FREEZE-DRYING: A RATIONAL APPROACH TO PROCESS DEVELOPMENT AND PRODUCT FORMULATION USING MODEL POLYMERIC PROTEINS AND DRUG MICROCARRIER SYSTEMS

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SUMMARY

A great deal of empiricism exists in pharmaceutical freeze-drying (lyophilisation), where the use of this process is reported to be increasing and its importance in the stabilisation of biomolecules becoming ever greater. While many workers have described specific applications of the process, few have set out to characterise the process itself. The present study addresses current issues in freeze-drying with regard to four key areas: the thermal characteristics of freeze-dried excipients and model pharmaceutical products, the stabilisation of model polymeric proteins, the use of lyophilisation in the preparation and stabilisation of sterically stabilised liposomes and the development and optimisation of lyophilisation cycles. Thermal analysis of a range of pharmaceutically relevant excipient mixtures containing mannitol demonstrated the thermal instability of many of these mixtures and the observed thermal data supported the existence of amorphous mannitol. The solid-state glass transition temperatures of all mixtures were below 26°C, which may have implications on the thermal stability of products containing such mixtures. Studies carried out to examine the effects of a range of saccharides on the freeze-dry stability of the model protein L-asparaginase revealed that the protective ability of each additive used was similar when assessed on both a weight basis and on the basis of hydroxyl group frequency. The use of PEG as a protective additive was also established, but with the conclusion that PEG operated *via* a different mechanism to the saccharides. Molecular modelling enabled assessment of the pattern of available highly polar residues (HPR) present on the L-asparaginase molecule in both monomeric and tetrameric forms, which allowed predictions to be made as to the theoretical amounts of additive required to provide protection to L-asparaginase. These theoretical values gave a close approximation to those found experimentally to provide maximal levels of protection to L-asparaginase in the present work. In addition, the levels of stabilisation conferred on a second polymeric protein, lactate dehydrogenase (LDH) by additives during freezing, freeze-drying and storage in the lyophilised state were attributed to the crystallising characteristics, reducing nature and chemical reactivity of the additives studied. Freeze-drying was also applied to the preparation of sterically stabilised liposomes (SSL). The use of simultaneous loading-rehydration was shown to be feasible for the encapsulation of the amphiphilic polypeptide drug polymyxin B (PXB) into SSL, although lower levels of drug encapsulation were achieved compared to those where a conventional method was employed. This was attributed to possible drug-lipid bilayer interaction operating within the chosen system. In addition, it was observed that the presence of excipients during preparation and subsequent storage of such preparations in the lyophilised state exerted little effect on either formation or stability of these PXB-loaded preparations, in contrast with previous reports describing the effects of such excipients on the stability of conventional liposomes. Freeze-drying cycle development studies for model crystallising and non-crystallising formulations demonstrated that cycle efficiency could be significantly increased and drying time considerably reduced by the adoption of non-conventional drying programmes based on observed physical data and freezing behaviour of formulations. The use of comparatively high shelf temperatures and chamber pressures were successfully employed to effect more efficient drying, whilst maintaining products below their characteristic collapse temperatures during processing.

Keywords: L-asparaginase, lactate dehydrogenase, differential scanning calorimetry, freeze-drying, polymyxin B, process development, product formulation, sterically stabilised liposomes.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisors at Aston University, Dr. H. O. Alpar and Professor W. J. Irwin, for their encouragement, support and guidance throughout the course of this project. I would also like to thank my industrial supervisor at CAMR, Mr. Gerald Adams, for his enthusiasm, wisdom and encouragement and for the useful discussions and advice over the past three years.

I also wish to gratefully acknowledge the Engineering and Physical Sciences Research Council (EPSRC) and the Centre for Applied Microbiology and Research (CAMR) for financial support of this project and Biopharma Process Systems Ltd. for initial funding of the Total Technology studentship.

I would like to take this opportunity to thank Dr. Melanie Duffield at CAMR for the molecular modelling work, Drs. Peter Lambert and Ian Baxter at Aston for their valuable help with protein analysis and Dr. Mark McAllister for the helpful advice on the liposome studies. I would also like to express my gratitude to the technical staff in the Department of Pharmaceutical and Biological Sciences at Aston for their expert laboratory assistance during the past three years.

Finally, I would like to thank my family and my friends for their support and encouragement throughout my years of study.

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ABBREVIATIONS

ΔH	change in enthalpy
AHA	L-aspartylhydroxamic acid
CAMR	Centre for Applied Microbiology & Research
Chol	cholesterol
DMPC	dimyristoylphosphatidylcholine
DRV	dehydration-rehydration vesicles
DSC	differential scanning calorimetry
DSPE	distearoylphosphatidylethanolamine
FDM	freeze-drying microscopy
FPLC	fast protein liquid chromatography
FT-IR	Fourier-transform infrared
HPR	Highly polar residue(s)
KF	Karl Fischer
LDH	lactic (or lactate) dehydrogenase
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
mPEG	methoxypoly(ethylene glycol)
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
P_c	chamber pressure
PC	phosphatidylcholine
PCS	photon correlation spectroscopy
PEG	poly(ethylene glycol)
PHG	polar headgroup(s)
POPC	palmitoyldioleoylphosphatidylcholine
PXB	Polymyxin B
RM	resistivity measurements
SDS	sodium dodecyl sulphate
SSL	sterically stabilised liposomes
SUV	small unilamellar vesicles
T_c	collapse temperature
TEM	transmission electron microscopy
T_{eu}	eutectic melting temperature
T_g	glass transition temperature (solid-state)
T_g'	glass transition temperature of maximally-concentrated frozen matrix
TGA	thermogravimetric analysis
T_m	gel- to liquid- crystalline transition temperature
T_s	shelf temperature

1. INTRODUCTION

1.1. THE USE OF FREEZE-DRYING IN THE PHARMACEUTICAL INDUSTRY

1.1.1. Historical Background

Man has always sought to preserve precious foodstuffs and natural products from the effects of decay and spoilage. Advances in technology have enabled the development of a wide range of techniques for the preservation of labile biomaterials. Perhaps the most widely-used method of preserving foodstuffs in the Western world is freeze-preservation. However, there are three major disadvantages of using freezing alone for stabilising and storing pharmaceutical formulations, namely the high costs involved in the maintenance of frozen stocks, problems associated with transportation of frozen samples and the potential risk of total loss of high-cost material in the event of freezing-plant failure (Adams, 1991). These disadvantages have encouraged the adoption of alternative methods including desiccation. Conventional drying methods traditionally use high temperatures in order to dry the product and invariably cause physical and/or chemical alterations in the product formulation, resulting in a reduction in biological activity for sensitive bioproducts such as enzymes and hormones, or organoleptic changes in foods. Following the rapid growth of biotechnology industries during the past decade, freeze-drying (lyophilisation) has now become a standard method for the stabilisation of labile products isolated from biological materials. As such, it is now a well-established alternative to conventional drying for preserving labile materials, particularly when formulating novel therapeutic products (Nail & Gatlin, 1993).

Evidence of freeze-drying as a method of preservation can be traced back to prehistoric times, when, for example, the Eskimo preserved fish by dehydration in the cold Arctic winds. The fundamental principles of freeze-drying were understood by the beginning of the twentieth century by workers such as Altmann and Shackell, although practically, the process was not used industrially until the 1930s, when the need arose to preserve large volumes of heat-sensitive blood products and the newly discovered antibiotics (Flosdorf & Mudd, 1935; Greaves, 1946). Advances in the fields of refrigeration and vacuum technology have since enabled the development of reliable commercial freeze-dryers so that the lyophilisation

process has now essentially become fully integrated within the pharmaceutical industry. Indeed, it is now one of the most common methods used in the production of solid dosage forms, diagnostics and oral dosage forms, where subsequently a rapid dissolution rate is required. One feature of the freeze-drying process is that it allows heat-sensitive drugs and biologicals to be dried at low temperatures. The avoidance of high temperatures normally associated with traditional drying methods can help to reduce the extent of decomposition or loss of activity of a bioactive agent. A second feature of freeze-drying is that a lyophilised product has a very high specific surface area, which enables it to be reconstituted into solution rapidly and completely. This is particularly valuable in the case of emergency vaccines or antidotes, which need to be solubilised and administered in as short a time as possible. Thirdly, freeze-drying is more compatible with the production of particle-low pharmaceuticals, compared to dry powder filling (Nail & Gatlin, 1993). In this case, a solution can be sterile filtered immediately before being transferred into vials and freeze-dried. In short, freeze-drying offers a range of advantages to alternative methods in addition to simply stabilisation.

1.1.2. Current Status of Freeze-Drying

In recent years, the application of freeze-drying has continued to expand to encompass the formulation of vaccines, steroids, vitamins, therapeutic enzymes and a wide range of diagnostic products and seed cultures, amongst many other products. With the discovery, development and introduction of an ever-increasing number of modern therapeutic agents through methods such as molecular design and recombinant DNA technology, the importance of freeze-drying in the formulation of these entities will invariably expand. Many of these products are proteins and peptides which are characteristically unstable in solution. In order to remain biologically active, the maintenance of secondary, tertiary or quaternary structure is an essential requirement for such products. Enzymes alternate between the active and inactive state, in response to subtle changes in their micro-environment. It follows, therefore, that severe changes in the aqueous medium during preparation and processing may prove extremely damaging. The stresses involved in freeze-drying will invariably result in changes in the aqueous medium. The development of such therapeutic agents to produce acceptable oral or injectable dosage forms therefore presents a major challenge to the

formulation scientist. Freeze-drying is likely to prove a necessary technique (Nail & Gatlin, 1993), particularly when applied to the field of novel drug delivery systems, for example the stabilisation of liposomal formulations (Crowe & Crowe, 1993). Much of the reported data for the effects of freeze-drying on liposome preparations has been from studies employing non-therapeutic marker molecules to enable simple examination of physical effects of liposomes themselves, rather than from full investigations into the stability of liposomal formulations of therapeutic agents. This is discussed further in chapter 5 of this report.

Despite being one of the most commonly-used processes during drug formulation, freeze-drying also remains perhaps one of the most poorly-understood (Franks, 1990, 1994). Lyophilisation is usually the ultimate step in a complex chain of production sequences which constitute the overall formulation process for a drug. The method is preceded by various extraction, fractionation and purification processes which usually result in an aqueous solution, where the active ingredient is often very dilute, in the presence of other co-solutes and buffers. Standard texts dealing with the isolation of biochemicals (for example, proteins) describe these various stages in detail. Such stages may involve extraction, precipitation, resolubilisation, chromatographic and electrophoretic methods, dialysis and ultrafiltration. Each of these steps will have been carefully optimised to produce a maximum yield. The description often ends with the sentence: "The aqueous solution is then lyophilised.",implying that lyophilisation is a bland process, requiring little technical understanding. However, low-temperature processing and drying are scientifically and technologically much more complex than spray drying, solvent extraction or ultrafiltration. It therefore seems surprising that few chemical engineering texts devote little more than one paragraph to the technology of freeze-drying. Much promotional literature distributed by the manufacturers of freeze-drying equipment does very little to promote a better understanding of the subject, and may even provide incorrect information. It is, perhaps, unsurprising that the formulation scientist often treats lyophilisation as a trivial, push-button process, where the optimisation of the formulation and freeze-drying protocol are often arrived at by trial and error. In these cases, little or no effort is made to optimise the freeze-drying process, and processing protocols are often devised by folklore. In reality, freeze-drying conditions should be established for every new product on a rational basis by scientific thought and planning. Freeze-drying often

results in a substantial loss of activity and even chemical degradation for many therapeutic agents; even when measures are taken to reduce this, the issue of shelf stability still remains (Pikal, 1990a,b). Factors such as the moisture content of the dried preparation and the reactivity of components present are often overlooked and consequently, many products are freeze-dried using identical conditions, irrespective of their individual requirements.

A number of recent reviews in the area of freeze-drying, some dealing with the physico-chemical principles of the process itself, others with the application to the stabilisation of biomolecules and drug formulations, have sought to introduce to the formulation scientist concepts and issues in the area which were previously not perhaps fully appreciated (MacKenzie, 1985; Adams, 1991; Arakawa *et al*, 1993; Franks, 1990; Pikal, 1990a,b; Izutsu & Yoshioka, 1995; Skrabanja *et al.*, 1994). The clear message common to many of these articles is that only by a basic understanding of the fundamental concepts and characteristics of the freeze-drying process, can a more thorough and logical approach may then be applied to the development of lyophilised therapeutic agents. When stabilising newly-emerging therapeutic proteins and drug delivery systems by lyophilisation, several common factors may link many of these similar compounds and formulations. The diverse and complex nature of such novel products from biotechnological sources mean that when attempting to develop guidelines for freeze-drying formulations for such entities, there appear to be far more exceptions than there are rule-conforming cases. However, the development of formulation and processing guidelines which could complement current knowledge in the field of lyophilisation would be welcomed by, and of considerable interest to, the pharmaceutical industry. Indeed, it is common for individual workers to have some knowledge of the effects which freeze-drying has on their particular compound(s) of interest, although to gain some understanding of the broader principles of these effects on a wider range of products, the operator must first observe the process itself in closer detail. The following section of this report gives a basic overview of the sequence of stages of the lyophilisation process and provides an in-depth view of fundamental physico-chemical aspects of the stages themselves.

1.2. THE FREEZE-DRYING PROCESS

The underlying principles of the operation of a freeze-dryer are most conveniently discussed by dividing the process into a number of discrete steps, namely:

- Product preparation
- Pre-freezing
- Primary drying (sublimation)
- Secondary drying (desorption)
- Stoppering and removal
- Storage of dried product
- Reconstitution prior to use

Each of these individual stages should essentially be regarded as a discrete, potential source of damage to the product, if not performed in a manner which is sympathetic to the requirements of that product. This section seeks to introduce the underlying principles of each step of the lyophilisation process, as well as the physico-chemical principles, technical issues and practical implications.

1.2.1. Product Preparation

It is common for little consideration to be given to the manner in which the active component is prepared prior to lyophilisation. However, it must be appreciated that freeze-drying will never rectify any biological or chemical damage caused during the preparation of the product, but can only exacerbate any such damage (Adams, 1991). In the case of biologically sensitive materials, it is important that the active component remains stable in solution prior to freeze-drying. Poor formulation at this stage may result in a loss of biological activity or even damage to the physical structure of the active component during subsequent lyophilisation. Solutions for pharmaceutical use often contain protective excipients in order to minimise product damage. The selection of suitable additives should ideally enable the formulation scientist to maintain the stability of the active ingredient in a formulation, throughout all stages of the freeze-drying process and also during subsequent

storage as lyophilised products. Many compounds have been shown to afford protection to proteins and peptides in solution, as well as during freeze-thawing and freeze-drying, an issue which is discussed further in chapter 4. Parameters affecting the solution, frozen-state or solid-state stability of a particular product are dependent on a number of factors, although the more complex the structure and general stabilisation requirements of a particular active ingredient, the more difficult the task for the formulation scientist. The aim of this formulation exercise will be to balance conflicting requirements, in order to achieve an optimised formulation which can withstand the stresses of processing as well as those of storage. When formulating bioactive molecules, additives should be chosen on the basis of the physical stability which they confer to the dried plug, their stabilising effect on the biological activity of the active ingredient, while at the same time they should remain sufficiently chemically inert so that short- or long-term damaging reactions in the dried state are avoided. However, some of these issues are common to all formulations which are to undergo lyophilisation. A recent article by Hatley *et al.* (1996) describes the way in which a model, non-protein drug is formulated and stabilised by freeze-drying. The authors place particular emphasis on the choice of crystalline *versus* amorphous excipients, and the impact of this choice on the process cycle and the behaviour of the dried preparations. Differential scanning calorimetry proved an invaluable technique for determining process conditions for the freeze-drying cycle. However, the process of selecting excipients for inclusion into freeze-drying formulations is often not given sufficient consideration which its importance merits. These parameters will be discussed further in chapters 3-6 of this report.

1.2.2. Pre-freezing

Pre-freezing is an essential preliminary to the primary drying process, and serves three main purposes. Firstly, it minimises thermal degradation within the product. Secondly, it should ideally immobilise the components in the solution, thereby preventing freeze-concentration of solutes (as in the case of the formation of a eutectic mixture described below for a solution of sodium chloride), so that the solution components remain evenly distributed throughout the frozen mass. Finally, complete prefreezing should ensure that foaming is prevented when a vacuum is applied to the product (Adams, 1991), since foaming may result in a dried product with unacceptable appearance.

Freezing may be carried out by placing vials onto cooled shelves within the freeze-dryer. Alternatively, since many small-scale freeze-dryers are not equipped with an internal shelf-cooling facility, solutions are often pre-frozen before being transferred to the freeze-dryer for drying. In order to minimise resistance to vapour flow, pre-freezing should ideally result in the complete crystallisation of the solvent (usually water) and the solutes within the solution. This should result in the production of a cosmetically elegant, cohesive plug of dried product in a relatively short time-period, the ice crystals having been removed efficiently by sublimation through channels created in the microstructure, without causing collapse of the remainder of the dried solute microstructure. However, this is seldom achieved in practice and indeed, in the case of protein solutions, crystallisation of all solutes may not be desirable, since protein damage may result from exposure of a sensitive protein to a crystalline matrix (see Chapter 4). In such cases, suitable (mainly non-crystallising) excipients should be selected, although amorphous (and partially crystalline) behaviour of excipients will often lead to processing and storage stability difficulties, as described below in section 1.2.5.

1.2.2.1 Mechanism of Ice Crystal Formation

Ice crystal formation is a two-stage process. Initially, nucleation takes place, resulting in small ice crystals throughout the solution. After nucleation, these crystals then grow; the morphology of these crystals determines the structural form of the product. The initial nucleation of ice and the subsequent growth of the ice crystal is a complex phenomenon, influenced by the composition of the solution and the rate of cooling as well as other factors. Nucleation is encouraged by reducing temperature, until the viscosity of the mass becomes so high that further nucleation is inhibited. Conversely, crystal growth is encouraged by increasing temperature, thereby decreasing the viscosity of the mass until the melting point is reached and the frozen mass melts. Although it is possible for water to spontaneously nucleate (a process known as homogeneous nucleation), in practice, water will invariably nucleate heterogeneously by crystallising around microscopic impurities in the solution (MacKenzie, 1977). Pharmaceutical solutions which have been sterilised by filtration may prove reluctant to freeze, due to their reduced particulate content. Frequently, a solution will persist in the liquid phase when cooled below its equilibrium melting point, a

phenomenon known as supercooling (or undercooling) (Franks, 1982). A detailed study of the behaviour of solutions prior to nucleation was presented by Hobbs (1974).

While a detailed description of the complex physico-chemical parameters which determine ice nucleation and crystal growth are outside the scope of this study, the consequences of freezing behaviour on the lyophilisation process have to be considered when formulating a freeze-dried pharmaceutical product. Thus, this introduction shall restrict itself to the description of the practical implications of understanding the freezing process for the purposes of lyophilisation alone. The article by MacKenzie (1977) provides a succinct review of the mechanics of the process.

It is often observed that within a batch of vials, individual solutions appear to nucleate in a random manner, the contents of some vials may crystallise slowly up from the base, others may nucleate suddenly, resulting in a diversity of crystallisation patterns. These differences in freezing behaviour between neighbouring vials within a batch may be ascribed to differences in supercooling characteristics from sample to sample. The extent of supercooling achieved before ice and crystalline solute(s) nucleate are of great practical significance. A slow cooling rate will induce the formation of large ice crystals, extending from the base to the top of the vial. These open, porous crystals act as vapour paths from the frozen mass, providing optimal conditions for sublimation. A fast rate of cooling and/or significant supercooling will result in the formation of a large number of small ice crystals, forming a crystal array which is not conducive to sublimation. Small ice crystals are thermodynamically unstable and may be induced to recrystallise into large ice crystals which are more conducive to sublimation, by extended freezing or by warming the frozen mass (see heat annealing, chapter 3). A number of workers have carried out studies into the behaviour of solutions during freezing, many of these studies making use of microscopic techniques and involving the examination of the freezing characteristics of thin layers of solutions during drying (MacKenzie, 1976). In this comprehensive review, MacKenzie presents evidence which describes the existence and relevance of many different forms of ice crystal structure in freeze-drying, as shown in Figure 1.1. The precise freezing pattern of a solution will be governed by the solutes present in the solution, their relative proportions, initial concentrations and individual responses to freezing.

FIGURE 1.1: Scheme representing different forms in which ice crystallises in various aqueous solutions, according to solute concentration and freezing temperature. Typical "snowflake" hexagonal forms appear in zone 1, "irregular dendrites" in zone 2 (in which crystals branch at angles other than 60°), finely-divided "spherulitic" forms in zone 3, and "evanescent" spherulitic forms in zone 4. Ice does not crystallise during cooling in zone 5. Precise behaviour depends on the solute(s) present. (Reproduced from MacKenzie, 1976).

1.2.2.2 Behaviour of Solutions during Freezing

This section highlights the fundamental issues which have to be considered when describing the freezing of solutions. Regardless of the precise mechanism of ice formation, as the ice crystals proliferate in the solution, there will be an increase in concentration of solute(s) within the cooled mass. We shall firstly consider the simplest case of freezing behaviour: that is a simple sodium chloride solution, which readily crystallises into solute and ice. Figure 1.2 describes the eutectic phase diagram of aqueous sodium chloride system, while Figure 1.3 illustrates how this simple phase diagram may be translated into a description of events occurring during pre-freezing. The eutectic point is the temperature at which a residual liquid phase is in equilibrium with the solid phases. In this case, cooling from room temperature to 0°C is followed by supercooling prior to ice crystallisation, resulting in the concentration of remaining sodium chloride solution.

