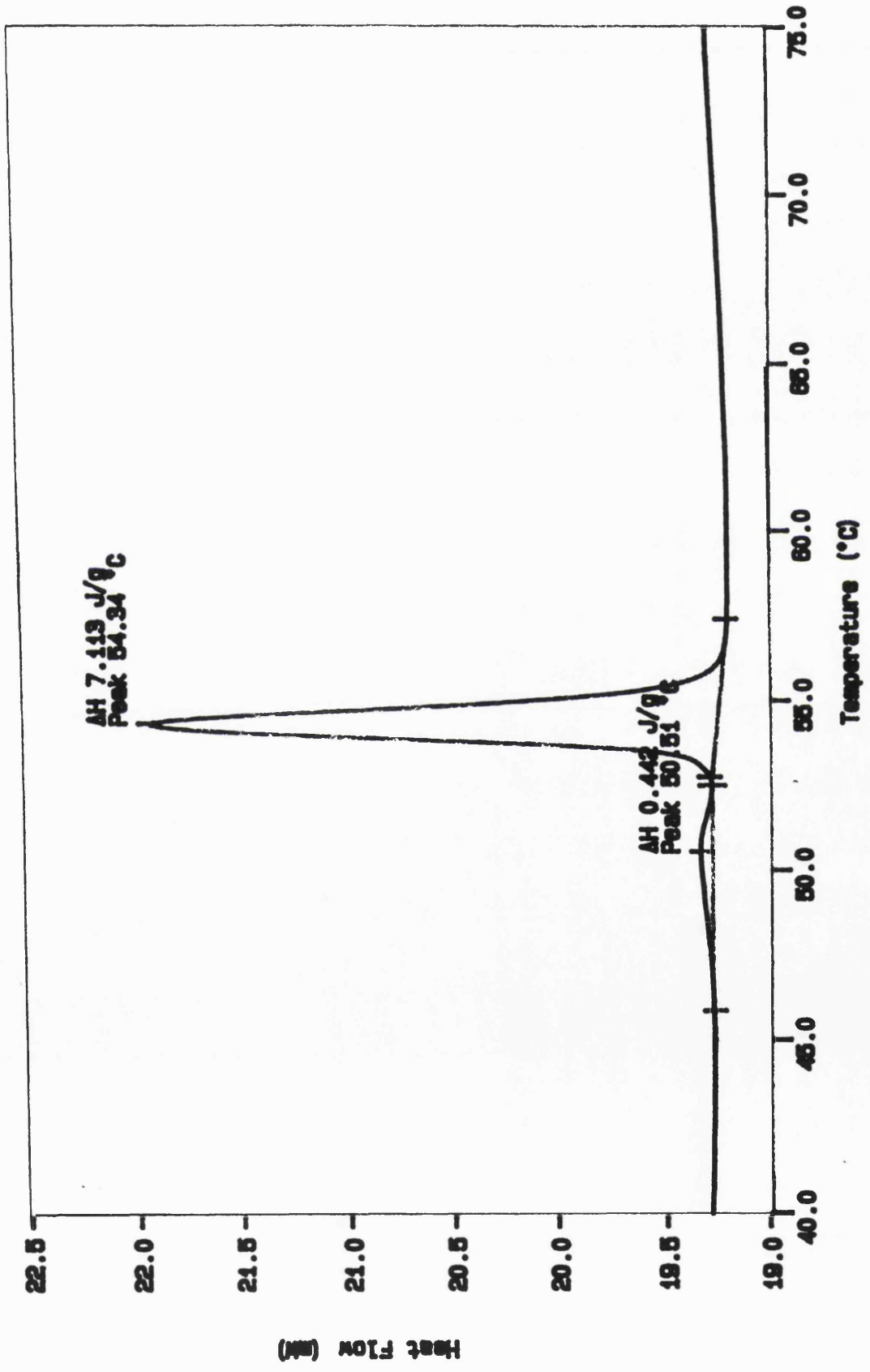


Table 4.5. Mean transition temperature and enthalpy values (± standard deviation) of DSPC liposomes (100 mg/ml) containing BDP.

BDP concentration	Pre-transition		Main transition	
	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
0 mole %	47.94 ± 0.44	3.72 ± 0.58	51.82 ± 0.418	71.04 ± 1.18
1 mole %	48.19 ± 0.39	2.10 ± 0.40	52.76 ± 0.29	64.15 ± 3.63
2.5 mole %	ND*	ND*	52.61 ± 0.38	57.52 ± 6.68
5 mole %	ND*	ND*	52.37 ± 0.15	59.86 ± 1.83

* ND denotes not detected.

The incorporation of BDP into DSPC liposomes produced a significant decrease in the enthalpy of the pre-transition at 1 mole % BDP ($p < 0.05$), and no significant change to the temperature ($p > 0.05$) (Figure 4.8.). In excess of this concentration, the pre-transition was abolished. The effects on the main transition temperatures of DSPC liposomes was negligible, whilst enthalpies decreased up to the addition of 2.5 mole % BDP (Figure 4.8.). Measurement of the HHW values showed that the pre-transition peak significantly increased in width ($p < 0.05$) on the addition of 1 mole % BDP (Figure 4.9.), but the main transition peaks were not significantly affected ($p > 0.05$) by an increase in BDP concentration.

The elimination of the pre-transition peak at concentrations above 1 mole % BDP, compared to 5 mole % for DPPC, suggests phospholipid chain lengths are accountable for the observed differences, since both phospholipids have the same head-group. However, it is unclear as to how similar concentrations of BDP should produce different effects on DSPC and DPPC bilayers. Hydrocarbon chains in a bilayer have restricted mobility (Hinz and Sturtevant, 1972), and it may be that molecules in a bilayer with longer chain lengths (DSPC) produce a larger effect in terms of chain motion, than in bilayers of shorter chain lengths (DPPC), which may explain the discrepancy. The width of the DSPC pre-transition increased on the addition of 1 mole % BDP (Figure 4.9.), indicating perturbation of the bilayer by BDP molecules at this concentration.

Figure 4.8. Pre- and main transition temperatures and enthalpies of DSPC liposomes containing BDP.

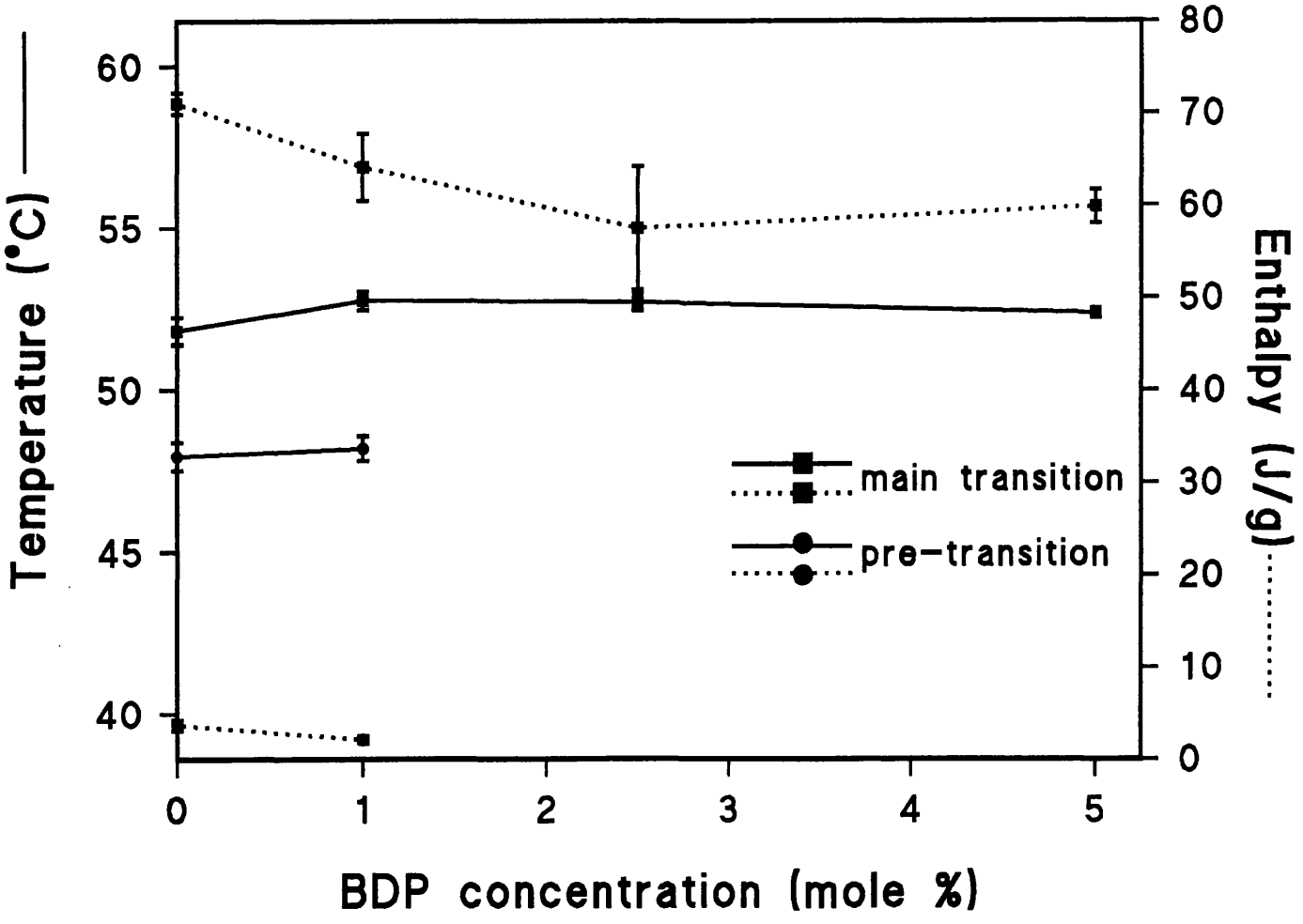
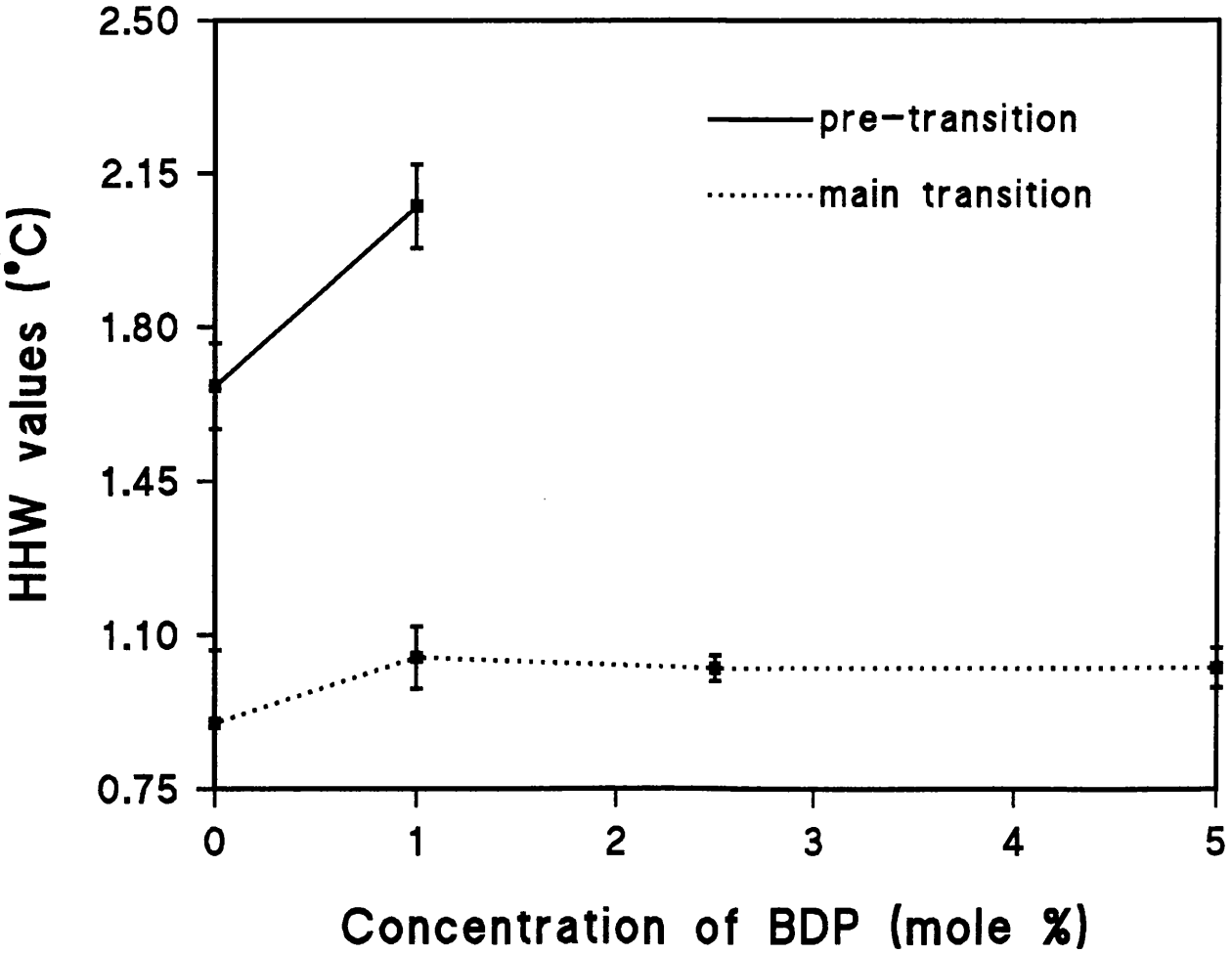


Figure 4.9. HHW values for pre- and main transition of DSPC liposomes containing varying amounts of BDP.



BDP produced no significant changes to main transition temperatures, enthalpies or HHW values. This is consistent with the theory that BDP is localised in the disordered terminal regions of chains. The addition of 5 mole % BDP to DPPC bilayers caused the main transition temperature to increase, indicating the presence of BDP in chain regions closer to the head-group. This was in accordance with the theory proposed by Jain and Wu (1977) that the nature of thermal profiles may differ with increasing concentration of the entrapped material. However this is not the case for DSPC. Samples containing 5 mole % BDP displayed transition temperatures similar to 2.5 and 1 mole % BDP. This suggests that thermal profiles at these concentrations are the same since the length of the DSPC alkyl chains is too long for additional BDP to affect the mid-chain region.

Since HHW values did not display a maximum value, it is not possible from these results to determine the concentration at which DSPC bilayers become saturated with BDP, nor is it possible to determine whether BDP domains form. However, it is probable that the longer chains in DSPC bilayers are able to accommodate higher concentrations of BDP before domain formation is observed, and this indicates that DSPC liposomes may be a more efficient vehicle with which to entrap BDP.

From the above results, it may be possible to predict the behaviour of BDP within DLPC bilayers. Table 4.6. shows the effects of BDP incorporation on the pre-transition of DLPC liposomes, and Figure 4.10. is a typical DSC trace for a liposomal sample composed of 100 mg/ml DLPC.

As mentioned in Section 4.3.4. only the pre-transition was measured, and whilst no peaks were observed for samples containing 1 and 2.5 mole % BDP, a peak was seen at 5 mole %. The abolition of the pre-transition at this low concentration may be explained in terms of the effects of the steroid molecule on bilayers composed of short-chained DLPC molecules. Since this transition is so sensitive to the presence of impurities, it follows that shorter chains undergoing this transition will be greatly affected by the presence of 1 mole % BDP near the terminal region of chains. If this transition is attributable to the tilting and/or motion of chains, then shorter chains will be affected more than longer ones. Therefore, the pre-transition of DLPC bilayers is abolished at only 1 mole % BDP, compared to 5 mole % BDP for longer-chained DPPC bilayers.

Figure 4.10. DSC thermogram of DLPC liposomes (100 mg/ml).