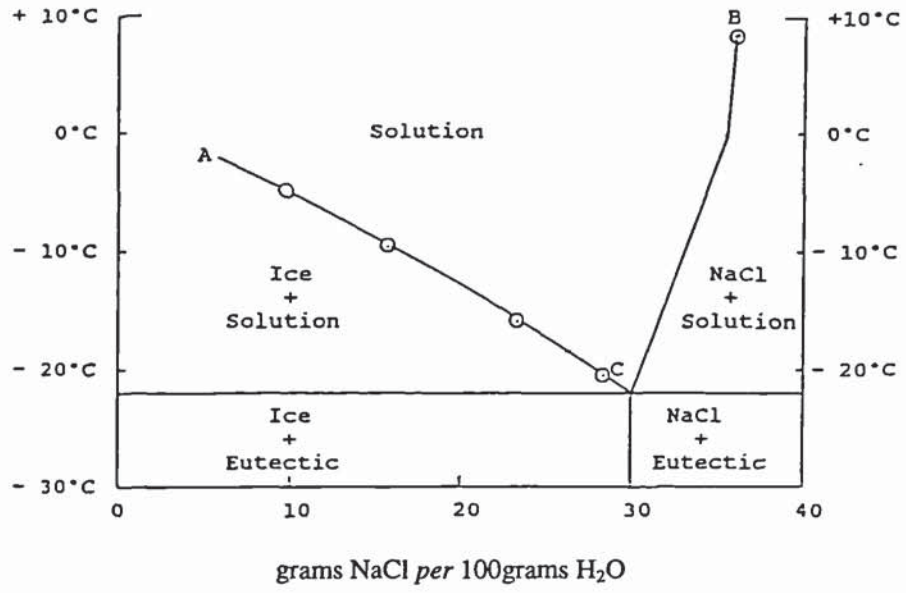


FIGURE 1.2: Eutectic phase diagram of sodium chloride solution

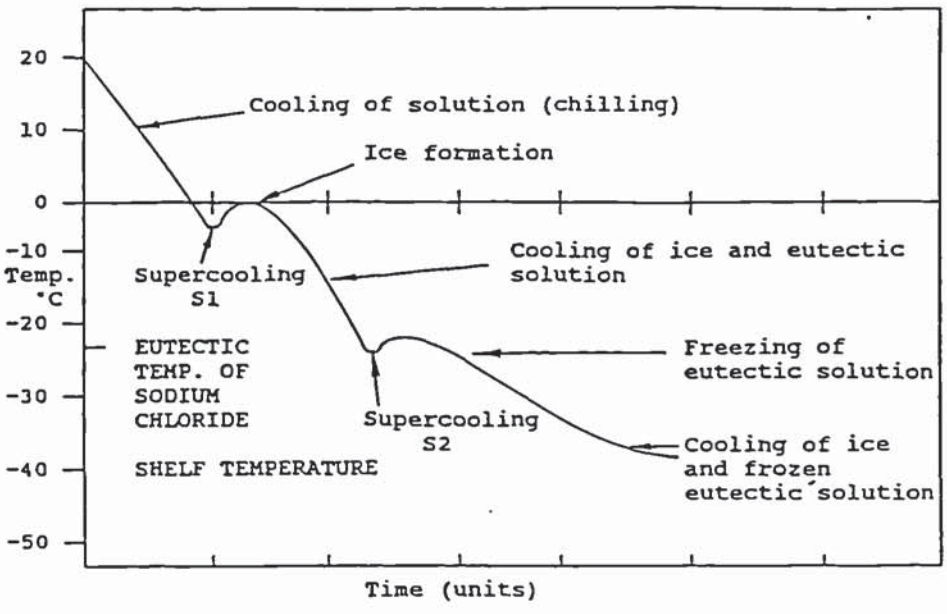


FIGURE 1.3: Pre-freezing cooling curve for an aqueous solution of sodium chloride

Reducing the temperature still further will increase the concentration of the salt solution during the eutectic transition. A second supercooling inflexion is followed by crystallisation of the remainder of the water and the salt at its eutectic point, resulting in a mixture of ice and salt crystals. Incomplete freezing may occur as a consequence of not allowing the product adequate time to cool to below its eutectic temperature (crystallising system) or collapse temperature (non-crystallising system), or due to a mechanical failure of the cooling system in the freeze-dryer. Complex solutions containing several crystallising excipients should be cooled below the lowest eutectic temperature in order to ensure complete freezing, while the behaviour of non-crystallising solutions can be variable, depending on the thermal history of the solution (MacKenzie, 1977). Examples of non-crystallising solutions are dealt with later in this section. Such solutions often require detailed thermal and freezing analysis studies before safe lyophilisation conditions may be found and it is important that the lyophilisation scientist has a knowledge of the characteristics of such solutions before suitable freeze-drying formulations can be developed.

As ice forms, the concentration of all solutes in the aqueous phase increases significantly. For a pharmaceutical product, it may be necessary to ensure that the stability of the active component is not adversely affected by high concentrations of co-solutes during freezing. When cooling conditions are controlled, the result should be that all solutes which were present in the initial solution are immobilised and distributed evenly throughout the frozen matrix. The consideration of the rate of freezing is a crucial factor in ensuring that freeze-concentration is avoided. In many instances, especially when laboratory-scale formulations are investigated, the type of freeze-dryer used may not be adequate so as to ensure reliable shelf-cooling and temperature control. In these cases, the rate of freezing cannot be precisely controlled by the operator. However, where the rate of freezing may be predetermined and monitored, this enables the operator to control the rate of freezing of the solution precisely.

Table 1.1 describes the five different types of freezing behaviour which a solution containing varying proportions of NaCl and sucrose was observed to adopt by MacKenzie (1985), who described the relevance of such behaviour to different complex solutions. Clearly, there are a number of freezing patterns which a solution may adopt, depending on the proportion of

crystallising solute(s) present, which will have implications on freeze-drying behaviour. Figure 1.4 focuses on the two extremes of freezing behaviour which a solution will adopt. In practice, a totally crystalline matrix is rarely achieved. Much more common, however, is the microstructure (b) resulting in the formation of an amorphous glass. A glass is a non-crystalline liquid with a high viscosity (>10^{14}Pas) which changes with temperature (Levine & Slade, 1987). This pattern of freezing behaviour is the most common in pharmaceutical products, for which an extensive treatment and discussion is provided by MacKenzie (1985).

***TABLE 1.1:** Five types of freezing pattern for a crystallising solute (from MacKenzie, 1985)*

***FIGURE 1.4:** Drawing of microstructure for a solute which (a) crystallises upon freezing and (b) remains amorphous upon freezing (from Nail & Gatlin, 1993)*

A glassy (vitreous) phase may be achieved in a number of ways. Firstly, if one or more components in the solution is reluctant to crystallise, then cooling such a solution will typically result in a partially amorphous phase, and the rapid cooling (quenching) of such a solution or will frequently result in an amorphous matrix. Even in glasses which appear to be solid, the constituent molecules will have some degree of movement, and can migrate through the glass, albeit at a slow rate (usually in the order of microns per year). It is also possible that a solution is frozen in such a manner that the resulting solid may be predominantly crystalline, yet also containing small 'pockets' of amorphous material. This is quite a common phenomenon, and may be essential for preserving biological activity. A mixed crystalline/amorphous structure may be heat-treated by annealing until full crystallisation of all components is achieved. However, in the case of products containing non-crystallising components such as proteins or certain polymers, it should be noted that full crystallisation will be unlikely, due to the reluctance of such components to crystallise.

In terms of microstructure retention and thus cosmetic acceptability during lyophilisation, crystalline solutes usually give an acceptable product. Thus, compounds such as many of the β-lactams (Pikal *et al.*, 1977, 1978) and cephalosporins (Gatlin & DeLuca, 1980) which have been reported to display better stability characteristics during shelf-storage when maintained in the crystalline state throughout processing, may be relatively simple to formulate, where the addition of traditionally-used crystallising excipients may prove sufficient to fulfil all processing requirements. The resulting product will be an elegant, cohesive plug containing the active ingredient which remains stable on reconstitution. However, many pharmaceutical solutions freeze to give amorphous glasses. Indeed, bioactive molecules may require the maintenance of an amorphous matrix in order that their biological activity be retained throughout the freeze-drying process (see chapter 4). Formulations which need to remain amorphous can often present problems in terms of structure retention. Since the viscosity of a glass changes with temperature, it is necessary to ensure that the glass does not soften during freeze-drying, as this may lead to processing defects such as collapse. The point at which a glass begins to soften and become flexible is termed its glass transition temperature. For a dried amorphous material, the glass transition temperature is denoted by Tg. The parallel transition temperature for a maximally freeze-concentrated solution is denoted by

Tg'. The value of Tg' (frozen state) will be significantly lower than that for Tg (solid state), since the water present in the frozen matrix will act as a plasticiser and consequently cause the transition temperature of the glass to decrease (Levine & Slade, 1987; Hancock & Zografi, 1994). This phenomenon is exemplified in the observation that the solid state glass transition temperature of sucrose decreases in a linear fashion with increase in moisture content (te Booy *et al.*, 1992). A similar correlation has been observed between collapse temperature and residual moisture content for a range of lyophilised excipient products (Adams, 1996). The heating of a product through its characteristic glass transition temperature (T_g or T_g') results in a concomitant increase in heat capacity of the sample. This enables T_g (and T_g') to be determined using differential scanning calorimetry (DSC) (see section 2.5). The values of T_g' (frozen state) and T_g (solid state) for a pharmaceutical formulation have significant implications on processing conditions and shelf stability, respectively. If the frozen or solid product is allowed to warm above its glass transition temperature, the material may exceed its collapse temperature (T_c). This is the point at which softening progresses to structural collapse of the material, a phenomenon which may be observed using a number of different techniques (MacKenzie, 1975). For a crystallising product, collapse will occur if the lowest eutectic melting temperature is exceeded.

Collapse of frozen material during freeze-drying, or of a lyophilised product during storage, can often lead to degradative processes within the product matrix; thus it is important to maintain a product below its characteristic collapse temperature both throughout and subsequent to freeze-drying (Pikal, 1985). One technique which has proved to be a valuable tool in lyophilisation technology is freeze-drying microscopy (FDM). This is a technique which allows a thin section of material to be examined by an on-line microscope, whilst being lyophilised (see section 2.4). In this manner, the collapse temperature may be observed at the sublimation interface as drying progresses through the sample. Numerous reports have described the success of FDM for this purpose. The information gained from this method may be used together with data describing, for example, crystallisation behaviour during freezing, annealing and drying, to provide practical information on suitable processing conditions for the frozen solution during drying.

To optimise freeze-drying conditions, products should be formulated to exhibit as high a collapse temperature (T_c) as possible, since increasing T_c will permit use of higher shelf temperatures, resulting in an increase in sublimation rate. The sublimation rate will also increase as the porosity of the drying matrix increases. Since the sublimation rate is related to shelf temperature and impedance to vapour migration by the dry layer, this rate may in turn be increased by incorporating excipients into the formulation which display high collapse temperatures, increasing cake porosity by using dilute solutions or reducing product fill depths (Pikal & Shah, 1990).

The collapse temperature of a product may be altered by changing the formulation to include excipients which afford greater thermal stability. For crystallising formulations, this would necessitate using excipients which raise the eutectic temperature (T_{eu}) of the overall system, whereas for a non-crystallising formulation, an increase in the solid state glass transition temperature (T_g or T_g') would be required. The present study concentrates on the behaviour of and partially- and non- crystallising systems, which are most representative of pharmaceutical formulations. DSC is a valuable analytical technique for the formulation scientist, since many potential freeze-dried formulations may be screened relatively rapidly, giving a value of T_g (or T_g') for each sample. This in turn enables a prediction to be made on the relative stabilities of a range of formulations tested, both during processing and for longer term storage. An example of a DSC profile for a glassy polymer is given in Figure 1.5. The glass transition is evident as a small endothermic rise and is represented by the midpoint of the rise measured from the extension of the pre- and post- transition baselines, that is, when the transition assumes half the value of this increase. The second thermal event in this profile is an exothermic peak, signifying crystallisation of the amorphous glass. The final event is a melting endotherm.

DSC is also useful in the evaluation of the long-term storage stability of a formulation. In some cases, the glass transition value for a particular formulation may decrease over a particular time period, indicating that the stability of that particular formulation decreases during long-term storage. Regular analysis of a sample over longer periods of storage may highlight changes in product structure over time. Some of the more important concepts

relating to thermal analysis and the stability of lyophilised products are discussed more specifically in chapter 3 of this report.

FIGURE 1.5: DSC thermogram for a glassy polymer (from Ford & Timmins, 1989)

In recent years, a number of technological developments in the field of thermal analysis have brought about new techniques such as isothermal microcalorimetry and modulated-temperature DSC. Isothermal microcalorimetry can provide useful information about many aspects of formulation stability in the solution state and also in the solid state. It may be suitably applied to the examination of possible interactions occurring between components present in a mixture, reactions in solution and in the solid state, and to the thermal stability of amorphous and crystalline products (and 'mixed' systems containing both crystalline and amorphous regions) held at a constant temperature. This technique is becoming increasingly more commonly used in the pharmaceutical industry (Koenigbauer, 1994; Buckton, 1995). It enables the formulation scientist to examine a lyophilised product with respect to the critical temperatures of the different regions present in the same sample, and to examine thermal events which accompany any structural changes occurring in the sample under conditions of defined and controlled temperature and humidity. Modulated temperature DSC (MT-DSC) is useful in that it can allow separation of complex events into their component parts. In this

technique, the temperature of the sample and reference are not increased in a linear fashion, but rather in a sinusoidal manner (Reading, 1993). The rate of temperature increase applied to the sample and reference fluctuates slightly above and below the line of average temperature increase. In this way, it is possible to resolve the reversible and non-reversible components which would normally appear as a single thermal event when analysed by conventional DSC.

1.2.3. Primary Drying (sublimation)

The primary drying process involves the sublimation of the solute (usually water) through the frozen matrix. Following freezing, the vacuum chamber of the freeze-dryer is evacuated to allow free migration of water vapour from the frozen mass. Figure 1.6 shows the phase diagram of water and demonstrates the conditions under which ice may sublime directly to give a vapour, provided that sufficient heat is applied to the product to compensate for latent heat losses due to the sublimation process.

As stated in the previous section, a number of techniques are available to ascertain the collapse temperature of a product throughout processing. Freeze-drying microscopy (FDM) is becoming a more widely-used technique, since it provides the observer with visible evidence of product collapse. This offers advantages over techniques such as DSC, which provides a measure of eutectic melting or glass transitions through the detection and interpretation (extrapolation) of secondary events such as a change in heat capacity of a sample which accompanies the primary event itself. However, on the practical level, DSC is particularly efficient in terms of high-throughput analysis.

Bellows & King (1972) were among the first observers of collapse using microscopy, reporting a loss of structure in the dried region adjacent to the sublimation interface. Vials of product displaying collapse will often be discarded simply due to lack of cosmetic acceptability, without regard to the biological activity of the product. Thus, it is important that collapse be avoided. Lyophilisation technology has long since reached the stage where many of the aspects of the freeze-drying process have been quantified and the relationship between each variable involved in the process defined mathematically, including the primary

drying (sublimation) phase especially, since the variables governing this stage are complex and generally less well understood than those for freezing (see Liapis & Bruttini, 1994). Most recent studies in this area are based on earlier works by MacKenzie, whose articles remain perhaps the most comprehensive publications describing the physico-chemical basis of lyophilisation (MacKenzie, 1966, 1975, 1976, 1977, 1985).

FIGURE 1.6: *The experimental phase diagram for water (adapted from Atkins, 1989)*

For the freeze-dryer operator, the major concerns are essentially to select the appropriate variables, where equipment permits, in order to achieve an acceptably dry product in a reasonable time. It is, of course, not possible for every operator to become an expert in the field of freeze-drying; although, the appreciation of the fundamental concepts will enable more efficient cycles to be developed. The experiments detailed in chapter 6 of this report provide a practical example of how the understanding of such parameters and the use of available analytical techniques were put into effect in the devising of more efficient freeze-drying cycles for model crystallising and non-crystallising solutions. In particular, the information gained by the use of freezing resistivity measurements and freeze-drying

microscopy enabled lyophilisation parameters to be selected confidently. However, if such methods are not available, then efforts should at least be made to ascertain the collapse or glass transition temperature of the frozen matrix, in order that this temperature is not exceeded during the drying phase. The primary drying step of the lyophilisation cycle may be more effectively understood by considering the stage in terms of heat and mass transfer. Primary drying involves the sublimation of the solvent from the frozen product, a process which is effected by the transfer of the vapour from the frozen mass (mass transfer). In order for this process to occur, energy must be applied to the system (product) to compensate for the latent heat losses due to sublimation (heat transfer). Both transfer processes will be governed by the variables in the system, including shelf and product temperature, chamber pressure, microstructure of the frozen matrix, impedances within the system, cake porosity and product fill volumes. Unless this balance between heat input and vapour extraction is maintained, drying may cease or conversely the product may melt or collapse. The aim of this section is to outline the practical implications of understanding these factors, rather than to consider their mathematical significance (see Mellor, 1978).

One of the most crucial factors which determines the success of the drying process is probably the avoidance of product collapse during the process. There are a number of techniques available to allow successful monitoring of important parameters during the lyophilisation process, as outlined in section 1.2.6 of this report. Such techniques allow the operator to ensure that the product temperature (T_p) does not exceed the product collapse temperature (T_c). Throughout the drying process, T_p will be governed by the heat and mass transfer characteristics of the system. It can perhaps now be seen that the rate and eventual success of the lyophilisation process is dependent on a complex set of variables.

Figure 1.7 illustrates the different mechanisms of sublimation which may occur during the drying process. It has been estimated that for each gram of ice subliming from a frozen mass under reduced pressure, typically 1000 litres of water vapour may pass through the drying product cake, escape from the partially stoppered vial and migrate to the condenser chamber under (Pikal, 1991a). To maintain constant product temperature, the heat transfer process must balance the energy input to a vial with energy lost from the product in the escaping

vapour. In an ideal situation, this balance would be achieved, with a constant energy input to the product from the shelf being matched by a constant rate of sublimation. Practically, since impedance to vapour flow is constantly changing as the dry layer increases in depth, the heat and mass transfer equation can only be maintained by varying heat input throughout the run. Some of the factors which determine the ease and rate of sublimation cannot be precisely controlled by the operator once drying has begun.

***FIGURE I.7:** Diagrammatic representation of the four principal sublimation mechanisms (from MacKenzie, 1965)*

As described above, the microstructure of the frozen matrix will have a significant effect on the rate of sublimation. The resistance to vapour flow through the dried region of the product will be dependent on whether the matrix adopts a crystalline, partially crystalline or

totally amorphous matrix. This will in turn depend upon the individual and combined behaviour of the components present in the initial solution, their relative proportions, as well as the overall (total) concentration. Pikal & Shah (1990) reported that primary drying of povidone was observed to be much faster in a 2% solution (1.4mm/hr at -30°C) than in a 10% solution (0.13mm/hr at -30°C). The authors also found that the collapse temperature was 2.5°C higher for the 2% solution than the 10% solution, and suggested that this may well have been a drying rate effect. In addition, it was reported in the same study that a product based on lactose as an excipient showed a collapse temperature of -30°C by a spectroscopic method, whereas the same product dried in vials at product temperatures up to -28°C showed no signs of collapse. However, such relatively minor differences in collapse temperature may equally be attributed to the difference in temperature measurement techniques utilised in each of the two methods described.

A further phenomenon which may provide resistance to vapour flow during drying is that of 'skin' formation during processing. In particular, mannitol is often observed to apparently crystallise out of a multi-component solution upon warming during the initial stages of freeze-drying, forming a thick layer on the top of the frozen matrix, a common result of this being vial breakage; indeed, Williams & Dean (1991) devote an entire report to the study of this phenomenon. When a skin layer is formed, this causes the flow of vapour to be impeded, resulting in a decrease in the rate of mass transfer from the frozen product. As the energy (heat) transfer from the shelf to the vial is no longer matched by the mass transfer, the product temperature in the vial rises. This increase in temperature can encourage crystallisation of mannitol as well as any unfrozen water present in the mass, which expands downwards through the vial and may lead to vial breakage. This problem may be circumvented by the inclusion of an annealing step in the freezing stage of the lyophilisation cycle (Pikal, 1991a). Since the eutectic melt of a mannitol solution occurs at around -1.4°C (MacKenzie, 1975), annealing such a frozen mass to give full crystallisation may be carried out with little danger of causing the mass to melt. However, many excipients commonly used in freeze-drying formulations exhibit relatively low collapse temperatures (for example, the Tg' of sucrose is -32°C), which increases the risk of collapse of these systems during annealing. For these formulations, it may be necessary to examine their relevant thermal

characteristics using, for example, differential scanning calorimetry (DSC), freeze-drying microscopy (FDM) or freezing resistivity measurements prior to lyophilisation, in order to predict freeze-drying behaviour.

1.2.4. Secondary Drying (desorption)

Primary drying usually results in a product which has a water content of approximately 10% (w/w). However, this is not sufficiently low a level to ensure good stability of many bioproducts over long periods of time. For many products, the increased mobility of components in the lyophilised cake resulting from such high moisture levels would facilitate potentially damaging reactions which could compromise the stability and activity of the product. In such circumstances, a secondary drying step is employed.

In contrast to primary drying which involves the sublimation of the frozen solvent (usually water), secondary drying involves the removal of the solvent from the sample by desorption. To facilitate desorption drying, it is customary to raise the shelf temperature and reduce chamber pressure after primary drying (Mellor, 1978). However, in his review of the freeze-drying of proteins, Pikal (1991a) has suggested that it is generally inefficient to evacuate the drying chamber to a pressure of less than approximately 0.2mbar, assuming that water is the solvent being removed from the system, as this will not usually increase the efficiency of the drying process, irrespective of the fill volume of the vial or the components present in the frozen matrix. This value is a function of the property of water and its sublimation characteristics. Therefore, it is a variable which will apply to most freeze-drying equipment, and should thus be taken into account when optimising freeze-drying cycles (see chapter 6). However, as discussed in chapter 6 of this report, there are further issues which dictate that a lower chamber pressure does not necessarily lead to increased efficiency of the drying process, and the optimum pressure for each particular product will need to be calculated according to these factors.

Premature increase of the shelf temperature before all ice has been removed will risk collapse or eutectic melt in those samples containing residual ice. Consequently, it is important that the operator is able to determine when primary drying is complete. The most commonly-

used method to achieve this is 'product temperature response'. Here, the shelf temperature is increased for secondary drying only when product temperatures approach that of the shelf temperature. Many freeze-dryers allow continuous monitoring of sample temperature throughout the process by the use of probes placed into a number of vials at different shelf positions. As a general rule, to avoid loss of product structure and to minimise damage to the active ingredient, conditions should be controlled so that the product temperature never rises above the collapse temperature during secondary drying. It should be noted that the glass transition temperature of a sample increases as the moisture level in the sample decreases. For a frozen solution, T_g' is the critical temperature, but for a solid, dried sample, the solid state T_g is the critical temperature. At all other levels of moisture, the maximum permissible temperature of a sample will lie somewhere between T_g' and T_g, as demonstrated by Hancock & Zografi (1994).

The presence of residual water may have a detrimental effect on product stability and care should be taken in the determination of the length of the secondary drying process and the desired moisture levels of the final product. Moisture levels of typically less than 3% (w/w) are achieved after secondary drying. However, it is possible that 'overdrying' may lead to damage of some biologically sensitive materials (Hsu *et al.*, 1991). In the case of proteins, the water required to keep the protein in its native state may be lost through overdrying, resulting in conformational changes in the quaternary or even tertiary structure of the protein (representing the separation of sub-units, or unfolding, respectively), which brings, as a consequence, loss of biological activity and even degradation (see Arakawa *et al.* (1993) for review). The concept of protein formulations having characteristic optimum levels of residual moisture after freeze-drying was the topic of a discussion by Hsu *et al.* (1991) and has also been dealt with more recently by Towns (1995). The effect of residual moisture on the stability of proteins in particular is discussed in chapter 4 of the present report.

1.2.5. Stoppering and Removal

The optimal water content of the lyophilised product should be established before the vacuum is released in the drying chamber and the product is removed. For example, some materials may need to be stored under vacuum, or under an inert gas. Many pharmaceutical

formulations are freeze-dried in vials with vented lyophilisation stoppers, as shown in Figure 1.8 below. Freezing and drying is carried out with the stopper placed into the neck of the vial at the freeze-drying position (a) to permit water vapour to escape from the vial. When the lyophilisation process is complete, the stoppers can be pushed down fully into the vial necks (b) by means of a shelf-stoppering mechanism, where the shelves in the drying chamber are pushed together.

FIGURE 1.8: *Diagram of the ventilated stopper used in freeze-drying, showing mode of action (from Adams, 1991)*

Pharmaceutical products are often sealed under nitrogen, to minimise oxidative degradation of the dried product during storage. In this case, dry nitrogen gas is admitted to the drying chamber (instead of air) before vials are fully stoppered. For freeze-dried pharmaceutical products, sealing under a 'partial vacuum' is common. and in this instance, an inert gas such as nitrogen is admitted into the drying chamber to partial pressure before the vials are sealed, so that the vial contents are maintained at a slightly negative pressure under the inert gas. The maintenance of a partial vacuum in a vial often allows more rapid rehydration of the product prior to use, compared to when this is carried out at atmospheric pressure. Sealing

under full vacuum (*ca.* 10^{-1}mbar) is often not desirable, since foaming of the product will occur upon reconstitution, resulting in difficulties when the entire contents of the vial need to be removed.