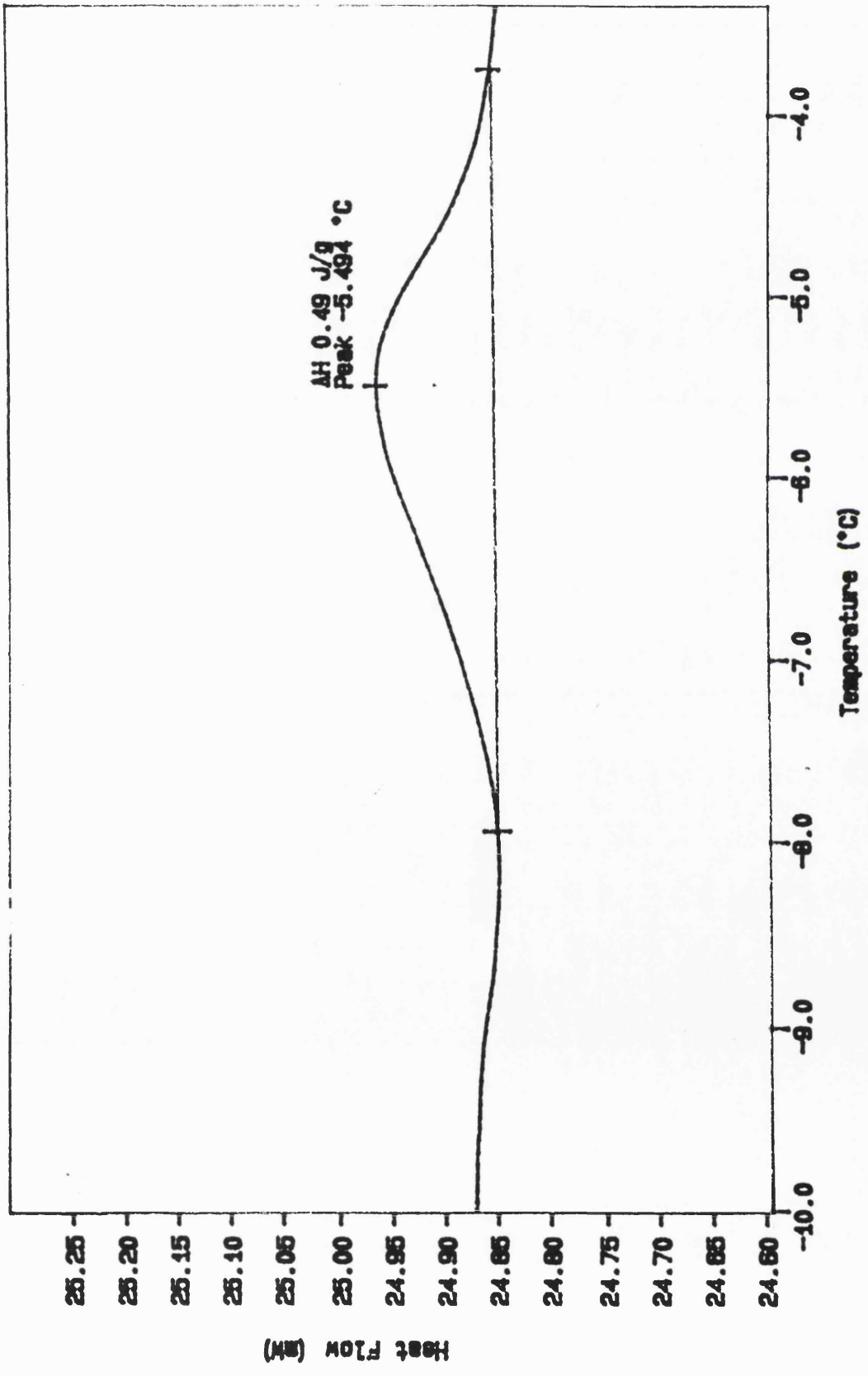


Table 4.6. Mean pre-transition temperature and enthalpy values (± standard deviation) of DLPC liposomes (100 mg/ml) containing BDP.

BDP concentration	Pre-transition	
	Temperature (°C)	Enthalpy (J/g)
0 mole %	-8.32 ± 0.20	5.69 ± 0.85
1 mole %	ND	ND
2.5 mole %	ND	ND
5 mole %	-8.80 ± 0.06	2.14 ± 0.29

It was not immediately apparent why the pre-transition should appear at 5 mole % BDP. Since HHW values were only available for 0 and 5 mole % BDP, a distinct relationship between peak width and drug concentration could not be determined. However, if we are to predict from the results on DPPC and DSPC liposomes that BDP is unlikely to be efficiently entrapped in DLPC bilayers, then it follows that domains of drug will probably form well below 2.5 mole % BDP (the limit for DPPC liposomes). If this is case, then additional drug may not simply attach to existing BDP within the bilayer, but may form a separate drug domain outside the liposomes. However, the instability of entrapped drug may result in BDP from liposomes agglomerating with BDP in domains. At concentrations of 5 mole % BDP empty (non-drug-containing) DLPC liposomes would exhibit a pre-transition again.

Studies on liposomes composed of DPPG were undertaken to investigate head-group size and charge on the effect of BDP on these liposomes. Figure 4.11. is a typical DSC trace for a liposomal sample containing 100 mg/ml DPPG. Since these liposomes did not exhibit any pre-transition peaks, Table 4.7. summarises the effects of BDP on the main transition of DPPG liposomes.

Figure 4.11. DSC thermogram of DPPG liposomes (100 mg/ml).

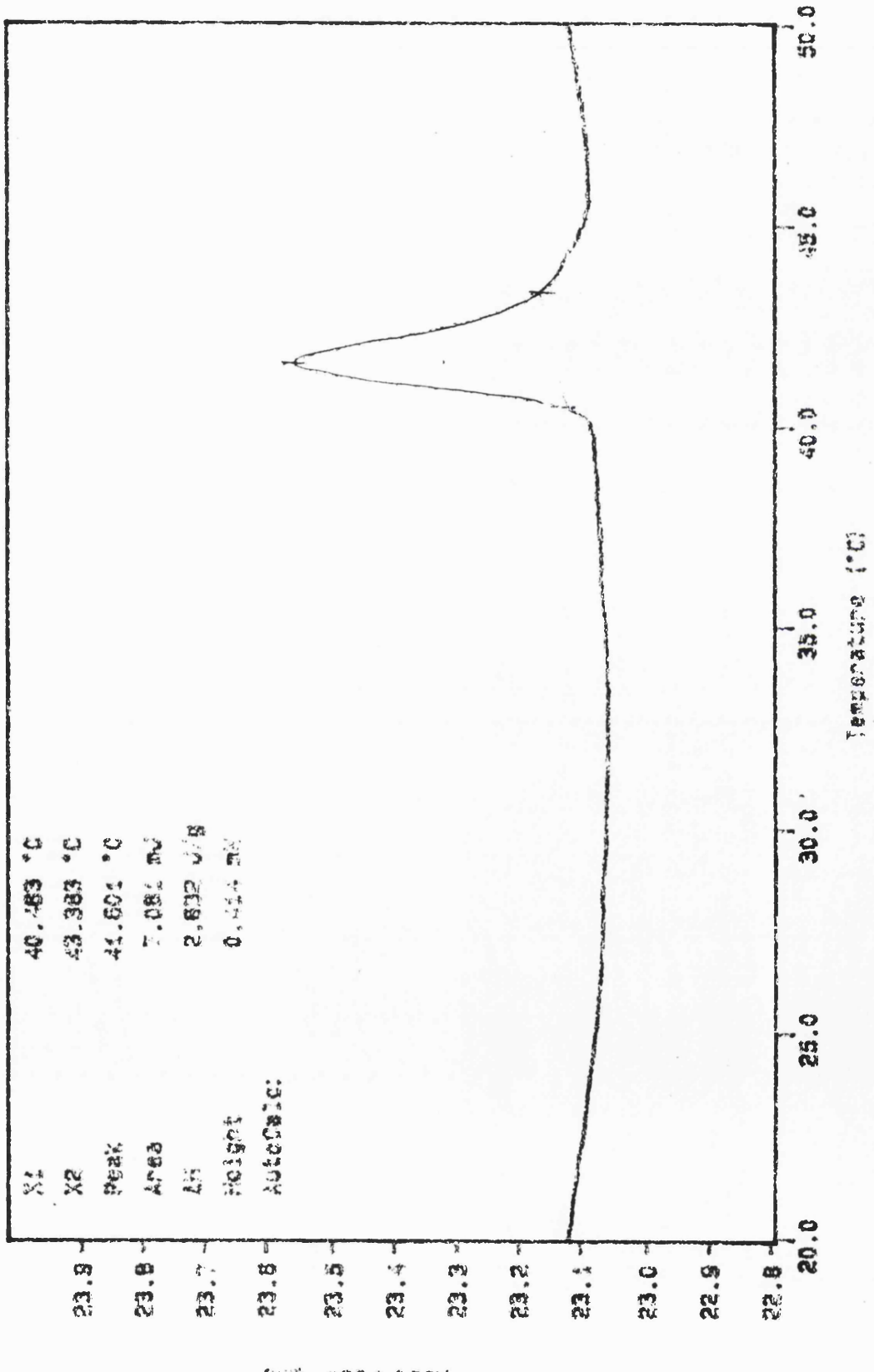


Table 4.7. Mean main transition temperature and enthalpy values (± standard deviation) of DPPG liposomes (100 mg/ml) containing BDP.

BDP concentration	Main transition	
	Temperature (°C)	Enthalpy (J/g)
0 mole %	39.671 ± 0.248	27.10 ± 1.24
1 mole %	39.800 ± 0.120	57.32 ± 22.40
2.5 mole %	41.198 ± 0.850	38.82 ± 8.77
5 mole %	41.031 ± 0.058	41.39 ± 7.13

Many difficulties were encountered in the preparation of these liposomal samples, such as achieving a solvent-free phospholipid film, and obtaining a homogeneous suspension. For these reasons, only two runs could be performed on samples containing 2.5 mole % BDP, and three runs on those containing 5 mole % BDP. The results indicated that increasing the drug concentration within liposomal samples had a small effect on the mean temperature of the transition (Figure 4.12.), but an increase in temperature was seen on the addition of 2.5 mole % BDP ($p < 0.05$). However, since these results were only duplicated they were disregarded. Enthalpy values showed a high degree of variation. HHW values for the main transition peaks were seen to increase with BDP concentration (Figure 4.13.) up to a maximum at 2.5 mole % ($p < 0.05$), after which they fell slightly.

Since DPPC and DPPG possess the same phospholipid chain length, similar thermal profiles may be expected from both sets of samples. It was thought the choline group of phospholipid head-groups is essential for the pre-transition to take place (Janiak et al, 1976), but it has also been reported that DPPG undergoes a pre-transition (detected using high-sensitivity DSC) due to its bulky head-group forcing chains to align into the rippled phase (Lo and Rahman, 1995). However, these studies were unable to detect a pre-transition for any sample composed of DPPG, which may be due to the fact that a high-sensitivity DSC instrument was not used to make the measurements.

Figure 4.12. Main transition temperatures and enthalpies of DPPG liposomes containing BDP.

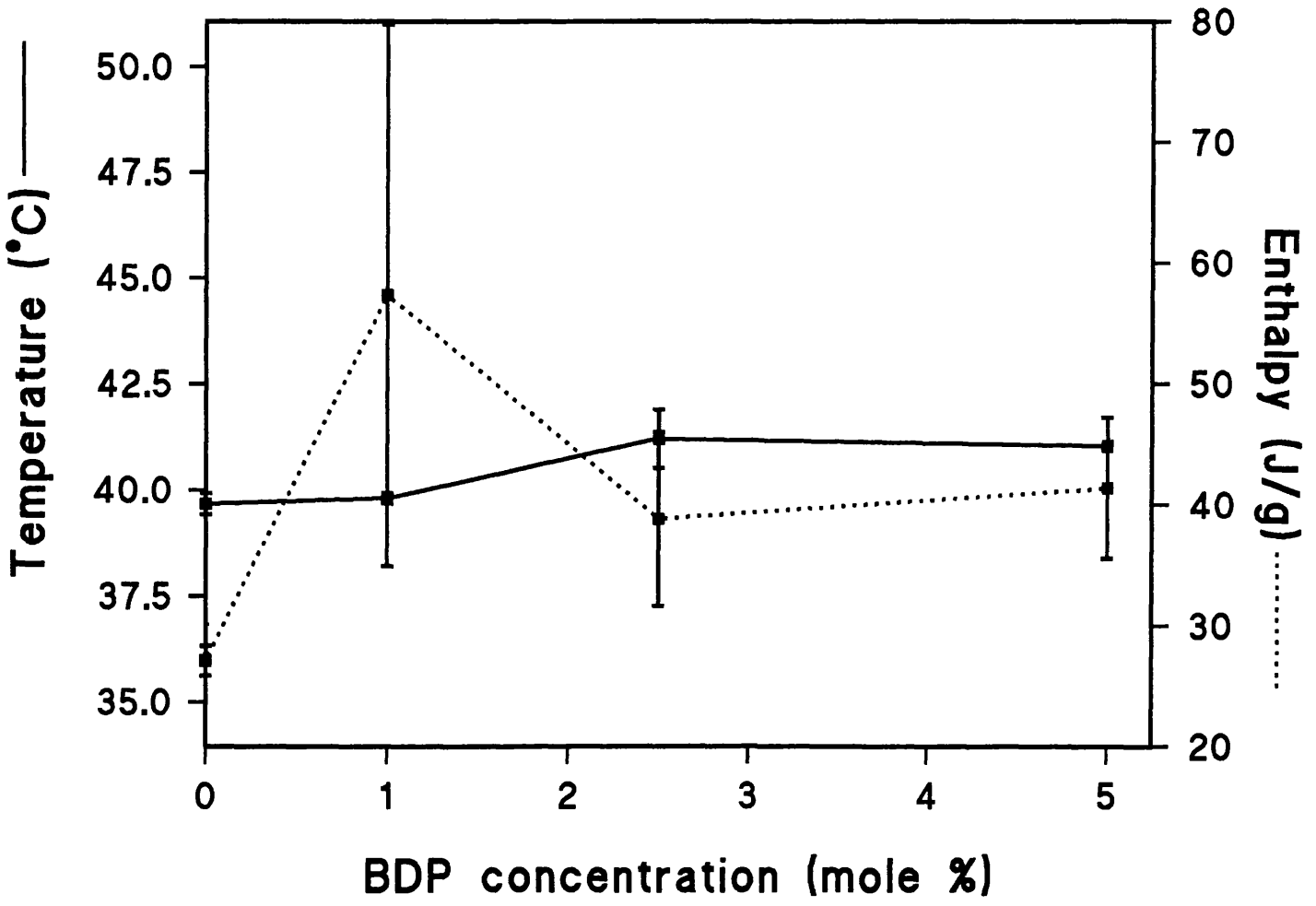
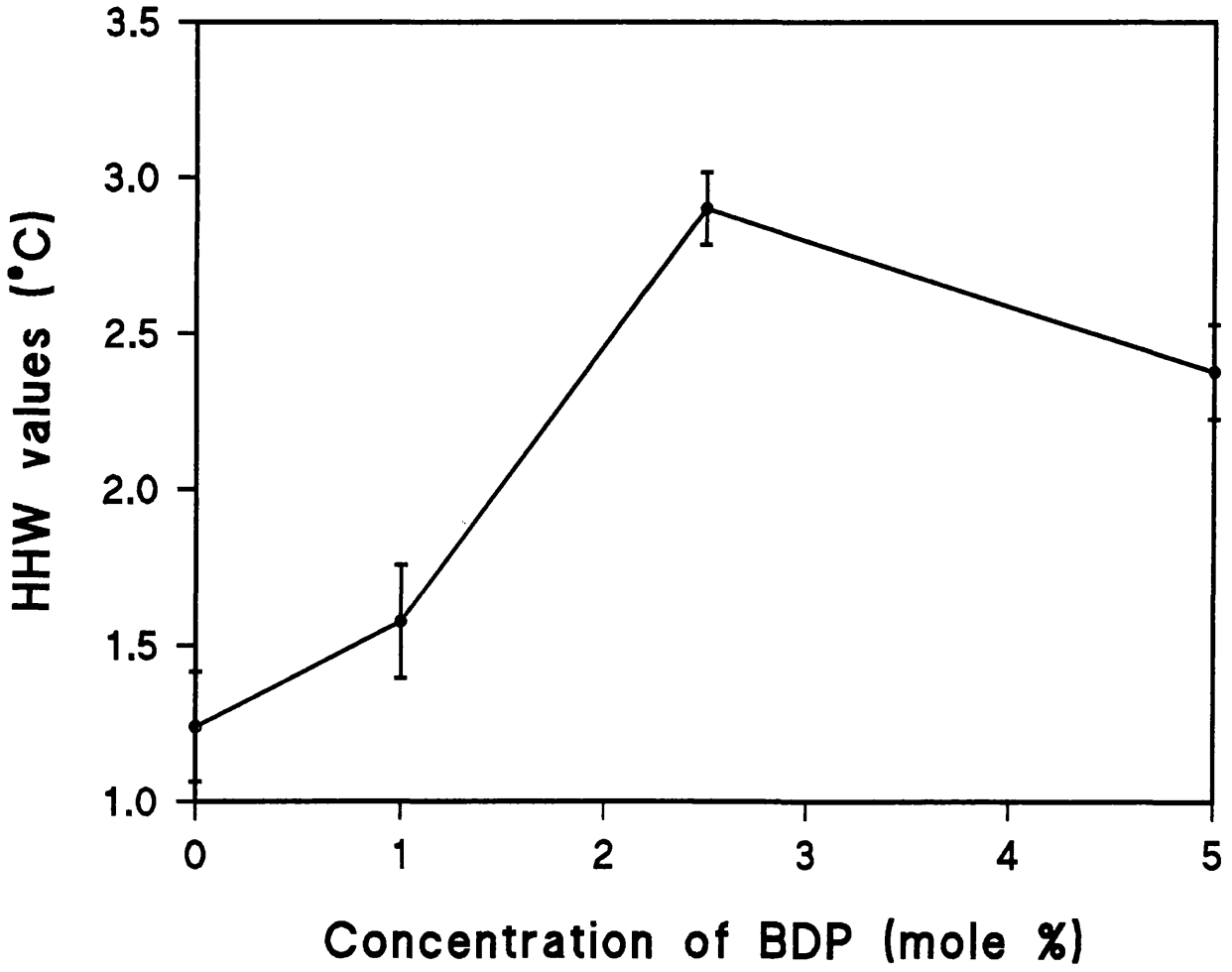