1.2.6 Storage and Stability of Lyophilised Products

It is often assumed that a freeze-dried product is immune to storage decay and is often expected to remain active under any storage conditions. Although freeze-dried foods and beverages store well at ambient temperatures, some pharmaceutical manufacturers recommend storage temperatures of 4°C or even -20°C for their lyophilised products. In such cases, there is probably a great deal of room for improvement of the formulation or processing conditions of the product by the manufacturer. In the case of products which currently require storage below 4°C, the solid state collapse- or glass transition-temperatures of such products are too low to ensure their stability at room temperature. The collapse temperature of a product may be altered by varying the combination and concentration(s) of excipients used in its formulation (Adams, 1996). Thus, the substitution of excipients within a product for alternatives which display higher collapse temperatures can increase the collapse temperature of the formulation, both in the frozen mass and in the dried product, which in turn will lead to greater product stability during processing and storage.

A further stability issue is that of product homogeneity. The presence of small amorphous regions within an otherwise crystalline matrix may be a crucial factor determining product stability. In a completely crystallising product, the solution-state and solid-state stabilities will be governed by the overall melting temperature of the frozen or solid masses, respectively. This is likely to be represented by a eutectic (or partial eutectic) temperature in most cases, but is also dependent on the physical compatibility of components present in the mixture. In either case, the melting point of the frozen or dry mass will be the most important factor to be considered when selecting suitable processing and storage conditions. However, in a product where complete crystallisation is not achieved, the stability of the amorphous regions within the product will be dependent on maintenance of product temperature below that of the frozen state or solid state glass transition temperatures (T_g' and T_g respectively) of these regions. In addition, one should note that in the lyophilised

state, it is likely that the amorphous regions will have a higher moisture content than the crystalline regions within the product. Thus, the glass transition temperature of the amorphous regions in the lyophilised product may be decreased due to the plasticising effect of residual moisture. In this case, the glass transition temperature of such a region will lie somewhere between T_g (solid state) and T_g' (Hancock & Zografi, 1994).

Since the morphology of the dried structure is determined during initial freezing, although this may alter to some extent during drying, the selection of excipients which provide the formulation with the desired freezing characteristics is essential. The freezing behaviour of a formulation may be altered by varying the combination of selected excipients and their relative proportions within the mixture. Many lyophilised products contain saccharides as bulking agents (*e.g.* lactose), agents which provide a crystalline matrix (such as mannitol), compounds which protect an active component from the stresses of the lyophilisation process (*e.g.* trehalose) *etc.* Often, a formulation will contain a number of saccharides in combination. Most saccharides display amorphous character during freezing in solution. Although this characteristic may be desirable, for instance, when used in combination with a protein where crystallisation of the matrix would result in a loss of activity, the unpredictable nature of glasses in terms of moisture retention and combinatorial behaviour (when several glass-forming components are included in the same formulation) can often result in processing and storage defects. To circumvent this problem, crystallising excipients such as mannitol or inositol are often added to solutions prior to freeze-drying, to maintain the rigidity of the product microstructure throughout the lyophilisation process and subsequent storage. However, such excipients are rarely able to induce crystallisation of a matrix. The behaviour of mixtures of crystallising and amorphous co-solutes is complex and will be considered in experimental discussions described in chapter 3 of the present report.

Techniques such as freeze-drying microscopy (FDM) and differential scanning calorimetry (DSC) are often employed to analyse the thermal behaviour of a frozen matrix or a dried product, to optimise processing and storage conditions. In addressing the issue of longer-term stability, however, one is faced with many other parameters, both physical and chemical, which must be taken into consideration. In particular, the long-term stability of proteins and

peptides is a very complex area, as described in a number of reviews cited for other purposes in the present study (Arakawa *et al.*, 1993; Wang & Hanson, 1988).

1.3. Technical Aspects of Freeze-drying Equipment

1.3.1 Types of Refrigeration System

The structure of a basic (single stage) refrigeration unit is shown in Figure 1.9. The system consists of a compressor, a condenser and an evaporator which are linked by pipes which carry the refrigerant material in a circuit. Compressed gas is forced through a pipe to a condenser (heat exchanger), where it condenses to give a warm liquid. This liquid is forced through an expansion valve or capillary to an evaporator, where it evaporates to give a gas. This is an endothermic process, which absorbs energy in the form of heat from the surroundings, thus cooling the evaporator. The gas is finally pumped back to the condenser by a compressor, before beginning the circuit once again.

FIGURE 1.9: *Diagrammatic representation of a single stage refrigeration system (from Sutherland, 1995)*

If such a system is used in a freeze-dryer, with the condenser (and/or shelves) acting as an evaporator, then this is known as a "direct expansion" system, since the refrigerant evaporates (expands) directly into the condenser or shelf. Some advantages of the direct expansion system are that it is simple to manufacture, energy efficient and can achieve low temperatures. It is suitable for the cooling of condensers, but is only usually accurate to within ~5°C, which makes it an unsuitable method of cooling shelves in the more advanced types of freeze-dryer used in research and development. In addition, due to the high pressures reached in the system, the pipes which carry the refrigerant need to be non-flexible and are usually constructed from stainless steel. This means that if such a system is used to cool the shelves of a freeze-dryer, then the shelves must be of a fixed position, and thus no shelf-stoppering system may be used (see section 2.1).

Many modern freeze-dryers use the direct expansion system to cool condensers. However, a separate system is often used to cool shelves. The method of "diatherm fluid circulation" is a more controllable method of refrigeration than direct expansion. In addition to the basic refrigeration system which it shares with the direct expansion system, the diatherm fluid circulation system also consists of a secondary circuit comprising a heat exchanger and a separate refrigerant. Shelf temperatures can be maintained to typically within ±1°C, in a temperature range of between -60°C and +60°C, depending on cycle parameters (or stage in cycle). One disadvantage of this system that it must necessarily include a pump, to force the diatherm fluid around the circuit. This pump will produce heat which is dissipated back into the system, thus reducing efficiency. A second disadvantage is that silicone fluid, which is the most commonly used fluid in this type of refigeration system, becomes highly viscous below temperatures of around -55°C, resulting in pumping difficulties. However, since most freeze-drying cycles do not require such low shelf temperatures, this method is often the method of choice for shelf cooling systems where strict temperature control is the main priority. Since the diatherm fluid is not subjected to high pressures, flexible tubing may be used which enables the movement of shelves within the freeze-dryer, thus allowing shelf-stoppering mechanisms to be used.

1.3.2 Temperature measurement

Product- and shelf- temperature monitoring are common and useful in freeze-drying, since they provide continuous feedback on the state of the lyophilisation process. A number of devices are available to enable these measurements to be made in freeze-drying. The choice of thermometer for a particular application depends on the temperature range to be measured, sensitivity, accuracy, reproducibility and cost. For example, a mercury-in-glass thermometer will be of no use for freeze-drying, since mercury freezes at -38°C. In addition, an electrical output is required for recording and controlling temperature. Nail & Gatlin (1993) provide a comprehensive review of the many types of device available for this purpose, although only the resistance thermometer and the thermocouple are generally used for product and process monitoring in freeze-drying (Adams, 1991).

The thermocouple is based on the principle that when a circuit is formed by joining the ends of two wires made from different metals, an electrical potential develops between the two wires which is dependent on the temperature to which the junction is exposed. Many different types of thermocouple are available, covering a wide range of temperatures and sensitivities. Thermocouples offer several advantages over resistance thermometers, in that they offer faster response times, are more rugged, are capable of withstanding higher temperatures, can measure temperatures at very precise locations (point sensing), are inexpensive and exhibit no self-heating error. The VirTis Genesis 25EL freeze-dryer used in the present study (see section 2.1) was equipped with type K thermocouples (chromel-alumel) which were used to monitor product temperature.

There are several aspects of product temperature monitoring which should be recognised by the freeze-drying scientist. Firstly, the presence of a temperature probe in a vial acts as a site for heterogeneous nucleation of ice, thereby reducing the extent of supercooling relative to non-monitored vials (Nail & Johnson, 1992). Since monitored vials freeze first, extra freezing time (known as soak time) is often allowed for the non-monitored vials to freeze fully before the primary drying cycle is begun. Secondly, since monitored vials freeze sooner and supercool less, the process of ice crystal growth in these vials occurs more slowly, resulting in larger ice crystals than in non-monitored vials. This leads to larger pores in the

drying matrix, less resistance to mass tranfer and a faster drying rate than in the non-monitored vials. Thus, the non-representative nature of monitored vials is also true during drying, as well as in freezing (Nail & Gatlin, 1993). These issues are discussed further in chapter 6 of the present report.

1.3.3 Pressure measurement

The monitoring of chamber pressure throughout the drying cycle is important, since pressure affects both heat and mass transfer, as described earlier in this chapter. There are a number of pressure monitoring devices for use in freeze drying, but the two most commonly fitted to freeze-dryers are the capsule gauge (rough gauge) and the thermocouple (or Pirani) gauge. The capsule gauge is capable of pressure measurement over a pressure range of approximately 10mbar to atmospheric pressure (*ca.* 1000mbar). A thermocouple gauge is often used in addition to a capsule gauge on a freeze-dryer, since it is capable of monitoring chamber pressure at high vacuum and is typically sensitive in the pressure range of $\sim 4 \times 10^{-1}$ mbar down to $\sim 1 \times 10^{-3}$ mbar (Adams, 1991). Used in combination, these two types of gauge allow continuous monitoring of pressure within the drying chamber throughout the entire drying process. The chapter by Nail & Gatlin (1993) presents a review of the types of pressure measurement device available, while details of the pressure monitoring devices used in the present study are given in sections 2.1 and 2.2.

1.4 Aims and Objectives of this Project

The primary objective of this project was to address four key areas in the field of pharmaceutical freeze-drying, as listed below. These four areas of study are addressed in chapters 3 to 6.

1. Assessment of the thermal characteristics of dried and non-dried excipients and model freeze-dried pharmaceutical formulations. The objective of this study was to examine the thermal characteristics of a range of single excipients, combinations of excipients and of model formulations in the light of their observed freeze-drying behaviour and stability (chapter 3).

2. Preservation of model therapeutic proteins by lyophilisation and the use of lyoprotectants. The aim of this study was to examine the lyophilisation characteristics of model polymeric proteins, with particular emphasis on the stabilisation of these proteins during the freeze-drying process by protective additives and the elucidation of the mechanism of stabilising interactions at the molecular level (chapter 4).

3. The use of freeze-drying in the preparation and stabilisation of stealth liposomes. The objective of this chapter of work was to investigate the application of the freeze-drying process to the preparation of sterically stabilised ("stealth") liposomes, since the successful use of the process has previously been demonstrated in the preparation of conventional liposomes. A further aim was to investigate whether freeze-drying could be utilised to stabilise such liposomes, once prepared. It was also anticipated that this study would be extended to include the investigation of the use of protective additives to preserve liposome structure during the lyophilisation process and in subsequent storage in the lyophilised state (chapter 5).

4. Freeze-drying cycle development and optimisation. The objective of this final area of study was to analyse the critical temperatures and freezing characteristics of model crystallising and non-crystallising formulations using thermal analysis and freeze-drying microscopy, to use this information to develop more efficient lyophilisation cycles for these products and to assess the practical implications of employing these revised cycle parameters in terms of cycle time and efficiency and product acceptability (chapter 6).

2. INSTRUMENTATION and METHODOLOGY

2.1. Lyophilisation of Solutions using the Edwards Modulyo Freeze-Drier

2.1.1. The Edwards Modulyo Freeze-Drier

The Edwards Modulyo is a relatively simple design of freeze-drier, consisting of a condenser and drying chamber, with vacuum created by an external pump, as demonstrated in Figure 1.9 (section 1.4). The drying chamber itself contains four shelves which cannot be cooled directly; thus samples must be frozen before being loaded into the machine. The total capacity of the condenser is approximately 3 litres. The particular model used in the studies detailed in this report was also equipped with a shelf heater (with one heat setting, approximately 30°C) and a mechanism which enables vials to be stoppered under vacuum. The condenser is cooled by a process known as direct expansion (see section 1.4), to a fixed temperature range of -55 to -60°C.

2.1.2. Procedure

Aliquots of aqueous solutions (typically 0.50ml or 1.00ml) were dispensed into glass freeze-drying vials (3 ml capacity) fitted with two-position butyl freeze-drying stoppers, as shown in Figure 1.8. Stoppers were inserted to the first position before samples were placed at -70°C for at least one hour, to ensure complete freezing. The Modulyo condenser was switched on and allowed to cool to around -60°C whilst the samples were freezing. Once fully frozen, the vials were transferred to the shelves of the Modulyo, and the shelf assembly placed within the thick-walled drying chamber. After ensuring that the chamber air admission valve and the condenser drain valve were fully closed, and that the rubber chamber seals were intact and in place, the vacuum pump was switched on. Once the chamber pressure had reached 0.2 torr, the shelf heater was connected to the shelf assembly unit and switched on. Since the Edwards shelf heating unit had only one heat setting (~30°C), this was switched off after approximately 4 hours for vials with a fill-depth of around 1 cm, so as to reduce the possibility of samples exceeding their characteristic collapse temperature during the drying process. After drying for typically 16-24 hours, the vials were stoppered under full vacuum before the chamber air admission valve was opened and the chamber pressure allowed to

reach atmospheric pressure. Dried samples were then remover from the chamber. Finally, the condenser was switched off and the frozen condensate allowed to thaw before the drain valve opened and the condensate released. Dried products were stored at +4°C, unless specifically stated otherwise.

2.2. Lyophilisation of Solutions using the VirTis Genesis 25EL Freeze-Drier

2.2.1. The VirTis 'Genesis 25EL' Freeze-Drier

The instrument used in this study was a four-shelf model of the VirTis Genesis 25EL, which has separate vacuum and condenser chambers, as depicted in Figure 1.10 (section 1.4). Each shelf measures 375mm x 250mm, giving a total shelf area (sum of 4 shelves) of 0.37m^2. Shelf heights (and thus vertical distances between shelves) may be adjusted to accommodate vials of up to 135mm high. The capacity of the condenser is approximately 25kg. The shelves, condenser and drying chamber are all constructed using stainless steel. Refrigeration is *via* a 'single cascade' unit, which cools both the condenser and the shelf by the use of a silicon oil diatherm system (see section 1.4). The silicon oil can be alternately cooled and heated externally, before circulating through the shelves.

The Genesis 25EL freeze-drier can be operated on manual mode or through a microprocessor-controller. The microprocessor has 5 programmable shelf cooling steps and 17 heating steps. Up to 10 programs can be stored in the microprocessor circuit. Chamber vacuum can also be controlled *via* an air bleed. The microprocessor controller enables heating and cooling rates to be programmed throughout a cycle. Stoppering is *via* a hydraulic mechanism: a valve between chamber and condenser permits chamber pressure to be monitored and sample temperatures monitored by Type K thermocouples. Shelf temperature is monitored by measuring the temperature of circulating diatherm fluid on entry to and exit from each shelf; the mean value is calculated and taken to represent the mean shelf temperature. As stated above, the circulating diatherm fluid method of cooling allows shelf temperatures to be more closely controlled than in driers which use the more crude method of direct expansion for shelf cooling. Pressure measurements completed by means of a capsule gauge ('rough gauge') which measures over the range 10mbar to atmospheric

pressure (*ca.* 1000mbar). A thermocouple gauge is also used for monitoring the chamber pressure at high vacuum (sensitive from $4x10^{-1}$ mbar down to $1x10^{-3}$ mbar). All data from monotoring devices may be plotted at selected time intervals throughout the cycle. Running conditions in the Genesis 25EL may be strictly controlled, with the chamber conditions and sample temperatures monitored continuously throughout the cycle. In addition, it is equipped with a shelf cooling facility, so that samples can be frozen to a desired temperature under chosen conditions.

2.2.2. Procedure

Solutions were dispensed into glass freeze-drying vials (10 ml capacity) fitted with butyl two-position stoppers. Sufficient vials were used such that an entire shelf tray was filled (shelf size 370mm x 250mm). Temperature probes were placed into eight of the vials in the tray, such that the end of the probe was in contact with the bottom of the glass vial. The microprocessor was then programmed to give the desired cycle conditions, before the cycle run was begun. A listing of each programmed cycle was made before starting each run, and all conditions were monitored and printed at five minute intervals and when the cycle entered a new phase (see sections 6.2 and 6.3 for examples of program listings).

2.3. Assessment of Freezing Characteristics of Solutions using Resistivity Measurements

2.3.1. The Edwards Freezing Resistance Analyser

The freezing analyser allows the user to observe phase changes by measuring the electrical resistance (resistivity) between two stainless steel electrodes 1 cm apart, placed into a glass measuring cell containing 5 ml of solution, whilst being cooled or heated. A Pt 100 (platinum) resistance thermometer is inserted between the probes, to measure the analyte temperature. Platinum is often chosen for such applications, since, conveniently, each 1 cm of Pt wire increases the resistivity by 100 ohms per 1°C. The sample is placed into a stainless steel analyser block, which can be heated or cooled within a chamber which can be evacuated, to mimic freeze-drying conditions.

The resistivity measuring range over which the analyser operates is 10^{+3} to $5x10^{+6}$ ohms (Edwards technical data). The principle of the method relies on a change in resistance as the material freezes (from low to high resistance) or thaws (from high to low resistance). Measurements are completed on the thawing cycle, to avoid ambiguities which may arise due to effects of supercooling. Chamber pressure and solution resistance can be measured on gauges and solution resistance and temperature are recorded on a chart. For conducting solutions, a tangent is drawn to inflexions on the resistance trace in order to extrapolate the onset temperature of any changes in resistance. A further line is then drawn horizontally across the resistance trace and the corresponding product temperature determined from the temperature scale, as shown in Figure 2.1.

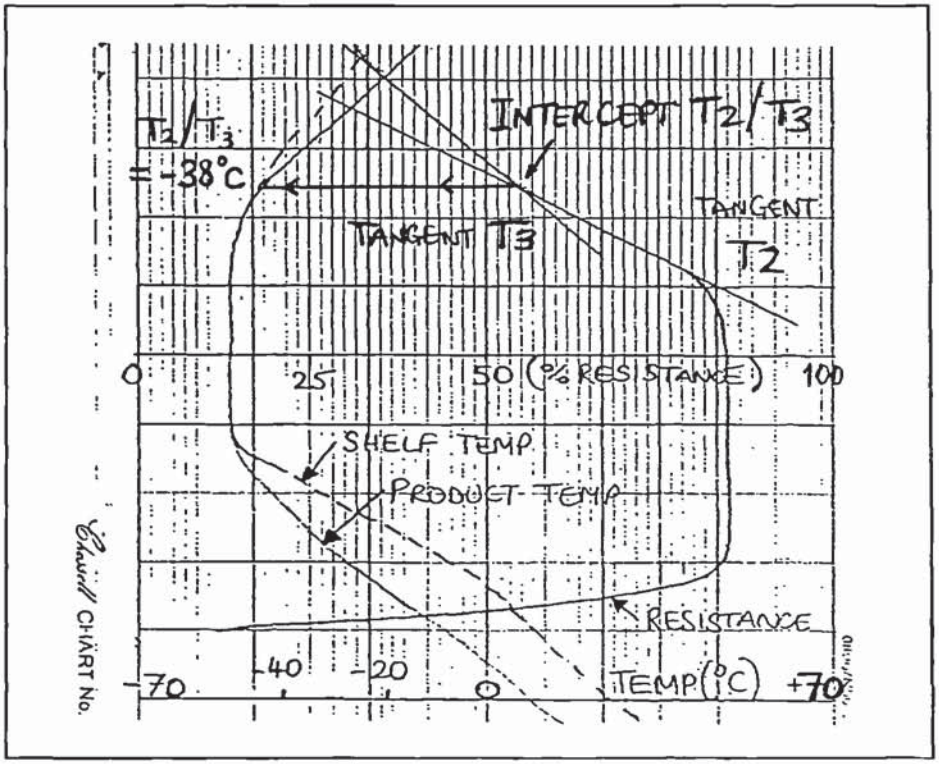


FIGURE 2.1: *Typical printout from the Edwards freezing resistance analyser*

2.3.2. Procedure

Firstly, it was ensured that the glass sample dish of the freezing analyser and the electrodes were clean and dry. An aliquot of analyte solution (5 ml) was dispensed into the glass sample dish, which was in turn placed into the stainless steel analyser block. The probe assembly was inserted into the analyte solution, the perspex lid placed over the analysis chamber, and

resistance measurements begun at room temperature. The cooling unit was switched on to cool the chamber to the desired minimum temperature. The block temperature, sample temperature and resistance were continually monitored and plotted on scrolling chart paper (χ-axis) against time (y-axis). Once the resistance of the sample had reached a maximum, the system was held at a constant temperature for a minimum of 10 minutes before a vacuum (0.1mbar) was applied to the chamber and the block set to heat at a constant rate of 60mW. Resistance measurements were taken whilst the sample was being warmed, to avoid effects of supercooling. Interpretation of a typical chart is given above in Figure 2.1.

2.4. Examination of Lyophilisation Characteristics of Frozen Solutions using Freeze-Drying Microscopy (FDM)

One of the earliest descriptions of a microscope set-up which enabled the researcher to observe a frozen sample whilst being dried was given by MacKenzie (1964). It was noted by the author that this technique allowed the elucidation of complex freezing characteristics as well as physical data such as eutectic freezing/melting and crystallisation. The freeze-drying microscope used in the present study was a custom-built construction as shown in Figure 2.2.

Based on an original design by MacKenzie (1964), the CAMR freeze-drying microscope consists of a small freeze-drying chamber fitted with optical windows, through which the behaviour of the sample during freeze-drying may be observed. The temperature of the drying product can be precisely controlled over the temperature range -120°C to +30°C. The collapse temperature of a product may be determined by cooling and warming.

2.4.1. Procedure

Firstly, a sample slide was prepared by placing two strips of glass onto the microscope slide of the freeze-drying microscope and covering them with a glass cover slip. Sufficient volume of the solution under investigation was dropped onto the microscope slide at the edge of the cover slip and allowed to be taken up into the slide by capillary action. The cover slip containing the analyte solution and a second blank glass plate were placed either side of the

stainless steel vacuum assembly (see Figure 2.2) and a slight vacuum pulled to immobilise the slips. The entire assembly was immersed into the methanol bath of the freeze-drying microscope (Figure 2.2) which had been precooled to the desired temperature. After having ensured that the analyte solution had become apparently solid, a vacuum of 0.2torr was applied to begin drying. Photographs were taken of the drying front (sublimation interface) at various stages and at a range of temperatures within the drying run, using a Wild Photoautomat MPS45 camera and control unit, equipped with light sensor and exposure-adjustment facilities. The freezing and crystallising characteristics of the solution were studied, and the temperature altered according to the behaviour of the solution.