Figure 4.13. HHW values for main transition of DPPG liposomes containing varying amounts of BDP.



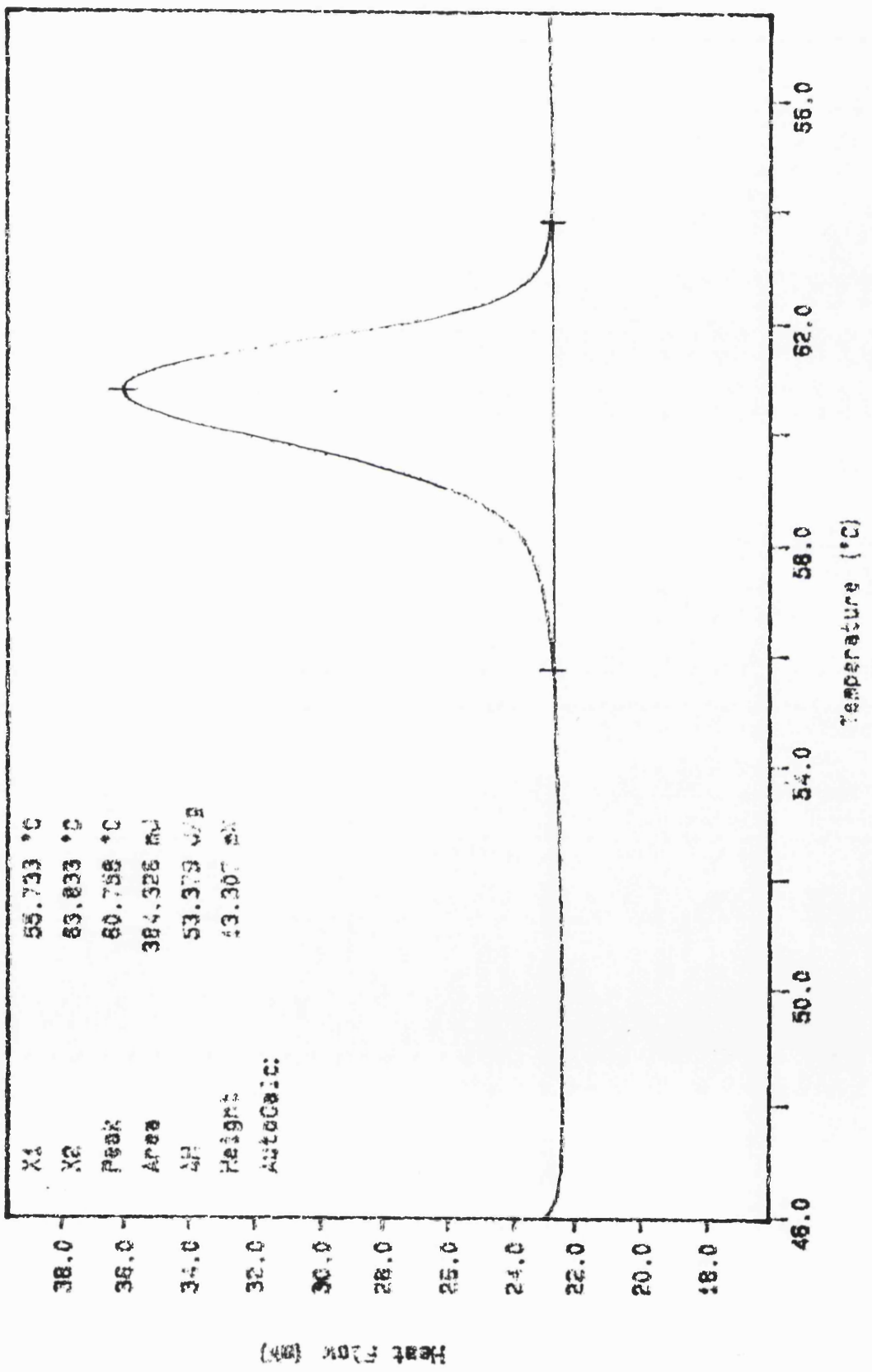
Another interesting feature is the fact that DPPG has a large head-group with a negative charge. As mentioned in Section 4.1.4., the nature of the head-group affects transitions depending on the degree of head-group ionisation and the pH of the surrounding solution (Biltonen and Lichtenberg, 1993). Therefore, whilst the main phase transition temperature has been quoted as approximately 41°C (Lo and Rahman, 1995) in an environment with pH=7, in a more acidic solution (pH=2) the transition temperature rises to 61°C (McElhaney, 1982), due to a decrease in repulsion between neighbouring negative groups. These studies utilised distilled water in the preparation of liposomes, the pH of which was not measured.

The effect of BDP on main transition temperatures may indicate the location of steroid molecules in the centre of the bilayer core. The larger head-groups may result in chains being further apart than in DPPC bilayers, particularly since there may be degree of repulsion between them. If this were the case, then BDP molecules may be situated at the terminal regions of chains without any effect on the actual chains (and therefore on the transition temperature profiles). Thus the addition of incremental amounts of BDP up to 5 mole % (excluding results from samples containing 2.5 mole % BDP) produced insignificant changes to the phase transition temperature, indicating that DPPG bilayers are able to accommodate more BDP molecules than corresponding DPPC bilayers. The effect on transition enthalpies was only significant on the addition of 1 mole % BDP ($p < 0.05$). Differences between liposomal samples containing greater proportions of drug were insignificant.

HHW values for the phase transition peak reached a maximum value at 2.5 mole %, indicating that the optimal interaction between BDP and DPPG occurs at this concentration. It is not known whether domain formation occurs immediately after this concentration has been exceeded, or whether domains do not affect thermal profiles until they are large enough in size to interfere with chain packing.

DPPA has a smaller head-group than DPPC, and as such would be expected to incorporate smaller amounts of BDP into its bilayers. Figure 4.14. is a typical DSC trace for a liposomal sample containing 100 mg/ml DPPA , and Table 4.8. displays the values obtained for the main transition of DPPA liposomes in the presence of different molar concentrations of BDP.

Figure 4.14. DSC thermogram of DPPA liposomes (100 mg/ml).



As with DPPG, DPPA liposomes were also difficult to prepare and since DPPA also does not exhibit a pre-transition, the effects of BDP on the main transition alone were investigated.

Table 4.8. Mean main transition temperature and enthalpy values (± standard deviation) of DPPA liposomes (100 mg/ml) containing BDP.

BDP concentration	Main transition	
	Temperature (°C)	Enthalpy (J/g)
0 mole %	66.88 ± 0.17	78.49 ± 4.85
1 mole %	57.66 ± 0.28	135.53 ± 5.88
2.5 mole %	56.66 ± 0.22	20.74 ± 6.15
5 mole %	60.29 ± 4.35	83.69 ± 32.76

The results obtained (Figure 4.15.) are inconsistent at 5 mole % BDP, but they suggest that 1 and 2.5 mole % BDP caused a significant reduction in the main transition temperature ($p < 0.05$). Transition enthalpies and HHW values (Figure 4.16.) reached a maximum at 1 mole % BDP.