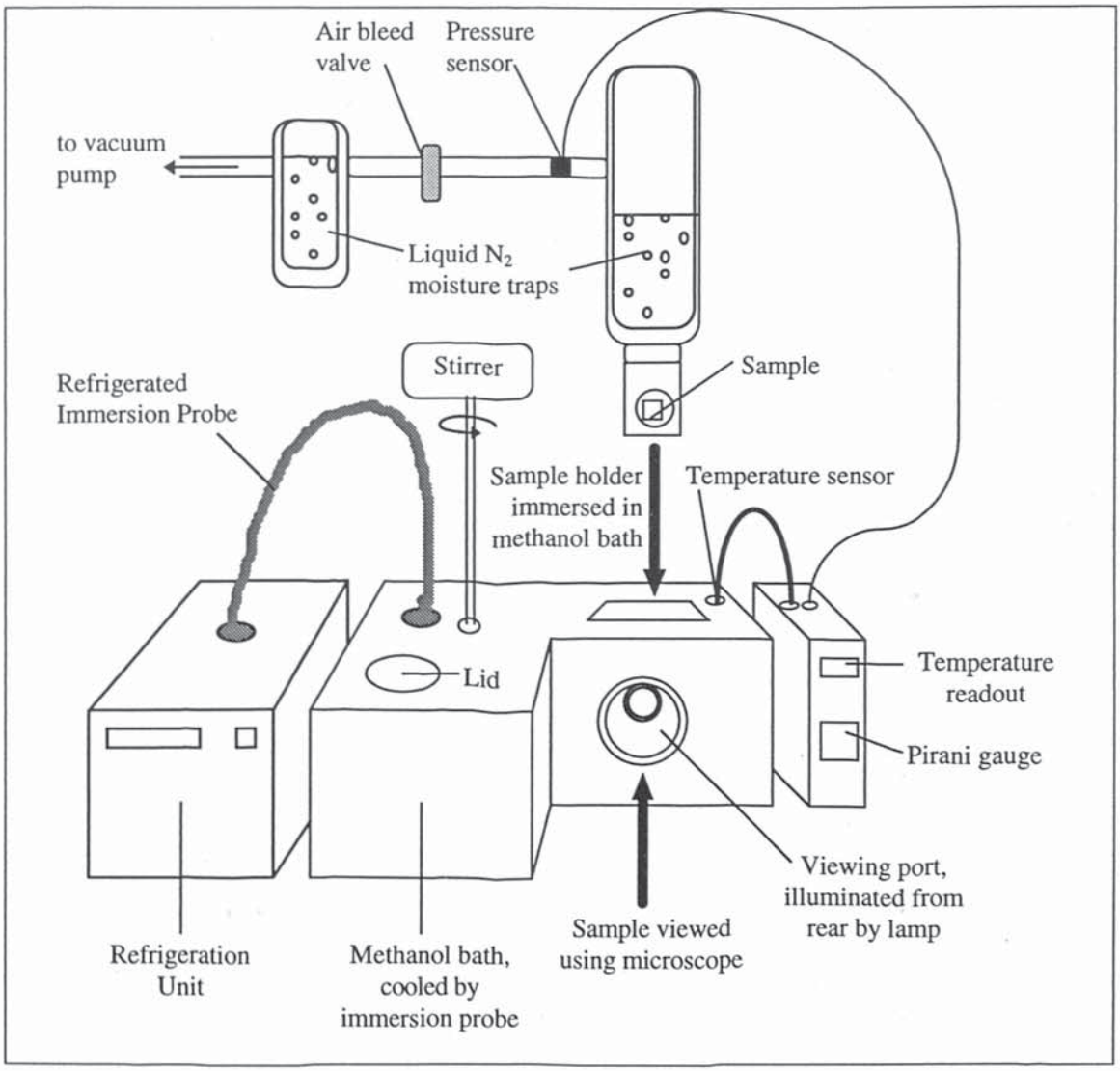


***FIGURE 2.2:** Schematic diagram of the CAMR freeze-drying microscope*

The temperature course followed during analysis by freeze-drying microscopy differed for each experiment carried out. In each case, it was necessary to increase or decrease the temperature throughout analysis, according to the thermal behaviour of the sample under observation. Drying was typically initiated at approximately -25.0°C. As the sublimation interface proceeded through the frozen matrix, it was ascertained whether the product was drying with collapse of structure, partial collapse or retention (see sections 3.4, 6.2 and 6.3 for examples). The temperature of the bath was then either increased or decreased, according to the structure at the given temperature and the expected behaviour of the sample.

2.5. Thermal Analysis of Solutions and Freeze-Dried Samples by Differential Scanning Calorimetry

2.5.1 Background

Differential Scanning Calorimetry (DSC) is a thermo-analytical method which measures the difference in energy (heat) inputs to a sample and a suitable reference as a function of temperature or time, whilst subjected to a controlled temperature programme. Heating is carried out at a constant rate, and the difference in energy inputs to the sample and a suitable reference is usually plotted directly against temperature. A deflection of the profile from the baseline is indicative of a change in the heat capacity of the sample; the European convention being to represent endothermic events by upward peaks, the American convention being the opposite to this.

In the present study, DSC was carried out using a Perkin-Elmer DSC-4 power-compensated system, comprising a calorimeter coupled to a microprocessor-controller *via* an interface unit. The system was accompanied by a refrigeration unit capable of cooling samples to -40°C. Routine data capture and analysis was performed using a Perkin-Elmer thermal analysis data station (TADS) system 3600, and plotted on a Perkin-Elmer plotter. Sealable aluminium sample pans (Perkin-Elmer code 0219-0041) were used for solid samples, and 'Volatile' pans (Perkin-Elmer code 0219-0062) for solutions.

Perkin-Elmer engineers designed and patented the first power-compensated calorimeter, the DSC-1, in the early 1960s. The DSC-4 system (see Figure 2.3) used in the present studies still utilises the Perkin-Elmer power-compensation system, but in addition, incorporates more recent features such as the use of a microcomputer to process the thermal data. The basic principle behind the power-compensation system construction relies on the fact that the sample and reference cells have separate heaters. The sample and reference are then maintained at nominally the same temperature, which is measured by the use of platinum resistance thermometers. The occurrence of endothermic or exothermic events (such as melting, crystallisation) in the sample will result in a slight variation in temperature of the sample pan, compared to that of the reference pan. This difference is compensated for by the system, which regulates the power output to the heaters and supplies different amounts of heat to each specimen in order to maintain both at the same nominal temperature. This difference in power output to the heaters is measured and plotted against temperature. This is in contrast with conventional (non-power-compensated) differential scanning calorimeters, which involves the sample and reference being heated at a constant rate by a single heater and the monitoring of differences in heat capacities of the sample and reference, which is then plotted against temperature.

FIGURE 2.3: *Schematic representation of a Perkin-Elmer DSC apparatus (from Ford & Timmins (1989), after Wendlandt (1986))*

Calorimetric accuracy and precision of ±1% and 0.1% respectively are claimed for the DSC-4 system (Perkin-Elmer, 1984), with temperature accuracy and precision of both 0.1°C being indicated. Heating rates of 0.1 to 100°C/minute are possible in 0.1°C increments with a temperature range of -70°C to 600°C.

2.5.2. Procedure

For solid samples, between 5-10mg was weighed accurately into an aluminium pan, which was sealed and placed in the sample chamber at room temperature. An empty pan, also sealed, was placed in the reference chamber. In the case of solutions or suspensions, typically between 15-20mg was used, depending on the concentration of the compound(s) of interest. If the sample was to be run from a temperature which was lower than ambient, the sample and reference chambers were initially cooled to the appropriate temperature via the microprocessor-controller unit. The controller unit and data station were programmed with the appropriate start and finish temperatures, together with the sample weight and the heating rate. The sample and reference were heated at the same given rate whilst the heating chambers were continually purged with nitrogen gas (20kg/cm^2). The difference in heat capacities was plotted against temperature and the raw plot saved onto a computer disk before all necessary data calculated from the obtained thermal profile (*e.g.* peak maximum/minimum temperatures, energies of endotherms/exotherms/glass transitions). A typical heating profile for a polymeric sample is shown in section 1.3.

2.6. Thermogravimetric Analysis (TGA) of Freeze-Dried Products

2.6.1. Background

TGA is a technique which enables the continuous monitoring of the mass of a sample whilst subjected to a controlled heating programme. The instrument used in the present study was a Perkin-Elmer TGS-2, which uses a null-point balance. Movement of the balance beam from its original position is sensed and a restoring force applied to restore the beam to its original position. The change in restoring force, proportional to the change in sample mass, is monitored. The configuration of the TGS-2 is with the sample hanging down from the

balance inside the furnace. The furnace itself has a low thermal mass to allow rapid linear heating and cooling rates (0.1 to 200°C/min in 0.1°C increments). At the time of production, the manufacturer claimed the microbalance to be the most sensitive in the industry (Perkin-Elmer technical data, 1986), capable of detecting weight changes as small as 0.1µg with 0.1% accuracy. This demands that the balance be resistant to ageing, temperature and vibration effects. The balance itself is also thermally isolated from the furnace to minimise interference.

2.6.2. Procedure

Thermogravimetric analysis was carried out using the Perkin-Elmer TGS-2 apparatus described in section 2.6.1 above. The microprocessor-controller unit was initially programmed with the appropriate start and finish temperatures of the run, together with the desired heating rate. The platinum sample pan was cleaned by heating in the blue flame of a Bunsen burner. The clean pan was placed into the pan cradle of the micro-balance of the TGS-2, the airtight chamber assembled around the cradle and the analyser weight set to zero. The chamber was disassembled, and a sample of typically between 0.5 mg and 5 mg was placed into the pan, which was then returned to the cradle, the airtight chamber re-assembled and the run started.

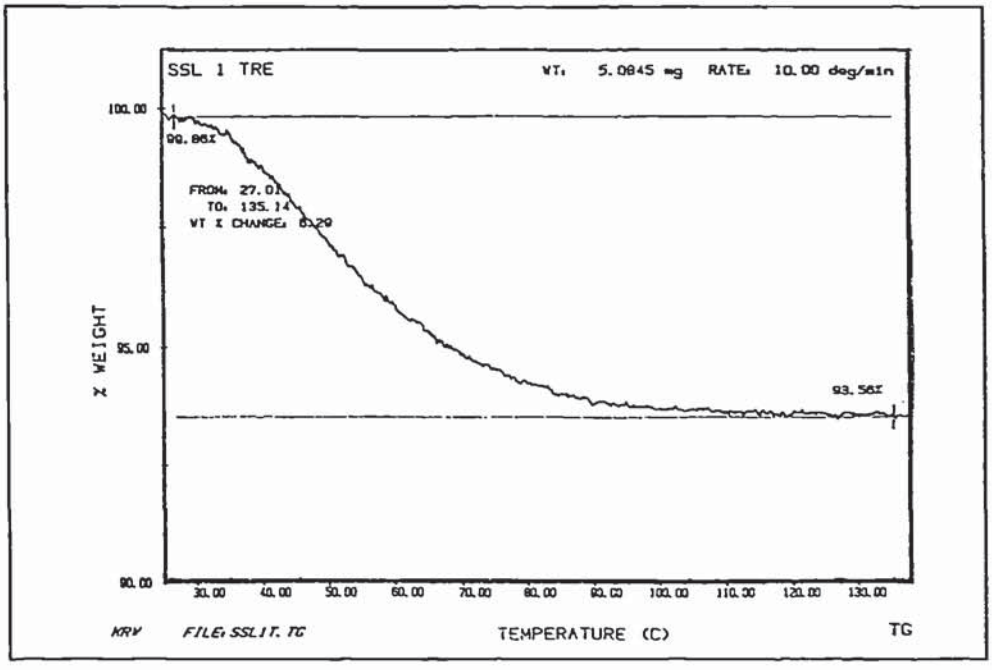


FIGURE 2.4: Typical TGA weight loss profile for a lyophilised product

The sample was weighed by the micro-balance before the heating programme begun, the sample weight loss monitored throughout the run and the % weight loss calculated continuously and plotted against temperature. All data capture and handling were performed using the internal software. A typical plot from TGA analysis is given in Figure 2.4.

2.7. Fast Protein Liquid Chromatography (FPLC) of Protein Solutions

FPLC was carried out using a Pharmacia system, comprising two piston pumps, a microprocessor control unit, a mixing device and a UV/visible detector. Two HR30/10 columns packed with Sepharose G-200 were connected in series in order to provide the resolution necessary for the present studies.

2.7.1. Calibration of columns

The columns were calibrated using a set of molecular weight markers (Sigma Chem. Co., Cat. No. MW-GF-1000), which contained blue dextran (MW 2,000,000) for determination of column void volume (V_0), β-amylase (MW 200,000), alcohol dehydrogenase (MW 150,000), bovine serum albumin (MW 66,000) and carbonic anhydrase (MW 29,000). A separate solution of each component was prepared to the specifications described by the supplier in Sørensen's glycine II buffer (pH10.0, 0.1M), and injected onto the column. The same buffer was used as eluent, at a flow rate of 0.40ml/min. Eluent was monitored continuously by spectrophotometric means at a wavelength of 280nm, and absorbance at this wavelength plotted against time (chart speed 2.0mm/min). The elution time was calculated by measuring the distance from the point of injection to the centre of the absorbance peak for each marker compound, and elution volume calculated from this value. The void volume (V_0) was found to be 13.71ml (±0.06ml, n=3). The mean elution volumes of the molecular weight markers are given in table 2.1. Normally, it would be expected that when V_e/V_0 obtained for each compound is plotted against the logarithm (base 10) of its molecular weight, then the result is a linear graph. However, in this case, it is evident from the data shown below, that a relationship clearly does not exist between the molecular weights of the marker compounds used here and their corresponding V_e/V_0 values. A possible explanation

of this observation may be found by the examination of the principles of FPLC and of the type of marker molecules used here. The rate at which a molecule progresses through the column packing material is determined chiefly by its hydrodynamic radius. Obviously, this will not only be linked to the molecular weight of the compound in question, but will also be dependent on the structure and folding behaviour of the protein in question, which, in turn, may be affected by the micro-environment to which it is subjected. Thus, the overall hydrodynamic radii of the proteins used as marker compounds in this study may have each been affected in different ways by the conditions employed. As a consequence, the expected logarithmic relationship between V_e/V_0 and molecular weight was not observed.

Marker	Molecular Weight	Elution Volume (V_e)	V_e/V_0
Blue dextran	2,000,000	13.71 ml	(1.00)
β-amylase	200,000	(not detected)	-
Alcohol dehydrogenase	150,000	22.80 ml (±0.15 ml)	1.66
Bovine serum albumin	66,000	21.52 ml (±0.13 ml)	1.57
Carbonic anhydrase	29,000	24.54 ml (±0.21 ml)	1.79

TABLE 2.1: Elution data for molecular weight marker compounds in the calibration of Pharmacia HR 30/10 columns, in Sørensen's glycine II buffer (pH 10.0, 0.1M)

Since the columns were to be used for the separation of tetrameric and monomeric components of L-asparaginase (MW 34,000 and 135,000 respectively, see chapter 4), and to simply allow a picture of the approximate proportions of each component in a mixture to be gained, it was decided that the 'non-calibrated' FPLC system should be sufficient for this to be carried out effectively.

2.7.2. Procedure

For solutions containing L-asparaginase, chromatography was carried out in Sørensen's Glycine II buffer (pH10.0, 0.1M), as this buffer had been reported to stabilise any monomeric L-asparaginase present against aggregation (Marlborough *et al.*, 1975). The flow rate was typically 0.40 ml/min, with an injection volume of 200µl. The absorbance of the eluent was

monitored continuously at 280 nm, and plotted against time. A typical plot is shown in Figure 2.5 below.

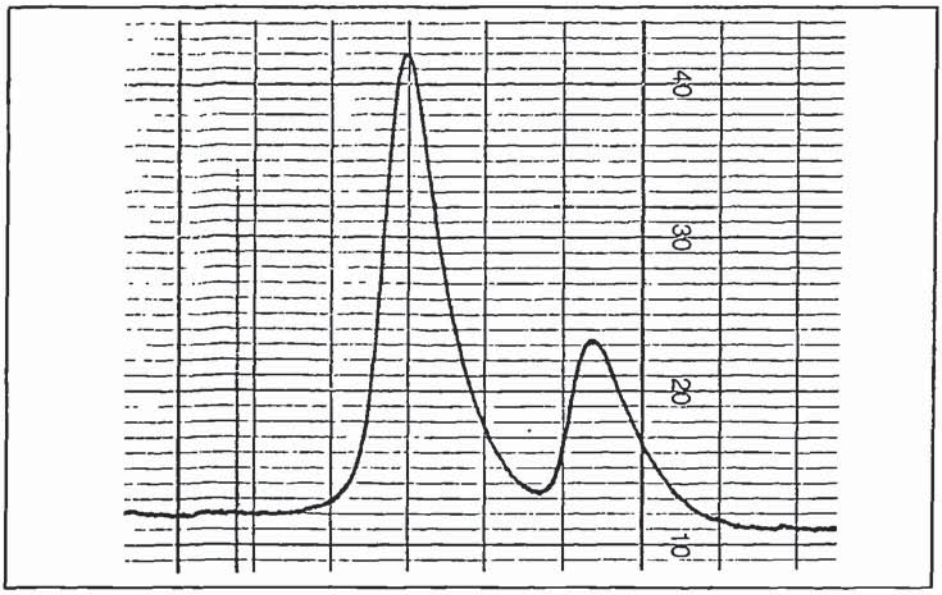


FIGURE 2.5: Profile showing tetramer and monomer peaks for a solution of lyophilised and reconstituted L-asparaginase eluted from FPLC columns

2.8. Determination of Total Protein Content in Aqueous Solution - the bicinchoninic acid (BCA) assay

The amino acid composition of PXB lends it to assay by protein assay methodology. The bicinchoninic acid (BCA) assay is both sensitive and reproducible and is particularly suited to working with small sample volumes (Smith *et.al,* 1985). The method is based on the classic Lowry protein assay (Lowry *et.al.*, 1951). Proteins, and PXB, react with copper (II) to form a copper (I) salt. The 4,4'-dicarboxy-2,2'-biquinolone (bicinchoninic acid), the key component in this assay (Smith *et al.*, 1985), forms water-soluble alkali metal salts. Thus, the reaction product of the assay is a water-soluble salt, whose presence can be detected by use of spectrophotometric measurement (absorbance) at 550nm. It is subject to less interference than the Lowry assay and is also more sensitive (to concentrations of approximately 10μg/ml of protein/peptide). Sample volume may be reduced to 10μl by

performing the assay in a microtitre plate (flat bottomed microtitre plates, 96-well, LIP Equipment and Services Ltd., West Yorkshire, UK.)

2.8.1. The Standard Assay Protocol

The scaled down microtitre version of the BCA assay involved the mixing of one volume of sample or standard (10µl, concentration 10 to 100µg/ml) with twenty volumes (200µl) of freshly-prepared protein assay reagent. Colour development proceeded at 60°C for 45 minutes, and plates were allowed to cool to room temperature before the absorbance of the contents of each well was determined (near simultaneously) at 550nm using a microtitre plate reader (Anthos reader 2001, Anthos Labtec Instruments, Austria). A minimum of three samples of each test solution were used. Reagents were prepared as follows:

Reagent solution A: aqueous solution of 1% (w/v) bicinchoninic acid, 2% (w/v) $Na_2CO_3.H_2O$, 0.16% (w/v) disodium tartrate, 0.4% (w/v) NaOH and 0.95% $NaHCO_3$. Adjusted to pH 11.25 with 50% NaOH solution or solid $NaHCO_3$.

Reagent solution B: 4% (w/v) $CuSO_4.5H_2O$, in double-distilled water.

The two reagent solutions were mixed together in the ratio of fifty volumes of solution A to one volume of solution B (typically 20.0ml + 0.40ml, respectively). Aliquots (200µl) of this mixture were added to each well of a 96-well microtitre plate, some wells containing standard solutions (10µl) of known concentrations, others containing solutions (10µl) of unknown concentrations. It was thus ensured that the standards and samples were analysed under similar conditions.

Once absorbance data had been obtained, a standard graph of absorbance versus protein concentration was constructed for the range of protein standard solutions used, so that the unknown concentrations of the test solutions could be determined by extrapolation from the graph (calculated by substituting absorbance values into the equation which was determined for the straight line standard graph). Figure 2.6 demonstrates the linearity of the relationship

between absorbance and protein concentration for a range of standard PXB solutions of between 25 and 200µg/ml.

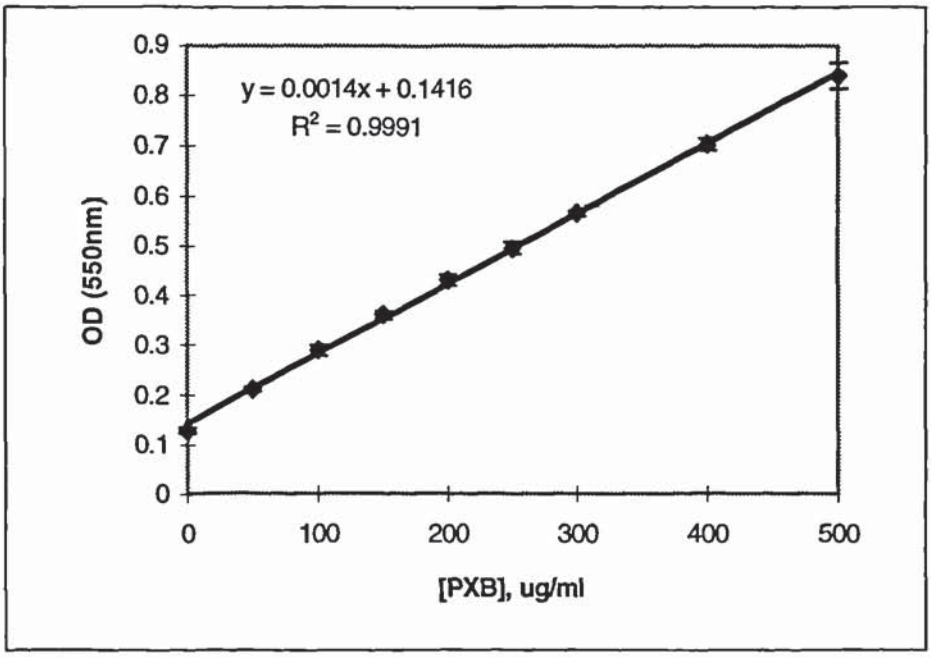


FIGURE 2.6: Typical calibration plot for PXB standard solutions analysed by BCA assay

2.8.2. The Microassay Protocol

For solutions where the concentration of protein was known or expected to be lower than 20µg/ml, the regular BCA assay described above did not prove sufficiently sensitive to allow precise evaluation of protein concentration to be made. However, with a modified BCA protocol which utilised more concentrated reagents, more dilute protein solutions (1.0 to 40µg/ml) could be assayed. The reagents for this more sensitive assay were prepared to the following specifications:

Micro-reagent A (MA): aqueous solution of 8.0% (w/v) $Na_2CO_3.H_2O$), 1.6% (w/v) NaOH, 1.6% (w/v) disodium tartrate, adjusted to pH 11.25 with $NaHCO_3$.

Micro-reagent B (MB): 4% (w/v) bicinchoninic acid (disodium salt) in double-distilled water.

Micro-reagent C (MC): 4% (w/v) $CuSO_4.5H_2O$ plus 100 equivalent volumes of MB.

Micro-working reagent (M-WR) was prepared by mixing equal volumes of MA and MC. It was necessary to prepare M-WR immediately before use, although solutions MA and MB were individually stable for several months at room temperature.

Samples and standards (100µl, 1.0 to 40µg/ml) were mixed with an equal volume of M-WR in the wells of a microtitre plate and incubated at 60°C for 30-45 minutes. A total of four samples of each test solution were assayed, and the mean absorbance determined for each group set. This average was then used to calculate the protein concentration in the test solution by reference to a calibration plot which was constructed from the absorbance values of standard solutions of known concentration assayed under identical conditions. The linear range of the calibration plot (5-40 µg/ml) was found to be suitable for the range of concentrations not covered by the standard assay protocol (section 2.8.1). Figure 2.7 shows a calibration plot for the microassay of PXB.