Since DPPA has a smaller head-group (than either DPPG or DPPC), this suggests that head-groups (and therefore alkyl chains) within bilayers of DPPA are closely packed. The addition of 1 mole % BDP caused a change in the main transition temperature (unlike DPPG or DPPC samples) indicating the ability of steroid molecules to affect bilayer fluidity at this concentration. Hydrocarbon chains interact via long-range attractive forces, van der Waals interactions and short-range repulsive forces (Jahnig, 1979), and gauche conformations are associated with an increased rate of inter- and intramolecular interactions (Chapman et al, 1974). The presence of BDP in the disordered region of the alkyl chains in DPPA bilayers, and the proximity of the chains to neighbouring phospholipid molecules, may results in BDP affecting chain motion co-operatively, and thus the thermal profile of DPPA at a concentration of only 1 mole % BDP.

Figure 4.15. Main transition temperatures and enthalpies of DPPA liposomes containing BDP.

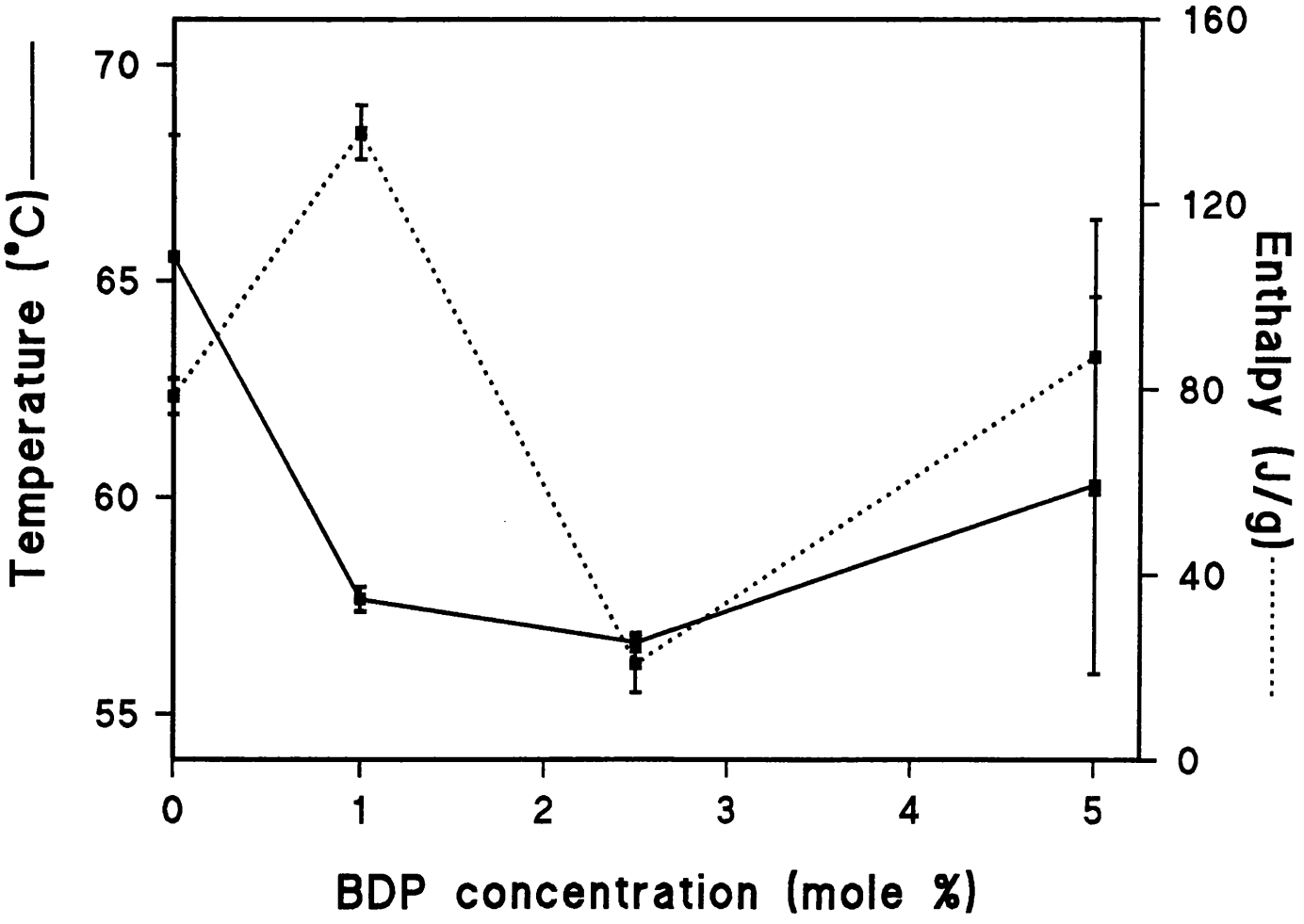
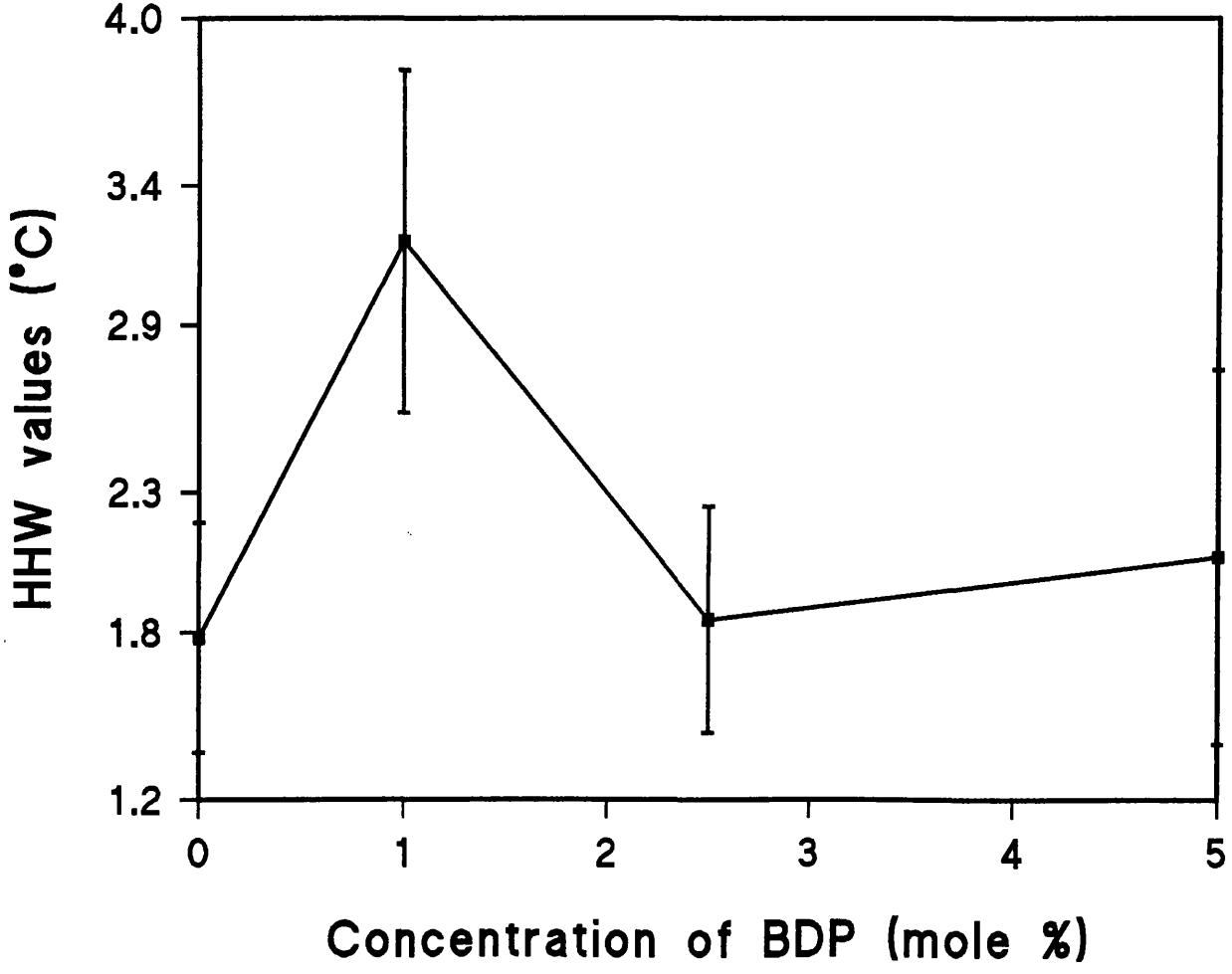


Figure 4.16. HHW values for main transition of DPPA liposomes containing varying amounts of BDP.