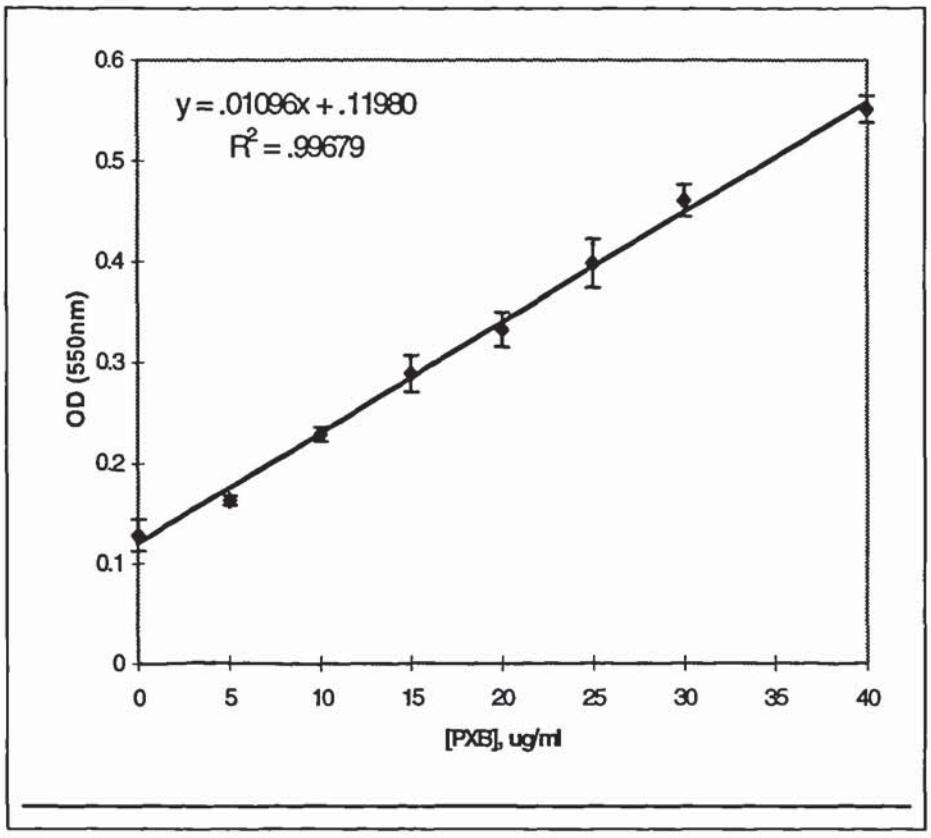


FIGURE 2.7: Typical calibration plot for PXB standard solutions analysed by microassay

2.9. Determination of Biological Activity of L-Asparaginase (L-ASNase) using L-Aspartyl-β-hydroxamic Acid (AHA) as Substrate

2.9.1. Theory & Method Development

Determination of the biological activity of L-asparaginase in the present work was carried out using L-aspartyl-β-hydroxamic acid (AHA) as substrate, using a procedure based on that described by Frohwein *et al.* (1971). The authors found that L-asparaginase was capable of hydrolysing AHA to yield aspartic acid and hydroxylamine; this alternative substrate allowed the avoidance of problems which are often inherent in more usual methods of enzyme analysis, such as the production of ammonia (indeed, L-asparaginase is usually assayed using the basic transamination reaction L-asparagine + water → L-aspartic acid + ammonia). The reaction could be stopped by the addition of an acidic solution containing ferric chloride; any remaining (unreacted) AHA forming a coloured complex with iron (III) under such conditions. This complex was found to obey Beer's law in the AHA concentration range of 0.1 to 0.6μmol/ml. The initial method which the authors described in their short communication was found to be time-consuming for the assaying of several dozen solutions of L-asparaginase, since each solution required individual analysis in a test tube and the absorbance of each solution measured manually by placing into a UV/visible spectrophotometer. This method was adapted so that it could be carried out in microtitre plates, which not only reduced analysis time, but also minimised variability within the experiment associated with manual absorbance measurement and allowed each standard and test solution to be analysed simultaneously under similar conditions. The original concentration of AHA substrate used by Frohwein and co-workers (10μmol/ml) was retained, with the volumes of each reagent solution scaled down to give a total working volume which was suitable for use in microtitre plates.

2.9.2. Procedure

Aliquots of analyte solutions (100μl) diluted 1 in 1000 with Sørensen's glycine II buffer (0.1M glycine + 0.1M NaCl, pH adjusted to 10.0 with NaOH) were pipetted into the wells of a 96-well microtitre plate, to which a solution of AHA (10μmol/ml, 25μl/well) was added. After an incubation period of 10 minutes at 37°C, a solution of ferric chloride reagent (25g

$FeCl_3.6H_2O$ + 4.50ml hydrochloric acid (specific gravity 1.16) made up to 500ml with filtered distilled water) was added to each well (125µl) to quench the reaction by forming a complex with excess AHA present. The absorbance of the resulting complex was analysed at a wavelength of 550nm using a microtitre plate reader (Anthos Instruments, Austria). Calibration standards were prepared by dilution of original L-asparaginase/additive solutions (pre-freeze-drying) and the retained activity of L-asparaginase in reconstituted samples expressed as a percentage of original activity. A 'blank' was prepared by replacing AHA with filtered distilled water (thus giving no complexation) and a positive control prepared by performing the addition of AHA to wells containing L-asparaginase solution after the ferric chloride reagent had been added (thus giving full complexation). Good linearity was demonstrated between optical density readings and complexed AHA remaining in calibration standards, as shown in Figure 2.8

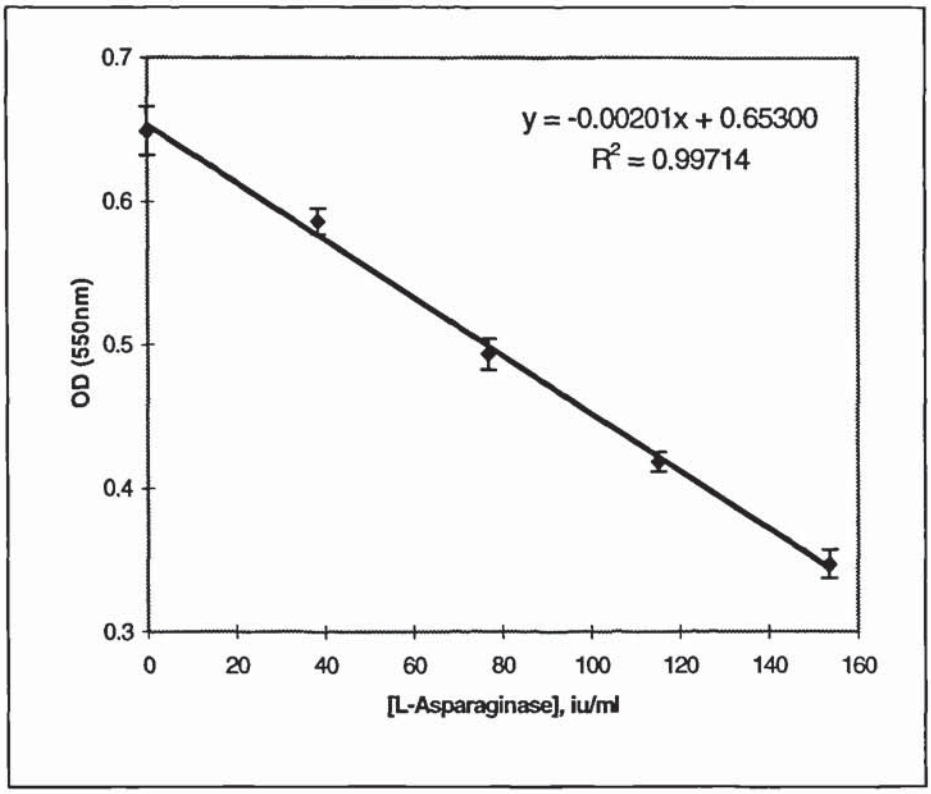


FIGURE 2.8: *Calibration plot for L-asparaginase standard solutions analysed using the AHA assay developed from Frohwein et al. (1971)*

2.10. Moisture Determination in Freeze-Dried Samples by a Coulometric Karl Fischer Titration Method

2.10.1 Background

The Karl Fischer method for moisture determination is commonly used in the assay of water-contents of lyophilised materials. Iodine, together with pyridine, sulphur dioxide and methanol form the Karl Fischer reagent which reacts quantitatively with water, according to equation 2.1 (Adams, 1990):

$$H_2O + I_2 + SO_2 + ROH + 3RN \longrightarrow 2RNHI + RN + HSO_4R \quad \text{....(Eq.2.1)}$$

If sample size is large enough (typically 40mg), then when dissolved in methanol and titrated with Karl Fischer reagent from a burette, a visual endpoint may be seen (US Pharmacopeia XXII/National Formulary XVII, 22nd ed., Mack Publishing, Easton, PA, USA, p.1619). A number of electrochemical methods have been devised which combine titration from a burette with electrometric endpoint detection; however, none of these circumvents the problem of having to use large sample weights. Karl Fischer instruments which use coulometry eliminate the need for a burette. Iodine is generated coulometrically to react with the water present in the added sample in the presence of pyridine, sulphur dioxide and methanol in a special reaction vessel (Meyer & Boyd, 1959). The ability of the coulometric Karl Fischer method to measure residual moisture in approximately 10mg of a freeze-dried biological sample makes it the most practical Karl Fischer method in the determination of residual moisture in lyophilised biological products in single dose final containers (May *et al.*, 1982). Coulometric systems offer several advantages over the traditional Karl Fischer method. Firstly, the system only releases sufficient iodine to react with the water in the sample. Secondly, the system can be made self-purging, with free iodine being constantly regenerated to dry the reagent. Finally, reagent may be used for a considerable time, since the iodine is regenerated (Adams, 1990). The Harvard-LTE AF7 Coulometric Karl Fischer (KF) titration apparatus used in the present study is equipped with a self-purging facility, thereby constantly drying the reagent within the vessel. Dry reagent can be removed from

the reaction vessel, injected into the vial containing lyophilised sample, left in contact for the solvent/water extraction period before being added back to the reaction vessel.

2.10.2. Procedure

An aliquot (1.00ml) of Karl Fischer reagent was removed from the reaction vessel and injected into a sealed vial containing lyophilised product. After full solubilisation of the sample was observed (giving a minimum time of 30 seconds), 0.80ml of the sample solution was added back to the reaction vessel. After a period of reaction time, the instrument gave a readout of the moisture content of the sample in milligrams. This was then converted to give a moisture content in units of % (w/w) for the original sample.

2.11. Determination of Enzyme Activity of Lactate Dehydrogenase (LDH)

Determination of the activity of LDH was carried out using a standard LDH assay kit (Cat No. DG1340-UV) supplied by Sigma Chemical Co. The principle of the assay and its procedure are described in the following sections.

2.11.1 The Sigma LDH kit assay

The principle of the Sigma kit assay is based on the following reaction (Eq. 2.2) which is catalysed by LDH:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+ \quad \text{.....(Eq. 2.2)}$$

During the reduction of pyruvate, an equimolar amount of NADH is oxidised to NAD^+. Since NADH absorbs light of wavelength 340nm, a decrease in absorbance at 340nm may be observed as the reaction proceeds. The rate of decrease in absorbance at 340nm is directly proportional to the amount of NADH in the solution, and it has also been shown that the rate at which this reaction occurs is directly proportional to the amount of lactate dehydrogenase in the sample (Sigma kit handbook).

The reagents in the kit were supplied as powders, which were reconstituted to a given volume with distilled water. These (optimised) reagents, once reconstituted, contained the following concentrations of active ingredients:

Reagent A (buffer/co-enzyme):

NADH	0.194mmol/l
Phosphate buffer, pH7.5	54mmol/l

Reagent B (pyruvate):

Pyruvate	16.2mol/l
Non-reactive stabilisers and fillers	

2.11.2 Procedure

The spectrophotometer used in this assay was a Philips UV-2700 model, equipped with a temperature-controlled cuvette compartment and rate kinetics software. The compartment temperature was programmed to 37°C and allowed to warm to this temperature before the assay was carried out. Once the preset temperature had been reached, the instrument was set to zero absorbance at wavelength 340nm, using a cuvette filled with double-distilled water.

Solutions of reagents A and B were prepared by the addition of double distilled water to the volume indicated on the labels (20ml for reagent A, 5ml for reagent B). Solutions were mixed gently by inversion. "Sample Start Reagent" (SSR) was prepared by mixing 0.40ml of reagent B solution with 10ml of reagent A solution (or multiples thereof). Any unused solutions were kept refrigerated at 4°C until required. Aliquots of SSR (2.50ml) were pipetted into individual quartz cuvettes which were placed in the heated cuvette compartment of the UV spectrophotometer. For each cuvette containing SSR, an initial absorbance reading was obtained before the reaction was started. An aliquot (100µl) of sample solution was added to the cuvette and the contents mixed thoroughly. The lid of the cuvette compartment was closed firmly before the kinetics software program was initiated. The absorbance of the test solution was monitored for at least 2 minutes and automatically plotted against time by the software program, from which the mean rate of decrease of absorbance was calculated. Where the reaction was complete (*i.e.* absorbance had reached a

plateau) within one minute or less, the test solution was diluted ten-fold using phosphate buffered saline (PBS, pH7.4) and re-analysed. The activity of the test solution was then calculated according to the following equation (Eq. 2.2) given in the Sigma kit handbook:

$$\text{LDH Activity (u/ml)} = \frac{\Delta A \text{per min} \times TV}{6.22 \times LP \times SV} \quad \text{....(Eq. 2.2)}$$

'here: ΔA per min = change in absorbance *per* minute at 340nm
TV = total volume
SV = sample volume
6.22 = millimolar absorptivity of NADH at 340nm
and LP = light path (1cm)

2.12. Preparation of Dehydration-Rehydration Vesicles (DRV)

The method of liposome preparation in the present study was based on the dehydration-rehydration method first described by Kirby & Gregoriadis (1984), which is succinctly summarised by New (1990). An overview of the process is given in Figure 2.9.

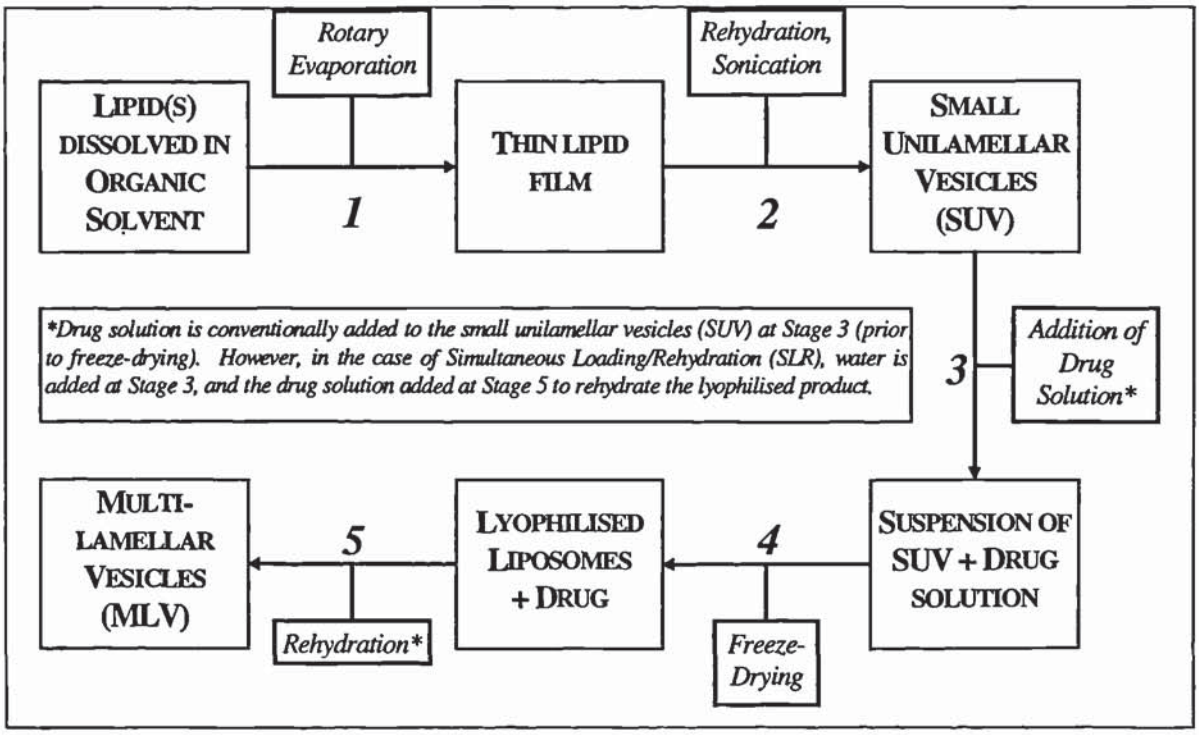


FIGURE 2.9: Flow diagram showing the main stages of the DRV process

2.12.1 Procedure

The appropriate proportion of lipid(s) (see individual experiments in chapter 5) and cholesterol (where used) were added to a round-bottomed flask (Quickfit, 100ml) and sufficient chloroform added (typically 5ml) to obtain a thin film. The lipid mixture was dried by rotary evaporation (Rotavapor, Büchi, Switzerland) under reduced pressure above the non-hydrated transition temperatures (T_m) of the lipid mixtures used, which were determined using differential scanning calorimetry as described in section 2.5. Remaining traces of solvent were removed by gentle drying under a stream of dry nitrogen. The resulting thin film was redispersed with sterile filtered double-distilled water by gentle agitation for 30 minutes at a temperature higher than T_m. The mixture was then briefly sonicated (Bath Sonicator, Kerry Instruments, Ireland) to ensure full dispersion of the lipids in the water. The resulting milky-white mixture was sonicated to clarity using a probe sonicator (probe diameter 14mm, Soniprep 150, MSE, UK) at a minimum probe amplitude of 18 microns for 10 cycles of 60 seconds on / 30 seconds off, before undergoing particle size analysis (see sections 2.13 and 2.14) to determine whether small unilamellar vesicles (SUV) had been formed. Preparations were cooled in an ice-water bath to disperse heat generated during sonication. The resulting suspension of SUV was centrifuged (Beckman JII, Beckman Instruments Ltd., Bucks., UK) at 400 x *g* for 10 minutes, to remove large lipid aggregates and titanium particles. Aliquots of the SUV suspension were diluted with appropriate solutions containing any required solutes such as model drug and/or protective additive (in the case of 'remote-loaded' preparations, only water was added at this stage, the drug solution subsequently used to rehydrate the freeze-dried plug as described below). The diluted mixture was then freeze-dried under appropriate conditions (given individually for each experiment, see subsequent chapters). Rehydration (to give multilamellar vesicles, MLV) was always carried out in the same manner, as follows. First, the dried plug was reconstituted to one-tenth of the pre-lyophilisation volume with distilled water (which had been incubated at a temperature above the T_m value of the dried plug, wherever its T_m value was higher than room temperature), adding dropwise until the plug had fully collapsed. After gentle mixing, the rehydrated plug was allowed to stand for 30mins at a temperature above its T_m value, before a further aliquot of water was added slowly to make the suspension to half of the pre-lyophilisation volume. After a second period of incubation above T_m, the

remainder of the distilled water was finally added, to dilute the suspension to original volume. The suspension was then diluted to the desired volume. Liposomes were separated from non-entrapped drug/co-solute(s) by centrifugation (18,000 x *g*, 30 mins, Beckman JII, UK). The supernatant was retained for analysis, the pellet resuspended in aqueous phase and the centrifugation was repeated. The second supernatant was also retained fo further analysis and the final liposome pellet resuspended in aqueous phase for storage at 4°C until required. For liposomes which contained radiolabelled material, entrapment was also assessed by direct assay of lysed vesicle samples (see section 2.16).

2.13. Determination of Particle Size by Laser Diffraction

2.13.1 Background and instrumentation

Laser light diffraction may be used to determine the size distribution of liposome preparations (Woodle & Papahadjopoulos, 1989). Particles pass through a laser beam and the light scattered by the particles is detected over a range of angles in the forward direction. In the particular instrument used in these studies (Mastersizer E, Malvern Instruments, UK), the beam source was a helium-neon gas laser with a wavelength of 0.633μm. The angular intensity distribution of the scattered light varies with particle size. The particles pass through an expanded and collimated laser beam in front of a lens in whose focal plane is positioned a photosensitive detector, consisting of a series of concentric annular rings. The unscattered portion of the laser beam is brought to focus in the detector plane and is usually allowed to pass through an aperture at the centre of the detector and effectively out of the system. However, the annular rings centred around the axis of the incident laser beam record the intensity of the light scattered over a range of angles and the distribution of scattered intensity is analysed by a computer software package to yield the particle size distribution. For a polydisperse sample of particles, the detector measures the integral angular scattering across the complete size distribution. Several thousand particles may be illuminated at any one time and the diffraction pattern will change in time as the number distribution of particles in each size class varies with particles entering and leaving the beam. This temporal variation is usually integrated to give an average for a truly representative sample of the particles

present. The Malvern Mastersizer E (Malvern Instruments, Malvern, UK) uses reverse Fourier optics to enable size distribution to be measured in the sub-micron range. Analysis of small particles is not possible using the simpler and more widely used Fraunhofer scattering theory, as it requires particles to be large compared with the laser wavelength. Fraunhofer theory also fails to recognise that light can pass through the particles. Mie theory predicts all paths of light around and through the particle and copes with partially absorbing particles including those which are totally opaque or clear. Therefore, it may be applied over the entire range of particle types and sizes to predict scattering at any angle from forward diffraction to back scatter. This combination of reverse Fourier optics with Mie theory allows the Mastersizer E to measure liposome size distributions over the range 0.1-80μm. The volume mean sample reproducibility of the system is given as ±0.5% for a stable sample under recirculating conditions in liquid suspension. The Malvern laser diffraction technique generates a volume distribution for the analysed light energy data. The amount of light scattered by a particle is proportional to the sixth power of its diameter. Therefore, in the 0.1 to 1.0μm range, the ratio of scattered intensities is 10^6. The data may be heavily skewed by a small number of large particles or aggregates (Malvern Mastersizer E Handbook, Malvern Instruments, Malvern, UK). For this reason, in the present study, values are reported as the equivalent number mean (d[5,5]) plus or minus the standard deviation.

2.13.2 Procedure

A quantity (200μl, or sufficient to give the required obscuration) of liposome dispersion was vortex mixed with 10ml of either filtered double distilled water or filtered buffer (0.2μm polycarbonate filter, Millipore, UK) and sized using the Mastersizer E. Liposome diameters were determined from both volume and number size distributions. A typical printout from the Mastersizer E is shown in Appendix I.

2.14 Determination of Particle Size using Photon Correlation Spectroscopy (PCS)

2.14.1 Background

PCS (also referred to as quasi-electric light scattering or dynamic light scattering) may be used to measure the size distribution of particles in the sub-micron range. Colloidal particles in a liquid undergo random motion due to multiple collision with the thermally driven molecules of the liquid (Brownian motion). Light scattered by these particles will display a characteristic dependence on the diffusion coefficient of the particle, which can be related to particle size. Small particles diffuse more rapidly than large particles and the rate of scattered light intensity varies accordingly. These changes in the intensity of scattered light are detected as the particles move under Brownian motion. PCS determines particle size by assessing the fluctuations in intensity of light over time, caused by the diffusing particles. By examining the signal pattern at set time intervals and comparing these data with each other (autocorrelation), the time dependency of these fluctuations can be evaluated, resulting in an autocorrelation function from which particle size and size distribution may be determined. For a monosized distribution of spheres, the function is an exponential function of time. For a polydisperse system, multiple values of the correlation coefficient are determined and a first-, second- and third- order fit of the data yields a size distribution and standard deviation. The translational diffusion coefficient can be measured, which in turn can be used to determine the mean hydrodynamic radius of the particle using the Stokes-Einstein equation, viz:

$$D = \frac{kT}{6\pi\eta r} \qquad \text{.... } (Eq.\ 2.3)$$

(where D=diffusion coefficient, k=Boltzmann's constant, T=absolute temperature (K), η=solvent viscosity, r=mean equivalent spherical hydrodynamic radius)

In the Sematech correlator, the time increments separating the two copies of the signal to be processed are termed channels. The overall time separation between channels can be varied by the operator by selecting channel sample time from the appropriate pull-down menu on the display screen and entering the desired value. The choice of sample time is critical in

achieving meaningful results. Although the Sematech instrument can be set to automatically determine the optimum sample time by comparing calculated and measured background values, this parameter was set manually before analysis in the present study. Commonly, a sample time of between 5 to 10μs was selected, according to the relative anticipated particle size, as well as the concentration and opacity of the suspension. A longer sampling time was used for larger particles, to compensate for the greater number of slower moving vesicles. A total running time of 60sec was used for all experiments. Similar parameters for PCS sizing of liposomal preparations have been reported by McAllister (1995) and Elorza *et al.* (1993). PCS is the most widely and commonly used method to determine size distribution of liposomes in the sub-micron range (Barenholz & Amselem, 1993). The limitations of PCS concern the size range over which the validity of the technique is maintained. The range of PCS measurement is usually given as 3nm-3μm, although for most instruments the practical upper limit is 1μm. As with laser light diffraction, the presence of a few large particles may obscure smaller particles and result in skewing of the size distribution towards the higher size range. The Sematech multiangle goniometer used throughout this study is equipped with a high power laser source (Helium-neon 5mW, wavelength 632.8nm) and advanced photomultiplier detection system, combined with data analysis by a singular system algorithm. It is claimed that this specification enables particle size distributions to be determined over the 5nm-5μm range, even for polydisperse samples (Sematech Multiangle Goniometer Handbook).

Results from PCS sizing in the present study are quoted as the (number) mean hydrodynamic diameter of the analysed sample, which compensates for any large particles aggregates which may otherwise skew the data (as for particle sizing using the Malvern Mastersizer, see previous section). The Sematech algorithm also generated a value of polydispersity which is similar to the value used in the Malvern Mastersizer software. The value of polydispersity varies between 0 and 1, with low values representing monosized systems and higher values indicative of multimodal distributions or polydispersity.

2.14.2 Procedure

PCS sizing of stealth liposome formulations was performed using a Sematech multi-angle granulometer (Sematech, Nice, France). Samples were prepared by diluting a quantity (typically 100μl) of liposome suspension with double distilled water which had been passed through a 0.22μm filter. The diluted sample (approximately 1ml) was transferred to a sample tube which was placed in the analysis chamber of the PCS instrument. The size measurement was carried out as described in the above section (2.14.1). A typical printout from the PCS analysis is shown in Appendix II.

2.15. Zeta Potential Analysis for Liposome Preparations

2.15.1 Background

The surfaces of a particle in a liquid are typically electrically charged, due to the adsorption of specific ions or to the ionisation of groups on the particle surface. For preparations of liposomes, this surface charge is an important factor in determining stability, drug loading/adsorption and the fate of therapeutic liposome preparations *in vivo* (Gregoriadis, 1995). When such a system is present in an aqueous electrolyte environment, the net surface charge will affect the distribution of ions in the immediate vicinity. Oppositely-charged ions (counterions) are attracted to the particle surface, while ions of like charge (co-ions) are repelled. The surrounding region of a charged surface where unequal concentrations of counterions and co-ions exist is known as the electrical double-layer (EDL), as depicted in Figure 2.10. The inner part of the EDL is made up of strongly-adsorbed ions, whilst the outer part consists of ions which are more diffusely-distributed according to a balance between electrical forces and random thermal motion. In this way, an electrical force exists between similar particles in an aqueous environment.

The potential at the surface of shear (Figure 2.10) between the charged surface and the electrolyte solution is called the electrokinetic or ζ (zeta) potential. The exact location of the shear plane is an unknown feature of the EDL and is, in reality, a region of rapidly changing viscosity (Shaw, 1991).

FIGURE 2.10: Schematic representation of the structure of the electrical double layer (from Shaw, 1991).

The zeta potential of particles may be determined by measuring the particle mobility in an applied electric field. The zeta potential can then be calculated from this mobility, by using equations of Smoluchowski, Henry or Debye-Hückel (Müller, 1991). The electrophoretic mobility (μ_E) is defined as the velocity of the particle under unit electric field and is usually expressed in relation to the particle velocity (v) and the applied field strength, as given in equation 2.7:

$$\mu_E = \frac{v}{E} \quad \text{....(Eq. 2.7)}$$

where v is measured in m/s, and E in V/m, so that μ_E has the units $m^2.s^{-1}.V^{-1}$ (Kayes, 1988). The equation used for the conversion of the electrophoretic mobility into zeta potential depends on the value of the Debye-Hückel parameter (k), which depends on electrolyte concentration and the particle radius (a) only. At large values of ka, the Smoluchowski equation can be applied. This was the equation used throughout the course of the present study, due to the conditions of the measurement employed (aqueous medium, moderate

electrolyte concentration, typical mean particle size of 0.5-5μm). Under these circumstances, the Smoluchowski equation (Eqn. 2.8) defines zeta potential as:

$$\zeta = \mu_E \frac{4\pi\eta}{\varepsilon} \quad \text{....(Eq. 2.8)}$$

where η is the viscosity of the medium (in poises), and ε is the respective dielectric constant. The value of ζ is measured in mV. It follows from this equation that the electrophoretic mobility of a non-conducting particle for which *ka* is large at all points on the surface, should be independent of its size and shape, provided that the zeta potential is constant (Shaw, 1991). In the present study, zeta potential determinations were carried out using a Malvern Zetamaster (Malvern Instruments, UK.) with the parameter settings listed in Table 2.2.

Parameter	**Value**
Cell type	Zetamaster standard cell
Cell voltage	100mV (constant)
Current	buffer dependent
Conductivity	buffer dependent
Temperature	25.5±0.1°C
Dielectric constant	79.0
f(ka)	1.50

***TABLE 2.2:** Parameters used in zeta potential determinations carried out in the present study (from Malvern technical information)*

A laser Doppler anemometry instrument such as the Malvern Zetamaster consists of two coherent laser beams derived from the output of a low power laser. These beams intersect within the sample cell, forming a beam crossover pattern of interference fringes. Particles moving across the fringes in response to the applied electric field scatter light with an intensity which fluctuates at a frequency related to their velocity. The frequency of the scattered laser light differs from the frequency of the initial laser beam. This shift is caused by the Doppler effect and is a function of the particle velocity. The signal from individual

photons of scattered light are detected by a photomultiplier and analysed by a digital correlator to give a frequency spectrum from which the particle mobility and the zeta potential are calculated (McFadyen, 1986; Müller, 1991).

2.15.2 Procedure

Zeta potentials of empty and loaded vesicles were measured to investigate the effect of drug (PXB) loading on liposome surface charge. The buffer system used in all measurements was 0.001N potassium chloride (filtered through a 0.22μm filter before use), which as a weak electrolyte, provided a suitable environment for the measurement of zeta potential conforming to the conditions stipulated by the Smoluchowski equation (Eq. 2.5). Machine operation was checked periodically using a latex standard with a defined electrophoretic mobility. A carboxy-modified polystyrene latex (AZ55, Malvern Instruments, Malvern, UK) was diluted in a supplied buffer and measured using the parameters listed in Table 2.2 above. The defined zeta potential of this standard diluted in the supplied buffer was -55mV at 25°C, and the acceptable deviation from this value was ±5mV. Liposome samples were diluted in 0.001M KCl solution prior to analysis and the resulting zeta potential data subjected to interpretation by use of the system software (Malvern Zetamaster software version 2.1) using the standard template designed for lipid in water systems. A typical data report from the Zetamaster is shown in Appendix III.

2.16. Radiolabelling and Scintillation Counting of Polymyxin B

2.16.1 Background

Radioactive isotopes such as tritium (^{3}H) and carbon-14 (^{14}C) interact with matter in two ways, which gives rise to both ionisation, which forms the basis of Geiger-Müller counting, and excitation. The latter effect leads to the emission of photons by the excited compound (or fluor). This fluorescence, or scintillation, can be detected and quantified by means of a scintillation counter comprising a photomultiplier tube which detects light pulses from the radioactive source. With liquid (or internal) scintillation counting the sample is mixed with a scintillation cocktail containing a solvent and a number of fluors. The electric pulse which

results from the conversion of photons to electric energy in the photomultiplier is directly proportional to the energy of the radioactive event. However, quenching (optical, colour or chemical) can interfere with the energy transfer process and reduce the efficiency of counting. Samples may be corrected for quenching effects by monitoring the extent of quenching agent present in the sample and expressing activity in absolute units of disintegration *per* minute (DPM). This requires the determination of the counting efficiency and the conversion of counts *per* minute (CPM) which are the units of measurement of the scintillation counting process. The relationship between DPM and CPM are as shown in equation 2.6:

$$\text{Counting Efficiency} = \frac{\text{CPM} - \text{background counts}}{\text{DPM}} \times 100(\%) \quad \text{.....(Eq. 2.6)}$$

The samples channel ratio (SCR) method may be used to correct quench. In this method, two regions of the sample liquid scintillation (LS) spectrum are monitored in two channels and a ratio of the count rate determined. The assumption made when using the SCR method is that the entire LS spectrum will change in its distribution and shift downward due to quenching,which lowers the pulse height of many energetic decays. To obtain the DPM of samples by this method, a quench curve is required. A quench curve is a mathematical curve which relates the counting efficiency of a range of standards, whose activity is known, to the SCR (that is, the extent of the quench). Practically, this may be achieved by the counting of samples in the presence of increasing quantities of a quenching agent, typically chloroform. The DPM can be calculated from the CPM measured by the instrument and expressed as a percentage of the theoretical CPM. This gives the counting efficiency, which is then plotted (y-axis) against the theoretical CPM itself (x-axis) for the desired range of isotope activities (McAllister, 1995).

2.16.2 Use of Radiolabelled PXB and Sample Preparation

Tritiated PXB was prepared by NEN products (Canada) by catalytic exchange with tritium gas in aqueous solution at room temperature, followed by the removal of labile tritium with ethanol. This method is particularly suitable for the tritium labelling of compounds containing benzylic protons, such as PXB (Vaara & Viljanen, 1985). HPLC analysis of the

radiolabelled material (100μg/ml) showed the same peak pattern as the unlabelled drug, indicating that no degradative changes had occurred due to the radiolabelling process. The final activity of the supplied solution was 39.59MBq/ml (1.07mCi/mg). For the preparation of labelled solutions of PXB, a quantity of diluted label was added to a solution of unlabelled PXB to provide a sufficient level of activity for subsequent analysis; this was typically a 0.1-1.0% ratio of labelled to unlabelled PXB. It was assumed that the behaviour of labelled PXB was entirely representative of that of the excess unlabelled PXB present.

Levels of PXB loaded into liposomes, or that released into supernatant solutions from liposome preparations, was quantified by scintillation counting of aqueous PXB in scintillation fluid. Samples were prepared by mixing 20-50μl of tritiated sample together with 5.0ml of scintillant fluid (Optiphase Hisafe 3, LKB scintillation products, UK) in scintillation vials. Vials were counted for a minimum of 5 minutes each (Canberra Packard 1600TR scintillation counter, Berkshire, UK) and DPM derived from CPM by reference to a quench curve. Counts were corrected for quench using a set of external quench standards, prepared by adding increasing amounts of chloroform (0-5000μl)to a set activity of [^{3}H]-PXB. Vials were counted for three cycles of 10 minutes and a quench curve established. Maximum efficiency of counting of a 100μl sample of [^{3}H]-PXB in 5mls of scintillation fluid was found to be 64.99% (Figure 2.11).

Scintillation counting was used to determine loading efficiency of PXB in liposome preparations as well as of leakage of the drug over time. It was found that it was unnecessary to lyse liposomes prior to scintillation counting, since the scintillation fluid itself was observed to cause lysis of liposome preparations, this releasing vesicle contents into suspension.

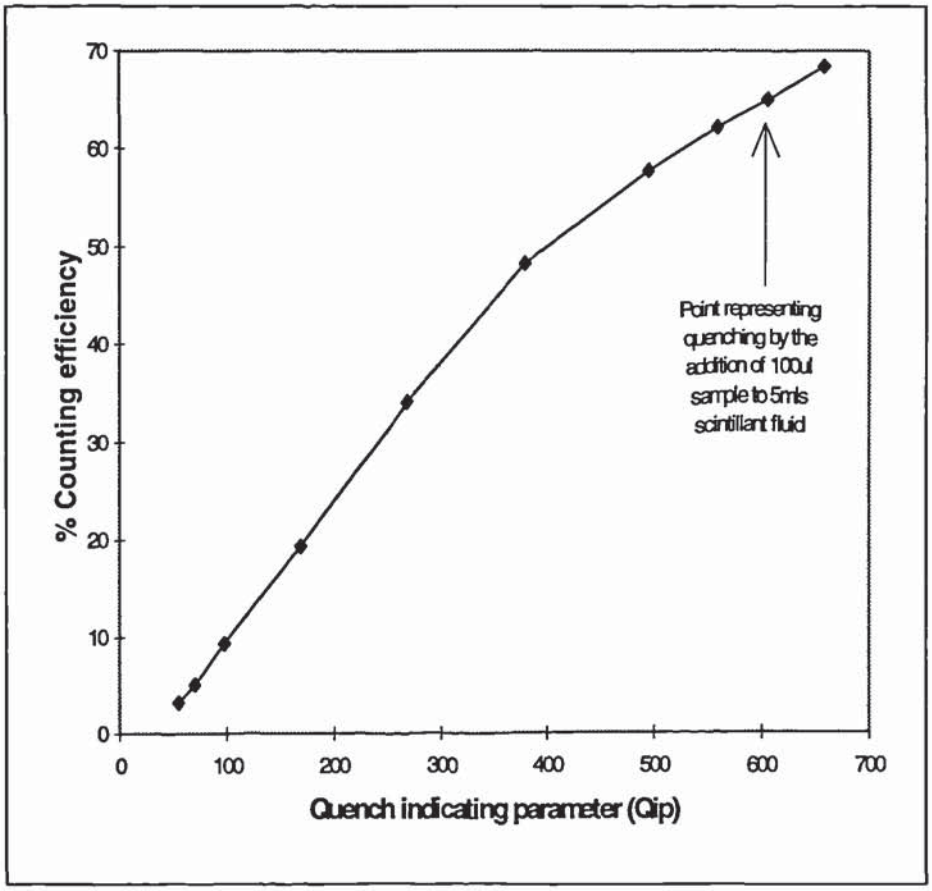


FIGURE 2.11: Quench curve for ^{3}H-PXB standards diluted in Optiphase Hi-Safe 3 scintillant fluid (5ml)

2.17 Transmission electron microscopy (TEM)

A variation on the 'drop method' was used to investigate gross liposomal morphology, membrane integrity and size. This method utilises a negative stain provided by a heavy metal salt which strongly scatters electrons. As electron scattering is directly related to atomic number, the phospholipid bilayers of vesicles, composed principally of carbon and hydrogen, will scatter fewer electrons than the stain and therefore appear in negative contrast (New, 1990). A single drop of freshly-prepared liposomes was placed onto a carbon-coated copper grid (200µm mesh size), and allowed to stand for one minute before being dried using a wick of filter paper. The stain (2% sodium phosphotungstate, pH6.6) was then dropped onto the grid and allowed to dry for a further minute. Fresh filter paper was used to remove excess stain from the grid. When dry, the sample was imaged with a transmission electron microscope (Jeol 1200 EX) operating at an accelerating voltage of 80kV with zero tilt.

2.18. Fourier-Transform Infrared (FT-IR) Spectrophotometry

FT-IR was carried out on potassium bromide (KBr) discs of dried samples. Discs were prepared by placing KBr (~300mg, dried at 60°C) and a small portion of sample (~15mg) into a small agate mortar and grinding until an intimate mixture of fine powder was formed. This mixture was subjected to high mechanical pressure in a casting die for 5 minutes, maintaining a pressure of approximately 5 tonnes, whilst a vacuum pump was used to remove moisture from the die. The resulting disc was carefully placed into the sample chamber of the FT-IR instrument for analysis, and a wavelength scan of the sample disc carried out. The resulting FT-IR spectrum was printed out using the system software (Mattson First, version 1.2).

3. THE USE OF THERMAL ANALYSIS IN THE DEVELOPMENT OF FREEZE-DRIED PHARMACEUTICAL PRODUCTS

SUMMARY

The thermal characteristics of a range of excipients and excipient mixtures were examined using DSC and related to lyophilisation behaviour and stability characteristics. Thermal analysis of a range of polymer solutions revealed that the values obtained for T_g' of the frozen masses corresponded closely with frozen-state collapse temperatures measured in an independent study. In a range of studies of a number of saccharide excipients and excipient mixtures, it was observed that quenched mixtures composed of two components often formed glasses which displayed solid-state glass transitions in the region of 15-25°C. In particular, it was apparent that mannitol, which typically crystallises during freeze-drying, could be induced into an amorphous glass by quenching a molten mixture of mannitol and a second, amorphous, component. Such products were all observed to exhibit a glass transition in the region of room temperature, which was believed to have significant implications on the suitable storage conditions of lyophilised products containing such excipient mixtures.

3.1 INTRODUCTION

3.1.1 General considerations for freeze-dried formulations

There are many different types of product which are routinely freeze-dried, as described in section 1.1. Some of these products are labile biomaterials or living organisms which require the maintenance of biological activity during and subsequent to drying. By far the largest user group in freeze-drying is that of products with limited biological activity. These include foodstuffs where organoleptic properties are important, together with pharmaceutical products such as enzymes, vaccines, hormones, vitamins and antibiotics. Products which are even more biologically sensitive may also be freeze-dried, including living organisms for seed culture or attenuated vaccines (Adams, 1995). In addition, the process of freeze-drying is often used simply to concentrate or dehydrate products of non-biological origin, which may otherwise be reactive or prone to degradation.

As described earlier, freeze-drying is not a neutral process, but is potentially highly damaging, especially to labile materials such as enzymes. For all three categories of product described above, there exists the need for product formulation, in order to overcome the damaging effects and artifacts which may be introduced by the freeze-drying process (for example, collapse). Therefore, it is necessary to have an understanding of solutes and active components and to carry out pre-formulation investigations, such as thermal analysis, in order that suitable, stable formulations may be designed. Before full-scale production, it is also necessary to carry out small-scale freeze-drying trials, in order to assess the freeze-dry stability of the chosen formulation and to select suitable process parameters. The studies described in the present chapter involved the investigation of the thermal characteristics of a number of pharmaceutically relevant formulations and excipients.

3.1.2 Requirements of the freeze-dried pharmaceutical product

For a freeze-dried pharmaceutical product, there are a number of requirements which should be met in order that the product is acceptable. Ideally, the product should exhibit minimal changes during the process, remain clean and sterile (pharmaceutical), be cosmetically acceptable and uniform from vial to vial (and from batch to batch), it must be ethically

acceptable and simple to reconstitute, while the process itself must be economically practicable.

In order to allow the above criteria for freeze-dried products to be met, the excipients included in a pharmaceutical formulation should satisfy a number of criteria. They should bulk the product, preventing ablation, be compatible with the freeze-drying process, be inert to the bioproduct, dry to form a cosmetically acceptable product with a prescribed moisture content, be soluble in the chosen medium and provide good shelf-stability.

It is not expected that excipients will satisfy all the above criteria in any formulation, but may often satisfy many, resulting in a product which may be optimised, but not perfect. Since each bioproduct is unique, it follows that the selection and optimisation of formulation and processing variables should be carried out separately for each product, since each will respond differently to the addition of excipients and to the stresses of freezing and drying. While some materials will dry to give acceptably stable lyophiles even under non-optimised conditions, others may be more sensitive to the process and may only give a stable lyophile if particular criteria are fulfilled. For example, an enzyme may require the use of amorphous excipients in order to maintain its native confirmation during the lyophilisation process, yet such excipients may be less stable than crystalline excipients upon storage in the lyophilised state. Often, a combination of excipients will be used in order to satisfy the processing and storage requirements of a product, as described below.

3.1.3 Freeze-drying characteristics of crystallising and amorphous formulations

There are two basic types of formulation, namely: formulations which form a crystalline eutectic and those which form an amorphous glass (see Figure 1.4, section 1.2). The micro-structure of the frozen mass of a formulation determines the success with which freeze-drying takes place. The drying characteristics of these structures depends on the type of water present in the frozen mass. The structural characteristics of crystalline eutectic formulations and amorphous glasses relevant to the present study are described in Table 3.1 below.

Type of structure	Type(s) of water present in frozen mass	Mechanism of water removal	Resulting product structure
Crystalline eutectic	Water crystallised as ice Water adsorbed to bulk powder	Sublimation Desorption	Dry solute cake
Amorphous glass	Water crystallised as ice Unfrozen water associated with amorphous glass Adsorbed water in 'dried' sample	Sublimation Evaporation Desorption	"Dry" amorphous glass

TABLE 3.1: Drying characteristics of crystalline eutectic and amorphous frozen masses

Eutectics are simple to characterise, since they remain unchanged by the thermal history of the sample. They serve to provide bulk to the product and represent an ideal physical system. However, they may afford little protection to sensitive bioproducts during freeze-drying. Conversely, amorphous mixtures are difficult to analyse, since the structure of a glass will be dependent on its thermal history. They are generally unable to provide bulk to a product and the collapse temperatures of common excipients are close to the minimum temperature attainable in many freeze-dryers. Collapse of amorphous materials is often confused with other operational defects such as product melt, whereas collapse actually occurs at a higher temperature than initial softening of the mass (see chapter 1). It may be seen from Table 3.1 that amorphous systems are more difficult to freeze-dry than simple crystallising systems, since these structures require more rigorous drying cycles to remove water from the frozen mass. However, the persistence of an amorphous state may be a pre-requisite for the stabilisation of biological components (discussed further in chapter 4).

Table 3.2 records five of the most commonly-used excipients in freeze-dried formulations and describes the physical characteristics which have relevance to the freeze-drying process.

3.1.4 Analysis of freezing- and drying- behaviour

The two methods employed in the present study to examine freezing and drying behaviour were differential scanning calorimetry (DSC) and freeze-drying microscopy (FDM).

Excipient	M_r	Classification	Description
Mannitol	180.2	Monosaccharide alcohol	Crystallises readily, widely used in freeze-dried pharmaceutical formulations.
Glucose	180.2	Monosaccharide (Reducing)	Persists in the amorphous state, presents problems in freeze-drying due to low collapse temperature. Often included in formulation complexes.
Sucrose	342.3	Disaccharide (Non-reducing)	Persists in the amorphous state, widely used in freeze-dried pharmaceutical formulations.
Lactose	360.3*	Disaccharide (Reducing)	Widely used in tablet formulations as a bulking agent, and in freeze-dried products.
Trehalose (*=Monohydrate)	360.3*	Disaccharide (Non-reducing)	Persists in the amorphous state, widely used as a cryoprotectant, but not routinely in freeze-dried preparations.