HHW values showed a maximum at 1 mole % BDP, which suggests a favourable interaction between phospholipid and steroid occurs at this concentration. However, no evidence to support domain formation was obtained as DPPA does not display a pre-transition peak.

4.5. Conclusions

The thermotropic behaviour of any liposome sample (with or without drug) is sensitive to chemical and physical factors. Drug-phospholipid interactions result in an alteration in the shape of the thermograms obtained, compared to those of the pure phospholipids, and also on the values of the transition temperature, enthalpy and HHW measurements. These effects are not only highly dependent on the actual amount of drug entrapped within the bilayer, but also on the specific nature of the interaction between drug and phospholipid. In addition, the type of effect is related to the position of the drug within the bilayer.

There has been some uncertainty whether it is the steroid nucleus or ester side-chains that are principally involved with bilayer interactions. Fildes and Oliver (1978) postulated a theory, using DSC findings and previous results from monolayer studies (Cleary and Zatz, 1973), that the interaction between steroid and phospholipid consisted of the acyl chain of the steroid "dipping" into the bilayer. However, Arrowsmith et al (1983b) studied the interactions between steroid esters and phospholipids further, by investigating the effect of the ester chain length. They found that the extent of interaction increased with ester chain length, and it was suggested that some interaction between the steroid nucleus and the bilayer may occur.

Investigations into the effects of BDP on the pre-transition of DPPC liposomes, have concluded that this transition is attributable to chain motion and/or tilt. DPPC bilayers are thought to be saturated at 1 mole % BDP, indicating an optimum interaction between drug and phospholipid at this concentration. However, the addition of further drug is thought to cause formation of drug domains, which affect the enthalpy of the pre-transition at 2.5 mole % BDP, and the main transition temperature. Therefore it is probable that drug is located in the central core of bilayers, and further drug is added until the size of the domain affects the mid-chain region, and hence thermal profiles. It is probable that the structure (size and shape) of

the BDP molecule is responsible for its location in the bilayer core, and not any specific interaction with the liposomal bilayer. This instability may also be the reason as to why BDP does not incorporate into liposomes in any large amount, even though this steroid is hydrophobic.

We can also conclude that the interaction between BDP and DSPC is essentially the same as with DPPC. In other words, the central location of the drug within the bilayer affects the chain motion and/or tilt, resulting in the abolition of the pre-transition. However, at concentrations of up to 5 mole % BDP, there is insufficient drug present to affect the main transition temperature of the longer-chained DSPC. It is expected that studies using a higher concentration of steroid would have produced the same results as studies on DPPC samples. The results from analysis of DLPC samples containing BDP suggest that domains of drug probably form well below 2.5 mole % BDP, and that separate drug domains form outside the liposomes. The instability of BDP in DLPC bilayers results in accumulation of entrapped drug with that in domains. Therefore, at concentrations of 5 mole % BDP, empty (non-drug-containing) DLPC liposomes would exhibit a pre-transition again.

Studies using liposomes composed of DPPG suggest that the effect of a larger head-group is the creation of extra space in the alkyl chain region that may be occupied by BDP molecules or domains. This was reflected by HHW values, that displayed a higher maximum value than with DPPC liposomes. Conversely, the addition of BDP to DPPA liposomes indicated the ability of steroid molecules to affect bilayer fluidity at low concentrations. Whilst BDP is located at the ends of the alkyl chains, the proximity of neighbouring phospholipid molecules results in BDP affecting DPPA chain conformation. Since HHW values for DPPA and DPPC show a maximum at 1 mole % BDP, it was concluded that a favourable interaction between phospholipid and steroid occurs at this concentration.

Therefore, the thermal profiles studied suggest that the optimum chain length with which to achieve maximum BDP entrapment is C_{18} (distearoyl). However, further work is required to investigate the effects of higher concentrations of BDP in DSPC liposomes. Similarly, the optimum head-group to attach to this chain would be PG (phosphatidylglycerol). The possibility of producing liposomes composed of mixed phospholipids, such as PCs and PGs, in order to control liposomal entrapment, also requires further investigation.

HHW values were found to be a useful measurement by which the interaction of components could be assessed. Whether the maximum HHW value indicates bilayer saturation or optimal interaction between the two components, this result has important practical implications for achieving higher BDP entrapment efficiencies in liposomes. Therefore, future DSC scans may provide a predictive *in vitro* test for further understanding the properties of particular steroids, and their interactions with phospholipids.

5. GENERAL CONCLUSIONS

The aims of this project were to investigate the entrapment of BDP into phospholipid structures, namely monolayers and bilayers, in order to gain an understanding of the behaviour of this particular drug within these structures, and also to help optimise future steroid entrapment for drug delivery purposes. The solvate behaviour of BDP monohydrate was investigated to determine the nature of this steroid in phospholipid films and liposomal suspensions. The phenomenon of crystal formation was observed microscopically, and the concentration at which excess BDP crystallises out was determined by this method and by an HPLC drug assay (after unentrapped material was removed by centrifuging samples in deuterated water). The incorporation of BDP into monolayers constructed from various phospholipids were investigated, with a view to developing a predictive test for the behaviour of BDP in equivalent bilayer systems. Liposomes containing BDP were investigated using DSC to examine the effects of steroid entrapment on bilayer fluidity.

Investigations into the solvate nature of BDP in Chapter 2 found that unconditioned drug confirmed the characteristics of micronised BDP monohydrate, as determined by TGA and DSC analysis. In addition, BDP monohydrate was found to form a solvate when recrystallised from chloroform, but with a stoichiometry of less than 1:1 solvent: steroid. It was concluded that monohydrates exposed to methanol and acetone do not form solvates, and in aqueous environments these monohydrates lose their water of crystallisation, and transform into pseudoanhydrates. These results have implications regarding the incorporation of this drug into phospholipid films and liposomes, since these findings indicate that BDP is present as a chloroform solvate within the dry phospholipid film. However, it is not known what form BDP is in when hydrated in liposomes. Therefore, since the chloroform solvate was calculated to contain 1:0.15 moles of steroid:solvent (and an identical molecular weight to 1 mole of BDP monohydrate), future work involving phospholipid films and liposomes used the molecular weight of BDP monohydrate as representing 1 mole of steroid. As described in Section 2.2.1. the nature of the incorporated solvent may influence molecular shapes, which may in turn affect the incorporation of drugs into liposomes, since the bulkiness of the solvated molecule may dictate its entrapment profile.

Therefore, the effects of the solvate nature of BDP on entrapment of this steroid into liposomes may be related to the size and shape of the chloroform solvate.

Further work is required in order to investigate the effect of the liposome formation process (such as exposure to vacuum and increased temperatures) on BDP solvates and monohydrates, and to determine the structure of the chloroform solvate, particularly in phospholipid films. In addition, DSC and TGA studies of the chloroform solvate of BDP in the presence of water could not be undertaken because of the effects of water on both thermal profiles and TGA analysis. Therefore, further studies may be performed to determine the how BDP solvates may be affected by the presence of both phospholipid and/or water.

BDP crystals are present in excess in liposomal suspensions, and were found, in Chapter 2, to form upon the addition of water, rather than forming in dry phospholipid films. Hydrophobic drugs in aqueous environments cause structuring of water and promote self-association into crystals (Arrowsmith et al, 1983a), and this result (Section 2.3.1.) was not unexpected. However, if the solvated form of BDP is maintained in phospholipid films on hydration, this may account for the fact that BDP forms crystals easily in liposomal suspensions. Future studies in this area might concentrate on investigations into crystal formation by different solvates of BDP. Since the size and spatial fit of BDP into bilayers may be of prime importance in determining the stability of this steroid in liposomes, the optimisation of BDP entrapment could lie in the use of solvents that do not form solvates with BDP, since solvates have different structures.