TABLE 3.2: Five commonly-used excipients in freeze-dried formulations

3.1.4.1 Differential scanning calorimetry (DSC)

Thermal analysis is a widely-used instrumental technique in pharmaceutical formulation. Of the many thermal analytical techniques available to the pharmaceutical researcher, the two most commonly-used techniques are thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA is the standard technique recommended by the United States Food and Drug Administration (FDA) to test for moisture levels in dried formulations, but in addition, is used to provide information on the decomposition kinetics of a drug compound, and has scope for determining the compatibilities of various components in a formulation (Ford & Timmins, 1989). DSC, however, may be used to examine many other characteristics; indeed, a DSC thermal profile will provide information on the melting point of a formulation, glass transition temperatures (and associated energy changes), purity, compatibility, decomposition kinetics, polymorphic transitions of a sample, *etc.* The use of DSC for studying such parameters has become increasingly common within the pharmaceutical industry, and particularly in the field of freeze-drying, where knowledge of

the thermal behaviour of formulations is important in providing the formulation scientist with the necessary data, such as the eutectic melt (T_{eu}) or glass transition temperature (T_g or T_g') of a formulation, to select the correct freeze-drying and storage conditions for a product. More recently, techniques such as isothermal microcalorimetry and modulated temperature DSC (see section 1.2.) have been employed to provide additional information about the thermal stability and physical characteristics of solutions as well as frozen or dried samples. In the present study, DSC was utilised for the analysis of bulk solids and blends, solutions and freeze-dried products, to examine the thermal characteristics relevant to freeze-drying and subsequent storage stability; that is T_{eu}, T_g' (frozen state) and T_g (solid state). The principles of differential scanning calorimetry and the instrumentation used in the present study are detailed in section 2.5.

3.1.4.2 Freeze-drying microscopy (FDM)

Freeze-drying microscopy (see section 1.2.2.2) was also utilised in the present chapter in order to provide confirmation of data from DSC regarding the freeze-drying characteristics of a number of formulations. A description of the instrumentation and procedure followed in the present study is given in section 2.4.

3.1.5 Aims of the present chapter

The objective of this chapter was to examine the thermal behaviour (glass transitions, crystallisation) of a range of excipients, either alone or in combination, in order to correlate such behaviour with crystallising characteristics, shelf stability and moisture retention, which will have relevance to the practical aspect of formulation stability. The primary analytical method employed for these purposes was differential scanning calorimetry (DSC), but additional techniques such as thermogravimetric analysis (TGA) and freeze-drying microscopy (FDM) were employed to reinforce the conclusions drawn from the DSC analysis.

3.2 Analysis of T_g' for Aqueous Polymer Solutions

3.2.1 Background

Polymers are becoming widely used as excipients in pharmaceutical formulations, in drug delivery systems such as microspheres and bead systems, and have also recently been included in liposome formulations, notably sterically stabilised liposomes (see chapter 5). Poly(ethylene glycol) has also been shown to provide biological protection to sensitive proteins, both in solution and during freezing. Indeed, PEG has been reported to be the most effective cryoprotectant to date (Arakawa *et al.*, 1993). A major factor determining the success of polymers as excipients for freeze-dried formulations is that of the collapse- (T_c) and glass transition- (T_g') temperatures of the frozen mass containing the polymer. As stated in section 1.2, the values of T_c and T_g' for a formulation may be raised by varying the components and their proportions within that formulation; the use of excipients with high T_c or T_g' values will increase T_c or T_g' for the whole formulation. This is a particularly valuable exercise when it is necessary to freeze-dry a formulation of an active component which itself displays a low collapse temperature, since frozen masses which display low T_c values are more time-consuming to dry and are at greater risk of collapsing during the drying process. It has been observed using freeze-drying microscopy that individual solutions of four particular polymers exhibited collapse temperatures which were sufficiently high as to render the polymers potentially useful as freeze-drying excipients (Adams, unpublished data, shown in Table 3.3 below). The aim of the present study was to ascertain the T_g' values of these solutions in order to compare values of T_g' with the frozen state collapse temperatures observed by Adams.

3.2.2 Materials and methods

3.2.2.1 Materials

Polymers selected for this study were dextran (70kDa), poly(ethylene glycol) (10kDa), ficoll (70kDa) and poly(vinyl pyrrolidine) (40kDa), all of which were supplied by BDH, UK.

3.2.2.2 DSC of solutions

Polymers were dissolved in sterile distilled water to give solutions of 10% (w/w). Aliquots of solution were analysed by DSC, as described in section 2.5.

3.2.3 Results and discussion

The values obtained for T_g' of the polymer solutions are summarised in table 3.3 below.

Polymer solution	T_g', °C (midpoint)	T_g', °C (onset)	T_c, °C (Adams)
Dextran (70kDa)	-11.57 (±0.17)	-11.84 (±0.28)	-9
PEG (10kDa)	-10.96 (±0.32)	-13.56 (±0.30)	-13
Ficoll (70kDa)	-20.10 (±0.67)	-21.09 (±0.53)	-20
PVP (40kDa)	-21.33 (±0.17)	-22.09 (±0.06)	-22

TABLE 3.3: Glass transition temperatures measured using DSC and collapse temperatures observed using FDM, for polymer solutions (n=2; range given in parentheses)

It appears that the T_g' data observed in the present study are in close agreement with the T_c data reported by Adams. The extrapolated onset T_g' values have been included in the table, since these appeared to correlate better than the midpoint values with the collapse temperatures. Ford & Timmins (1989) define glass-transition temperature as the temperature at the midpoint of the increase in heat capacity of a sample, thus the midpoint is typically the value which would be taken to be the true T_g value. In the present study, it appears that the extrapolated onset values may be better indicators of the true T_g' values for the polymer solutions, since it follows that initial softening of the frozen mass (at T_g') occurs at a lower temperature than full collapse of the matrix (at T_c). However, it should be noted that both collapse and glass transitions occur over narrow temperature ranges, and that the onset T_g' value should not be selected simply on the basis that it correlates with the observed T_c values more closely than the midpoint value.

3.2.4 Conclusions

The polymer solutions tested in the present study display high glass-transition temperatures which were in good agreement with the collapse temperatures observed by Adams

(unpublished data) and that the temperatures described are far above the minimum processing temperature of modern freeze-dryers. Therefore, such polymers may be suitable for use as freeze-drying excipients, since their frozen state thermal characteristics indicate that they may raise the collapse temperature of - and thus provide structural stability to - a frozen matrix. All polymers tested here compared favourably with a frozen sample of trehalose solution, which was observed to exhibit a T_g' at -29.89 (±0.04)°C (midpoint) and onset of -30.96 (±0.11)°C. Trehalose is used increasingly in freeze-dried formulations, especially to provide biological stabilisation to sensitive proteins and enzymes (see chapters 4 and 5), but due to its relatively low collapse temperature, does not tend to raise the collapse temperature of formulations. As stated at the beginning of the present chapter, the ideal freeze-drying excipient will provide thermal stability in addition to biological stability. The results of the present study indicate that some polymers which are known to provide stabilisation to proteins during freezing may also have the potential to provide thermal stability to such products during freeze-drying.

3.3 THERMAL ANALYSIS OF SUCROSE-FRUCTOSE BLENDS

3.3.1 Background

Sugars are ubiquitous in nature, existing as monomers or as polymers and serve a wide variety of functions in plant and animal life processes, ranging from energy storage to the structural protection of plant cells against freezing and dehydration stresses. Many sugars are widely used throughout the processing industries, yet in some respects, their physico-chemical properties have been neglected (Franks, 1987). In the context of the present study, one of their most important physico-chemical properties is their ability to form glasses and Finegold *et al.* (1989) have described the thermal characteristics of a number of binary sugar blends, which will have relevance to the shelf-stability of these mixtures. The authors concentrated on the study of mixtures of three common sugars for industrial application, the monosaccharides fructose and glucose, and sucrose, a disaccharide composed of these monosaccharide residues. Blends had been prepared under a nitrogen shroud by heating the crystalline components together in an aluminium pan until molten, before quenching the resulting liquid to form an amorphous glass. A 5-10mg chip was sealed under nitrogen in a DSC pan and analysed by a Perkin Elmer DSC-2 calorimeter, similar to that described in section 2.5. The authors reported changes in glass transition temperatures for binary sugar mixtures when the proportion of each component of a mixture was varied. They also observed that although heat capacity values obtained individually for glucose and sucrose alone were in good agreement with literature values, those obtained for mixtures of glucose and fructose deviated from the expected values. It appeared that fructose was acting as a plasticiser, since it depressed the T_g values of glucose and sucrose. However, it was also concluded that because of the non-unique nature of the glasses, which is dependent on the method and the rate of cooling, good correlation of heat capacities of the glasses produced in this study compared with those reported in previous studies (Goldberg & Tewari, 1989; Nelson & Newton, 1941) could not be expected. The aim of the present study has been to investigate the thermal behaviour of fructose and sucrose blends in a range of proportions, in order to confirm the data reported by Finegold *et al.* (1989). The study was then extended to include trehalose-mannitol blends, as described below in section 3.4.

3.3.2 Materials and Methods

3.3.2.1 Materials

AnalaR grade sucrose (dihydrate)and fructose (monohydrate) were obtained from BDH (UK) Ltd.

3.3.2.2 Differential Scanning Calorimetry

DSC was carried out as detailed in section 2.5 of this report. Briefly, sucrose and fructose were weighed accurately into an agate mortar in the desired proportion. Following gentle mixing, approximately 5mg of the blend were transferred to a tared aluminium sample pan and weighed using an electronic balance to 5 decimal places. Samples were heated to melting point, before cooling (quenching) at 320°C/min to -40°C, in order to induce an amorphous matrix. Glass transitions were measured on a second heating step and the temperature and energy flow of the transition calculated using the TADS software.

3.3.3 Results & Discussion

An example of a thermal profile for a blend of fructose and sucrose is given in Figure 3.1. A summary of thermal data for each blend is given in Table 3.4. A graph of composition versus glass transition temperature is shown in Figure 3.2. In concurrence with the results detailed by Finegold *et al.* (1989), the present data indicate that fructose appeared to act as a diluent for sucrose in the current study, as exemplified in Figure 3.2. It is believed that these data will have a bearing on the solid state stability of such mixtures, or of mixtures containing amorphous regions of sucrose and fructose in any proportion. Since the glass transition temperatures of all mixtures in the present study were close to room temperature, the storage of dried products containing these sugars in any combination at room temperature may compromise the stability of such products. Therefore, even though these excipients may be selected individually for inclusion into pharmaceutical formulations (often for the preservation of protein structure and activity) a combination of both fructose and sucrose may lead to a dried product containing amorphous regions which display a low solid state glass transition (and thus collapse) temperature. It has been shown that in a number of cases that under otherwise identical conditions, reactivity of a particular substance in the

amorphous state is greater than that in the crystalline state (reviewed in Shalaev & Zografi, 1996). A further point to note in the present study is that it was not determined which proportion of these two sugars results in a minimum transition temperature, which, incidentally, may be well below the temperatures observed for the mixtures analysed here. It is also not known whether the use of quench-cooling in the present study resulted in intimate mixing of the two components; however, it was believed necessary not to grind the sugars, since it has previously been demonstrated that the grinding of solvated excipients can result in alterations in thermal properties (Irwin & Iqbal, 1991), which would have invalidated the data in the present study. Therefore, it must be concluded from the present data that although the possibility exists that lyophilised products containing both sucrose and fructose may contain amorphous regions where the glass transition is below room temperature, it is not known to what extent the present experimental conditions reflect those occurring in real freeze-drying conditions. Nevertheless, the arguments presented here provide a rational basis for the discussion of the selection of excipient combinations and lyophilisation conditions.

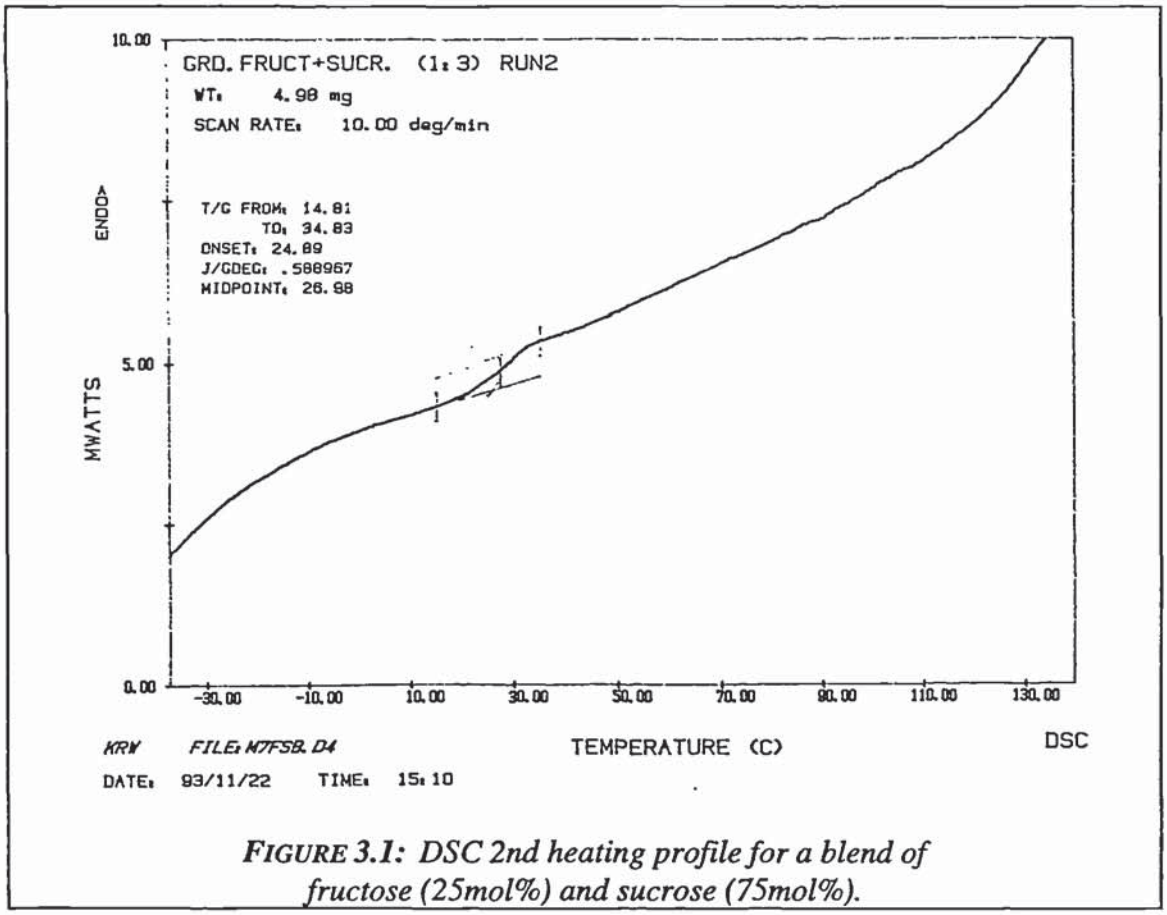


FIGURE 3.1: DSC 2nd heating profile for a blend of fructose (25mol%) and sucrose (75mol%).

COMPOSITION Fructose (mol%)	Sucrose	T_{melt} (°C)	ΔH_{melt} (J/g)	T_g (°C) (midpoint)	ΔH_{Tg} (J/g/°C)
0	100	189.8 (±0.9)	334.8 (±4.7)	70.7 (±1.8)	2.19 (±0.04)
25	75	122.0 (±0.5)	63.2 (±1.1)	26.8 (±0.2)	0.61 (±0.03)
39.1	60.9	121.9 (±0.2)	112.8 (±4.4)	24.8 (±0.2)	0.81 (±0.03)
65.5	34.5	122.5 (±0.5)	224.2 (±0.7)	26.1 (±0.3)	1.13 (±0.06)
100	0	124.2 (±1.7)	434.9 (±1.0)	13.6 (±0.1)	2.12 (±0.06)

TABLE 3.4: Thermal analysis data from DSC profiles for fructose and sucrose blends (n=2; range given in parentheses)

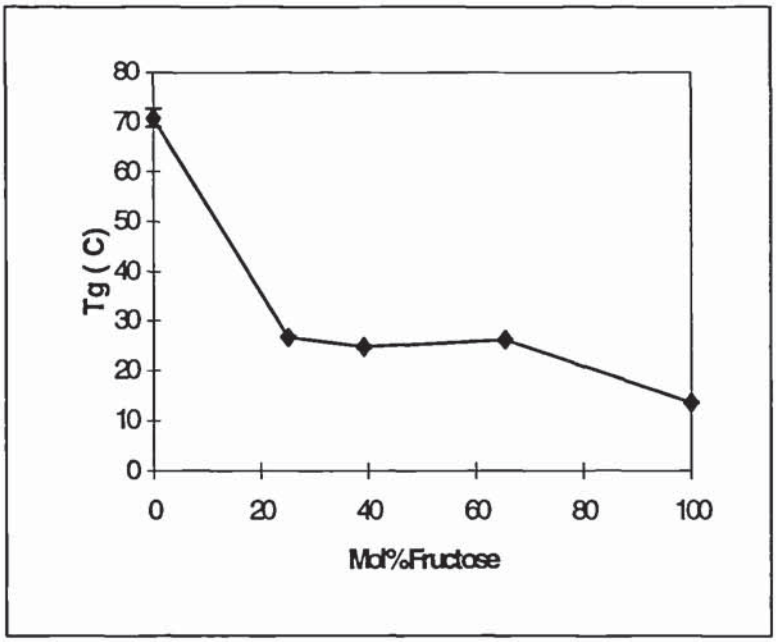


FIGURE 3.2: Graph showing the relationship between composition of fructose-sucrose blends and glass transition temperature

3.3.4 Conclusions

Simple mixtures of fructose and sucrose, which were melted together and quench-cooled, were observed to exhibit glass transition temperatures around 25°C (*cf.* sucrose 70.7°C and fructose 13.6°C, see table 3.4). These thermal characteristics were believed to have a

significant bearing on the solid-state stability of mixtures of these two components and that the storage of such products at, or above, room temperature would lead to increased mobility within the product and thus possible collapse. In addition, greater mobility may also lead to an increase in product degradation resulting from reactions between (highly concentrated) components within the matrix, as described by Levine & Slade (1988). These observations may also be applicable to lyophilised mixtures containing these excipients, with the implication that there may be possible risks of including such combinations in pharmaceutical formulations.

3.4 Thermal Analysis of Blends of Trehalose and Mannitol

3.4.1 Background

The study described in this section was an extension of the study described in section 3.3 above. Trehalose and mannitol are excipients which are often used in combination in freeze-dried formulations, particularly where biological stabilisation is necessary (see chapter 4). The aim of the present study was to examine the thermal characteristics of a range of blends of these excipients, in order to assess the effect of varying the proportion of each component on the glass transition temperature (and its associated thermal energy) of the mix. T_g was measured by inducing mixtures into a vitrified state, as described in the previous study. Examination of such mixtures in the solid state provided important information related to the shelf stability of compounds freeze-dried in these mixtures.

3.4.2 Materials and methods

3.4.2.1 Materials

AnalaR grade trehalose and mannitol (both anhydrous) were obtained from BDH (UK) Ltd.

3.4.2.2 Differential Scanning Calorimetry

DSC was carried out as detailed in section 2.5 of this report. The saccharides were weighed accurately into aluminium sample pans to give a combined weight of approximately 5mg

(exact molar ratios are given in Table 3.5.). Samples were mixed by heating to melting point, followed by quenching at 320°C/min to induce an amorphous matrix. Glass transitions were measured after reheating and the temperature and energy flow of the transition calculated using the TADS software.

3.4.3 Results and discussion

The mixtures of trehalose and mannitol displayed quite different thermal profiles to those for the fructose and sucrose blends described above. Mannitol alone remained in the crystalline form, giving identical profiles on reheating after quenching compared to thermograms obtained on initial heating. Trehalose, however, formed an amorphous glass after quenching from the molten state. The blends containing both components displayed amorphous characteristics, although no clear glass transitions were observed. Figure 3.3 shows the thermal (reheating) profile of the mixture containing 65.5mol% mannitol plus 34.5mol% trehalose (equivalent to a 1:1 (w/w) ratio of components). This is a profile which is typical for all mixtures analysed in the present study.

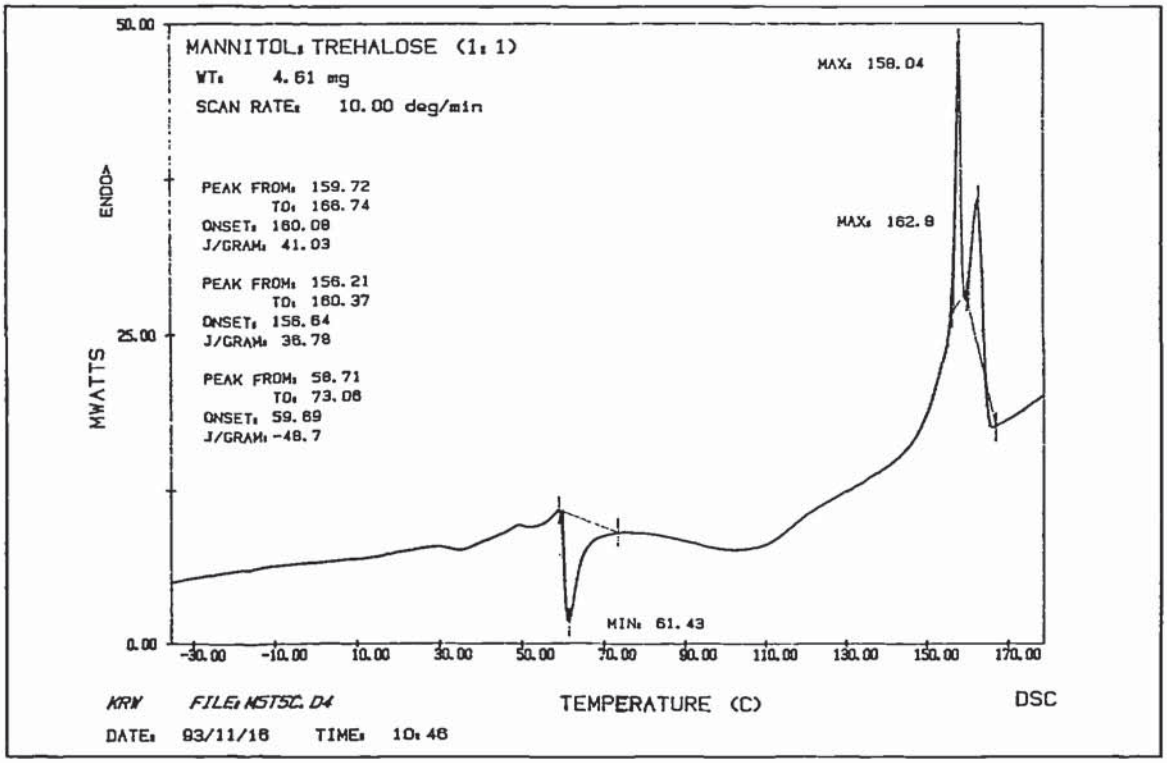


FIGURE 3.3: DSC reheating profile of a 1:1 (w/w) mixture of mannitol and trehalose

Characteristic features of reheating profiles included evidence of a glass transition (although difficult to quantify), a form of crystallisation exotherm, and a 'split' melting endotherm, which was believed to be due either to two discrete crystalline forms of mannitol or to one crystalline form of mannitol in association with a trehalose-mannitol co-mixture. Thermal events are summarised in Table 3.5. Each value is the mean of two independent sample runs, (range about mean given in parentheses).