Chapter 2 also concluded that the concentration at which crystals form in DPPC liposomal suspensions was approximately 1.5-2 mole % BDP, as determined by microscopy, indicating that the incorporation of less than 1.5 mole % BDP would result in 100 % entrapment efficiency. The fact that BDP crystals appear in liposomal samples only days after preparation, suggests that BDP entrapment probably involves a steric component, as suggested by Radhakrishnan (1991). DSC studies on DPPC liposomes containing BDP also indicated that the optimum interaction between steroid and phospholipid occurs at 1 mole % BDP, with domains affecting thermal profiles at 2.5 mole % BDP. The drug molecules appeared to be located at the disordered chain ends, and this may explain the instability of BDP in liposomal preparations. Future stability studies may be performed on liposomal preparations to confirm this.

The aims of Chapter 2 recognised that unentrapped crystalline material requires removal from liposomal suspensions, and centrifuging samples in deuterated water was identified as an efficient technique to accomplish this since BDP crystals formed a pellet, whilst liposomes (containing drug) floated on top. An HPLC assay was adapted from the original assay supplied by Glaxo Wellcome, and proved to be a useful technique for the determination of BDP, as others have reported (Devoiselle et al, 1992; Waldrep et al, 1994). This assay was found to be unaffected by the presence of DPPC, and dehydrated samples tested for entrapment values of BDP in DPPC liposomes yielded values (approximately 2.5 mole %) similar to those determined earlier by microscopy and therefore understood to be accurate.

Results from fluorescence studies of DPPC monolayers containing BDP in Chapter 3 indicated that increasing drug concentration increases the surface pressure at which phospholipid solid domains appear to form. This confirmed earlier findings that BDP increases the onset of the liquid-condensed phase of DPPC monolayers. Whilst there was a degree of miscibility between the steroid and the phospholipid in the liquid-expanded phase, the fewer number of domains and irregularity of domain shapes indicated that BDP and DPPC were not in equilibrium. The liquid-condensed phase meanwhile consisted purely of DPPC, with BDP molecules being ejected out of the closely packed monolayer.

The results from studying the effects of incorporating BDP into monolayers composed of various phospholipids (also in Chapter 3), were used to make predictions regarding equivalent bilayer systems. The high pressure (liquid-condensed) region is the most appropriate to examine for behaviour indicative of bulk systems and it is also where phase separation of mixed monolayers is most likely to occur (Blume, 1979). BDP is expelled out of closely packed monolayers of DPPC, DSPC, DPPA and DPPG, but it is not known whether drug molecules are ejected into the sub- or super-phase. One can predict from these results alone that the incorporation of BDP into liposomes composed of phospholipids with chain lengths of C_{16} or more will result in immiscibility of the two components (and hence low entrapment efficiencies). In addition, these results suggest that BDP is stabilised in closely packed monolayers of DLPC as no expulsion was detected by monitoring high surface pressure values.

It is unclear from these results why the behaviour of BDP in monolayers constructed from phospholipids with the same head-group, such as DLPC and DSPC,

is different, since it is the nature and size of polar head-groups that are important in dictating the behaviour of drug incorporation into phospholipid monolayers. One possible reason for this may be that the temperature at which these investigations were carried out was above the phase transition of DLPC, and therefore the chains were in the liquid-crystalline state and flexible, with respect to the presence of drug. DSPC chains on the other hand, at ambient temperature, would have been in the gel state and rigid.

If results from the thermal analysis of liposomes composed of different phospholipids (in Chapter 4) are taken into account, then another reason may be proposed as to why Langmuir studies of phospholipids with the same head-group should produce different results. It is thought that chain lengths are of prime importance in the determination of BDP incorporation into monolayers. Since BDP is a hydrophobic compound, it is feasible that on compression of the monolayer, drug molecules penetrate the chain regions of phospholipids. This expulsion into the super-phase would cause surface pressures of the respective films to approach that of the pure monolayer, and suggests that no drug molecules are present in the plane of the monolayer. The results obtained are consistent with the theory that BDP penetrates the chain region of longer-chained phospholipid monolayers, but the presence of BDP in phospholipid monolayers consisting of shorter chain lengths of C_{12} suggest that drug molecules are not ejected out of highly compressed DLPC monolayers. This may be a result of the fact that the drug molecules within the chain region may be close to the head-group region, thus affecting observed surface pressures. Therefore, it must be recognised that these studies cannot determine the effects of BDP on the hydrophobic region of these phospholipids. Whilst the Langmuir technique is a suitable tool with which one can study the compatibility of drugs or other molecules in a monolayer, in order to make predictions regarding liposomal systems, these can be confirmed by performing thermal analysis of liposomes composed of various phospholipids, and containing BDP. Future studies on phospholipid monolayers may be performed at temperatures above and below their phase transitions. In addition, fluorescent labeling of both components within a phospholipid monolayer may be useful when observing not only domain formation, but also the ejection of drug molecules into the super-phase.

Results from DSC investigations into the effects of BDP on phospholipid bilayers in Chapter 4 found that altered thermograms suggested the perturbation of bilayers by drug molecules. The effects of BDP are dependent on the concentration of drug entrapped, and the nature of the interaction between drug and phospholipid. In addition, the type of effect is related to the position of the drug within the bilayer.
The effects of BDP on the pre-transition of DPPC liposomes suggested that this transition is attributable to chain motion and/or tilt. DPPC bilayers are saturated with BDP at 1 mole % steroid, indicating an optimum interaction between both components (as suggested earlier in by microscopy studies). The addition of further drug caused the formation of domains, which affected the enthalpy of the pre-transition and the main transition temperature at 2.5 mole % BDP (the maximum amount of BDP that was found to be entrapped into DPPC liposomes, by HPLC). It was concluded that BDP is located in the disordered central core of bilayers, and that domains affect the mid-chain region (and hence thermal profiles). It is likely that the size and shape of the BDP molecule is responsible for its location in the fluid region of the bilayer core, and not any specific interaction with the liposomal bilayer. This instability may also be the reason as to why BDP does not incorporate into liposomes in any large amount, even though this steroid BDP is hydrophobic.

It was also concluded that the interaction between BDP and DSPC is essentially the same as with DPPC. In other words, the drug is located at the terminal chain region of bilayers affecting chain motion and/or tilt, and resulting in the abolition of the pre-transition. At concentrations of up to 5 mole % BDP, there is insufficient drug present to affect the main transition temperature of the longer-chained DSPC, indicating that whether drug domains have formed or not, this concentration of BDP within DSPC liposomes does not affect the fluidity of these bilayers. The results from the analysis of DLPC samples containing BDP suggested that drug domains form below 2.5 mole % BDP, and separate drug domains form outside the liposomes. The instability of this steroid within DLPC bilayers resulted in the agglomeration of liposomally-entrapped BDP with drug present in domains. Therefore, at concentrations of 5 mole % BDP, empty (non-drug-containing) DLPC liposomes exhibited a pre-transition again.

Studies on liposomes composed of DPPG concluded that the effect of a larger head-group size is the creation of extra space in the alkyl chain region, which may be

occupied by BDP molecules or domains. This was reflected by HHW values, that displayed a higher maximum value than with DPPC liposomes. Conversely, the addition of BDP to DPPA liposomes indicated the ability of steroid molecules to affect bilayer fluidity at low concentrations. Whilst BDP is located at the ends of the alkyl chains, the proximity of neighbouring phospholipid molecules results in BDP affecting DPPA chain conformation. Since HHW values for DPPA and DPPC show a maximum at 1 mole % BDP, it was concluded that a favourable interaction between phospholipid and steroid occurs at this concentration.

Thus, results from thermal profiles can be used to conclude that the optimum chain length with which to achieve efficient BDP entrapment is C_{18} (distearoyl), whilst further work is required to investigate the effects of higher BDP concentrations on DSPC bilayers. The optimum head-group to attach to this chain would be PG (phosphatidylglycerol), to create space in the chain region. These results have important practical implications for achieving higher BDP entrapment efficiencies in liposomes.

The main aims of these studies were fulfilled by the work undertaken in this project; a method by which the concentration of liposomally entrapped BDP may be accurately determined was successfully developed (by microscopic and assay techniques). In addition, theories as to the penetration of monolayers by BDP molecules, and the location of this steroid in bilayers, were proposed. Langmuir and DSC techniques were found to complement each other well, particularly when the development of profiles of phospholipid structures containing steroidal drug was required. However, investigations into the effects of BDP on phospholipid monolayers and bilayers warrant further study, as outlined above.

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Note

As the nature of solvent association with beclomethasone dipropionate in phospholipid films and bilayers was not established in this study, the use of the term "solvate" may be misleading. Hence, in this context, "associated solvent" is a more appropriate term.