Individually, both mannitol and trehalose describe high T_m or T_g exotherms after heating, which indicates that either excipient is likely to provide shelf-stable pharmaceutical formulations. However, mannitol, which would normally adopt crystalline behaviour, possibly exists in an amorphous phase when quenched with a secondary amorphous component, such as trehalose, thus giving a melting endotherm at reduced temperature. In the present study, the secondary thermal event for each mixture was in the region of 15-30°C, implying that storage above this temperature could lead to a change in the physical state of the product, possibly leading to increased risk of chemical degradation. As in the previous study (section 3.3), this may have a significant implications on the shelf-stability of products containing mixtures of trehalose and mannitol.

COMPOSITION (mol%)		**INITIAL HEATING**		**REHEATING PROFILE DATA**			
Mannitol	**Trehalose**	**T_{melt} (°C)**	**ΔH_{melt} (J/g)**	**T_g (°C)**	**T_{cryst} (°C)**	**ΔH_{cryst} (J/g)**	**Approx. Split Peak ratio (lower:upper)**
100	0	168.2 (±0.8)	722.0 (±1.5)	168 (melting endotherm)	-	-	-
85.1	14.9	166.1 (±1.1)	546.6 (±28.4)	ca. 15†	61.8 (±0.8)	-55.2 (±6.9)	1:30
65.5	34.5	163.3 (±0.5)	399.1 (±12.3)	ca. 15†	61.4 (n=1)‡	-48.7 (n=1)‡	1:1
38.8	61.2	161.0 (±0.2)	194.9 (±2.3)	ca. 30†	61.8 (n=1)‡	-3.0 (n=1)‡	20:1
0	100	212.4 (±0.2)	348.7 (±7.8)	117.7 (±0.6)	-	-	-

†Not calculable from either thermal profile. ‡Calculable from one of two profiles only

TABLE 3.5: *Summary of thermal data for trehalose/mannitol blends (n=2; range given in parentheses)*

3.4.4 Conclusions

The data presented here may have significant practical implications on the shelf-stability of products containing such mixtures. In the previous study, mixtures containing amorphous regions of sucrose and fructose in any proportion were believed to be thermally unstable at room temperature. Since an event resembling a glass transition occurred for all mixtures in the present study and that these events were close to room temperature, it was believed that the storage of dried products containing trehalose and mannitol in any combination under such conditions may compromise the stability of such products. It was concluded that the events resembling T_g for the trehalose/mannitol mixtures observed in the present study warranted further investigation and that the study should be extended to include other commonly-used amorphous freeze-drying excipients such as those shown in Table 3.2 at a range of proportions Such a study is described in section 3.5 below.

3.5 Thermal Analysis of Freeze-dried Single Excipients and Binary Excipient Mixtures containing Mannitol

3.5.1 Background

Many freeze-dried preparations for pharmaceutical application combine one or more saccharides (as illustrated in section 3.2 above), ostensibly to satisfy specific bioproduct requirements. However, formulations are often developed solely on a pragmatic basis, with little or no quantitative analysis of the physico-chemical behaviour of individual, or combinations of, excipients. Mannitol is commonly used as an excipient, since it displays a high T_m and its crystallising behaviour imparts structural rigidity and bulk to the dried product. Therefore, some of the potential problems associated with using amorphous excipients (*e.g.* dried cake structure softening over time) may be reduced. However, mannitol itself displays a complex crystallising behaviour, the nature of its behaviour dependent on a number of factors such as concentration, presence or absence of other solutes and the rate of cooling. Solutions which contain mannitol at low concentrations in combination with other solutes will not crystallise on cooling, even when temperatures below -40°C are reached. Practically, mannitol solutions can be induced to crystallise by heat-

annealing the sample. Heat-annealing (also termed thermal treatment or tempering) is performed by cooling the solution to freeze water as ice, then warming the mass to approximately -15°C to induce crystallisation of the mannitol, before finally cooling the mass prior to chamber evacuation. Heat-annealing may also be used to recrystallise small ice crystals into larger crystals which are more conducive to the sublimation process. The crystallisation of mannitol is accompanied by a concomitant release of lattice energy, which can be measured using differential scanning calorimetry (DSC). This knowledge of the crystallising behaviour of mannitol may be used to permit freezing of mannitol solutions to give a partially amorphous or crystalline microstructure. In terms of processing stability and overall collapse temperature in the frozen state, in cases such as this, where the frozen matrix is predominantly but not completely crystalline, the likelihood remains that any amorphous regions present in the frozen matrix may soften at temperatures far below the predicted eutectic melting temperature of crystallising mannitol solute. It is also possible that these solutions may exhibit differently structured regions within a lyophilised cake, each displaying different patterns of thermal behaviour. Therefore, the glass transition temperature of the amorphous region in such a product is an important issue, and is likely to be the critical temperature which determines the stability of the overall product as a whole. In addition, it should be remembered that glassy regions within a dried product have a far greater capacity to absorb and harbour moisture than any crystalline regions present (see section 1.2).

The primary objective of the present study was to examine the thermal characteristics of a range of freeze-dried products containing mannitol together with a second known amorphous component, in order to provide evidence of limiting stability factors for processing and storage. In particular, a glass transition temperature could then be determined for each dried product and these characteristics compared to those displayed by components freeze-dried individually. Freeze-drying microscopy (FDM) was also used to examine the freezing and crystallising characteristics of these mannitol systems containing co-solutes, to allow a more comprehensive understanding of the complex behaviour adopted by such solutions during the lyophilisation process.

3.5.2 Materials and Methods

3.5.2.1 Materials

Mannitol, sucrose, glucose, lactose, maltose, mannose and trehalose were all of AnalaR grade (or equivalent) from Sigma Chem. Co., Poole, UK., BDH, UK., or Fisons, UK.

3.5.2.2 Preparation of solutions for freeze-drying

Solutions of the chosen components were prepared in sterile distilled water for irrigation. Solutions of mannitol were prepared to final concentrations of 1% and 2% (w/w). Solutions containing mannitol together with a second component were prepared to give final concentrations of 2% (w/w) mannitol plus 1% (w/w) of the second co-solute.

3.5.2.3 Freeze-drying microscopy (FDM)

A solution of 2% (w/w) mannitol and 1% (w/w) glucose was analysed under the freeze-drying microscope, as described in section 2.4. Sufficient solution was pipetted onto a microscope slide under a cover slip supported by two glass strips (see Figure 2.5(b)). The slide containing the analyte solution under the cover slip was placed onto the stainless steel vacuum assembly (Figure 2.5(c)) and a slight vacuum pulled to immobilise the slide. The entire assembly was immersed into the methanol bath of the freeze-drying microscope (Figure 2.5(a)) which had been precooled to -25.0°C. After having ensured that the analyte solution had become apparently solid, a vacuum of 0.2mbar was applied to begin drying. Photographs were taken of the drying front (sublimation interface) at various stages and at a range of temperatures within the drying run, using the camera set-up as detailed in section 2.4. The freezing and crystallising characteristics of the solution were studied, and the temperature altered according to the behaviour of the solution. The temperature course followed for FDM is detailed below (section 3.5.3.2).

3.5.2.4 Freeze-drying

Freeze-drying was carried out as detailed in section 2.1, using an Edwards Modulyo freeze-drier. Aliquots of solution (0.50ml) were pipetted accurately into freeze-drying vials (3ml

capacity) and frozen for 1 hour at -70°C before being dried at ~0.2mbar for 16 hours, with shelf heating (~30°C) for the first 4 hours of drying.

3.5.2.5 Differential Scanning Calorimetry (DSC)

Thermal analysis of the dried samples was carried out using the Perkin Elmer DSC-4 system, as detailed in section 2.5.1, following the procedure described in section 2.5.2. Initial heating was from -40°C to melt, at a rate of 10°C per minute. The sample was then quench-cooled at 320°C/min to -40°C before being reheated to melt at 10°C/min. Glass transition temperatures were obtained on the reheating profiles. Analysis was carried out in duplicate on two independent samples, unless stated otherwise.

3.5.3 Results and Discussion

3.5.3.1 Moisture content determination

Typical moisture levels remaining in lyophilised products were calculated to be approximately 1% (w/w), as determined by a coulometric Karl Fischer method, as detailed in section 2.10.

3.5.3.2 Freeze-drying microscopy (FDM)

The temperature course followed for FDM was as follows. The sample was immersed into the methanol bath which had been pre-cooled to -25°C. The bath was cooled to -35°C before drying was initiated. As the sublimation interface began to proceed through the frozen mass, the product began to dry with collapse of structure (see Figure 3.4(a)). The temperature of the bath was lowered slowly and drying continued with collapse of structure until evidence of partial structural retention was observed at -38°C (Figure 3.4(b)). On further cooling, it was apparent that the matrix was beginning to dry with a greater degree of structural retention (-42°C, Figure 3.4(c)) which was believed to be due to the freezing of trehalose. However, it could not be determined whether the structure of the trehalose was crystalline or amorphous, but the latter is the favoured suggestion. On raising the bath temperature slowly, initial stages of collapse were observed at -39.5°C (Figure 3.4(d)), signifying the melting of the trehalose which was earlier observed to freeze at around this

temperature. Figure 3.4 (e) shows the extensive collapse of the structure when the product temperature had reached -32°C. The temperature of the bath was further increased, in order to determine whether mannitol would crystallise at a higher temperature. Figure 3.4(f) shows the progression of the drying front with partial structural retention of the matrix at -23°C, possibly indicative of initial signs of crystallisation of mannitol. By -18°C, it was apparent that the structure of the sample was drying with retention of structure to a large extent (Figure 3.4(g)) and that even dried regions which had previously collapsed had begun to regain structure. Figure 3.4(h) shows the sample at -11.5°C, where the sublimation interface had become broader and had begun to proceed through the matrix at a much reduced rate, which testified to the dense structure of the matrix at this temperature.

3.5.3.3 Differential Scanning Calorimetry

The main thermal events observed on the heating and reheating profiles of the freeze-dried samples are summarised in Table 3.6.

Sample Details	T_{melt} (°C)	ΔH_{melt} (J/g)	T_g (°C) (Midpoint)	$\Delta H_{(Tg)}$ ($J.g^{-1}\ deg^{-1}$)
Mannitol (1%)	167.8 (±0.9)	710.5 (±4.0)	166.4 (±1.5)†	700.3 (±4.5)†
Mannitol (2%)	168.3 (±0.9)	722.4 (±5.2)	167.9 (±0.9)†	713.9 (±3.3)†
Trehalose (1%)	213.1 (±2.5)	n/c*	115.8 (±2.3)	0.93 (±0.23)
Lactose (1%)	213.9 (±0.6)	n/c*	100.3 (±0.5)	n/c*
Maltose (1%)	n/c*	n/c*	99.4 (±0.0)	n/c*
Sucrose (1%)	189.8 (±1.2)	327.5 (±0.4)	66.6 (±0.3)	n/c*
Mannitol (2%) + Trehalose (1%)	158.5 (±0.2)	384.2 (±8.8)	27.1 (±0.3)	2.20 (±0.21)
Mannitol (2%) + Lactose (1%)	158.8 (±0.4)	377.5 (±1.8)	25.5 (±0.4)	1.84 (±0.23)
Mannitol (2%) + Maltose (1%)	158.6 (±0.0)	371.2 (±6.3)	23.2 (±0.1)	2.34 (±0.04)
Mannitol (2%) + Sucrose (1%)	154.3 (±0.8)	336.8 (±3.7)	19.6 (±0.4)	2.53 (±0.08)
Mannitol (2%) + Glucose (1%)	153.1 (±0.2)	323.6 (±9.5)	16.9 (±1.2)	1.86 (±0.08)

*n/c=not calculable from data obtained †data for second melting endotherms

TABLE 3.6: *Thermal characteristics of lyophilised solutions of individual saccharides and mixtures with mannitol, as determined by DSC (n=2; range given in parentheses)*

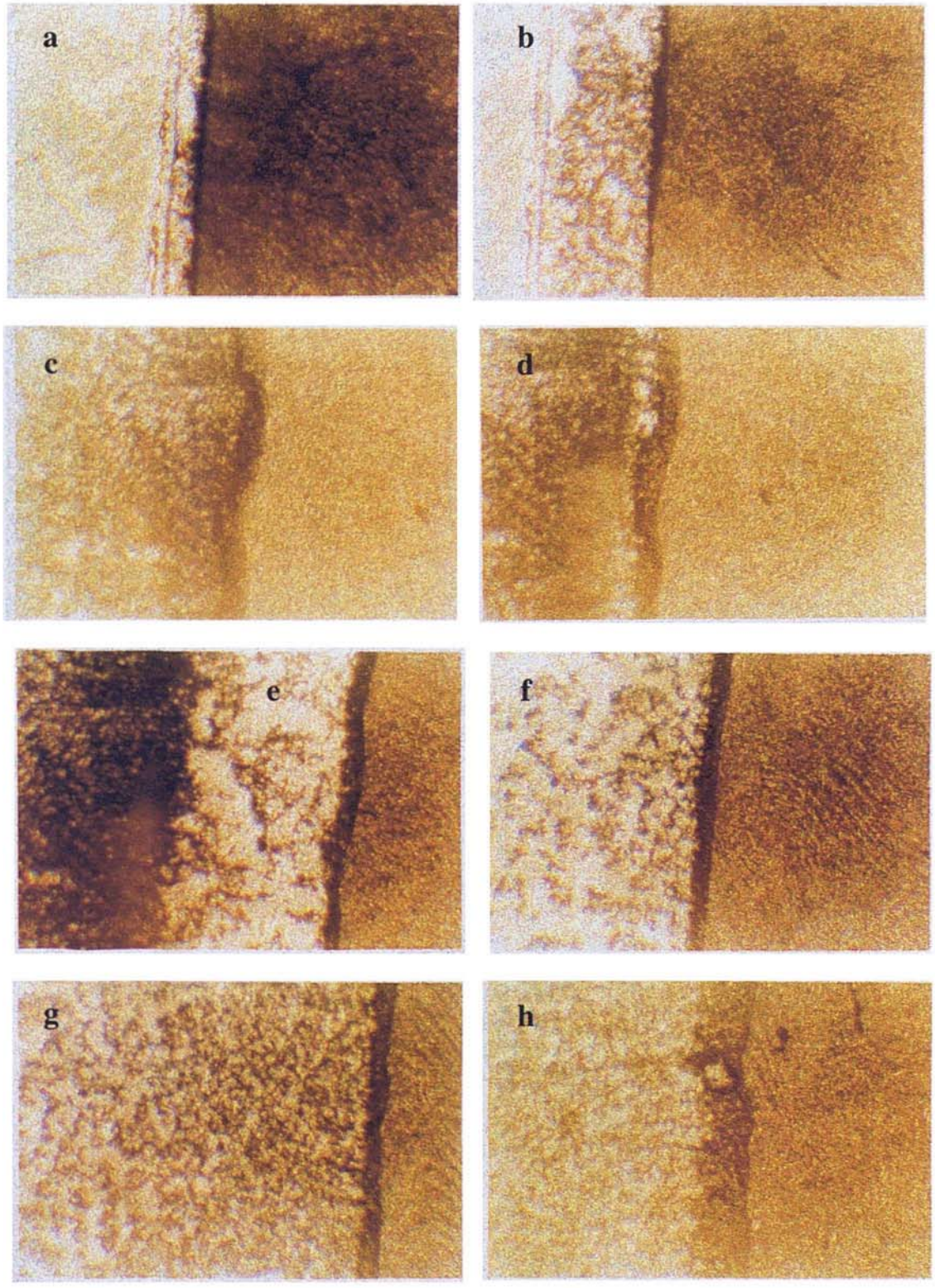


FIGURE 3.4: *FDM images of a solution containing 2% (w/w) mannitol and 1% (w/w) glucose. See text for description of observed events.*

It was noted that no glass transitions were observed on the initial profiles of any of the dried mixtures or for mannitol freeze-dried alone. Instead, each of the initial profiles contained a sharp endotherm, believed to represent the overall melting point of each mixture. The presence of just one apparent melting endotherm suggested that, in each case, the formation of a eutectic mixture may have been achieved. The existence of a eutectic would therefore preclude the existence of a glass, and thus explain why no glass transitions were observed in the initial profiles. The literature value for the melting temperature of mannitol is 168°C (CRC Handbook, 69th ed., CRC Press (1989)). This was observed experimentally for freeze-dried solutions of mannitol at initial concentrations of 1% and 2% (w/w), as shown at the top of Table 3.6. However, in all binary mixtures, the apparent melting endotherm lay between 153°C and 159°C, which suggests that the (thermally) predominant component in each mixture was mannitol, which could be said to be 'plasticised' by the second component in each case. The quenching of the mixtures at 320°C/min had led to the apparent formation of a glassy phase, for which a glass transition could be observed on reheating. It was appreciated that, by this stage, mixtures had already been subjected to heating and quenching, and that many changes in physical state had been brought about by this thermal history. However, although samples were not in the same physical state as they were immediately following freeze-drying, it was interesting to note the glass-transition temperatures of the induced glassy phases, since this may provide evidence as to the thermal characteristics of any amorphous regions present in dried formulations containing mannitol in combination with amorphous excipients, as is often the case in pharmaceutical formulations. This might have particular significance in terms of storage stability of freeze-dried products containing trehalose and mannitol, since the T_g values observed for all combinations in the present study were all at or below room temperature. Therefore, storage at ambient temperature may lead to the solid-state collapse of these products. As in the previous study, the amorphous components in the present study displayed individually high glass-transition temperatures compared to the co-mixtures tested (from 66.6°C for sucrose to 115.8°C for trehalose) with each being significantly lowered by mixing with mannitol.

It was hypothesised that the presence of a second component in combination with mannitol, coupled with the rapid quenching of molten samples after initial heating in the DSC

apparatus, had led to the formation of an induced vitrified state (glassy phase) in all samples tested. The peak energy values obtained for the crystallisation exotherms of the quenched mixtures are consistent with the high heat capacity of mannitol, thus implying that the apparent crystallisation of each mixture was chiefly due to the mannitol which may have been held in a vitrified state by the second component in each case. As the individual heat capacities of the second components were all found to be much lower than mannitol (see Table 3.6), it was not possible to determine from the DSC data whether the secondary components contributed to the exotherm in each case, since any event arising from the secondary component would be masked by the high thermal energy event(s) arising from mannitol itself. However, as solutions containing mannitol alone typically display crystalline behaviour at concentrations greater than 1% (w/w) (Hellman *et al.*, 1983), it appears that the secondary, amorphous component in each of the mixtures prepared here must have contributed to some extent to the formation of a glassy phase together with some of the mannitol present in each mixture.

Glass transition values observed for individually trehalose and the commonly used excipients lactose and sucrose freeze-dried individually correlated very well with the solid-state collapse temperatures (T_c) observed by Adams (personal communication), using a precise heating technique (see Table 3.7).

Excipient	Observed T_c value (°C)	Observed T_g value (°C)
Lactose	108 (±2)	100.3 (±0.53)
Sucrose	66 (n=1)	66.6 (±0.28)
Trehalose	115 (±3)	115.8 (±0.23)

TABLE 3.7: *Observed solid-state collapse temperatures (Adams, unpublished data) and glass transition temperatures for freeze-dried samples of commonly-used excipients (n=2; range given in parentheses)*

It was apparent from the observed data that freeze-dried products containing solely mannitol exhibited no glass transition when heated. It was thus believed that in the absence of other excipients, mannitol had freeze-dried to give a totally crystalline structure under the

conditions employed in this study. However, on closer examination of the initial heating profiles of these samples (mannitol freeze-dried from 1% and 2% (w/w) solutions), minor deviations from the baseline were noted at approximately 10°C below the melting temperature. It was hypothesised that these events may have been indicative of a small proportion of the mannitol persisting in an amorphous (vitrified) state in the dried product.

In addition, solid-state glass-transition values observed for the mixtures containing mannitol were also very similar to each other, but very different to those of the other components when freeze-dried individually. Further to this, it was apparent that, as the individual T_g values of glucose (Lit. values: 31°C (Levine & Slade, 1987), 32°C (Franks *et al.*, 1991)), sucrose, lactose and trehalose increased in that order, so the T_g values for the mixtures containing mannitol and each of these components increased in the same order. The T_g values observed for the mixtures (between 16.9°C and 27.1°C) may have been indicative of the theoretical value for the T_g of amorphous mannitol itself, since mannitol appeared to be the dominant component, displaying a far higher individual melting energy than any of the other components. However, a glass-transition temperature for mannitol alone does not appear to have been published, presumably since it has proven too difficult to isolate mannitol in an amorphous state.

3.5.4 Conclusions

The data obtained in the present study from the heating profiles of quenched mixtures of mannitol and amorphous excipients suggest that the solid state glass transition temperatures (T_g) of all mixtures are significantly lower than that of the individual amorphous components or, indeed, the melting temperature of mannitol. The precise proportion of mannitol remaining amorphous in each sample was not quantified in the present study, and therefore, possible correlation between T_g and composition of amorphous regions was not possible. The use of techniques such as SEM may reveal the extent to which mannitol remains amorphous; however, in order to produce quantitative data, the analysis of a set of calibration standards containing known (and unchanging) proportions of crystallinity would be necessary, which on a practical level, would be difficult to achieve. The use of a sensitive thermo-analytical technique such as microcalorimetry may also be used to provide more

detailed information about the behaviour of solutions containing crystallising and amorphous components during freezing, which could provide evidence as to the microstructure of the resulting frozen (and subsequently dried) matrix. The data reported in the present study may have implications on the shelf-stability of products containing mannitol together with amorphous excipients, since any isolated amorphous regions present in a formulation containing such excipients may contain different proportions of each component to those originally used, and may thus correlate with some of the proportions used here.

The following experiment builds upon the findings reported in the current section, by examining the effect of varying the proportion of mannitol and the secondary components in the mixtures. Further evidence for the ability of mannitol to adopt a partially amorphous microstructure upon freeze-drying has been demonstrated by Adams (unpublished data), who observed that at concentrations of less than 0.25mg/ml, mannitol remained amorphous when formulated with a model protein. This is discussed further in chapter 4 of the present report.

3.6 A STUDY OF THE EFFECTS OF VARYING COMPONENT PROPORTIONS ON THE GLASS TRANSITION TEMPERATURE OF THE INDUCED VITRIFIED STATE OF FREEZE-DRIED MIXTURES CONTAINING MANNITOL

3.6.1 Background

In the experiment detailed in section 3.5 above, all mixtures containing mannitol in combination with a secondary component were prepared to give a weight ratio of 2:1 of mannitol to the second component in each case. The aim of the present study was to investigate the effects of varying the ratios of mannitol and the chosen second component present in the initial solution on the glass transition temperatures of the induced glassy phases produced from melting and quenching the lyophilised product of each solution.

3.6.2 Materials and Methods

3.6.2.1 Materials

All chemicals were supplied by Sigma, BDH or Fisons, UK, and all were of AnalaR grade, or equivalent.

3.6.2.2 Preparation of solutions

Solutions for lyophilisation were prepared in sterile distilled water, to the concentration specifications given in Table 3.8.

3.6.2.3 Freeze-drying

Freezing and drying of the solutions was carried out as described for the previous study, using an Edwards modulyo freeze-drier, in accordance with the standard procedure given in section 2.1.

3.6.2.4 Differential Scanning Calorimetry

Differential scanning calorimetry of dried products was carried out as detailed in section 2.5 of this report.

3.6.3 Results and Discussion

The glass-transition temperatures observed for the quenched samples on reheating are summarised in Table 3.8. Each value is the mean of T_g values for two independent samples, unless otherwise stated. Values for individual components are taken from the study detailed in section 3.3.

Composition of Original Solution	Mol% mannitol	T_g, °C (mean ± range)	$\Delta H_{(Tg)}$, $J.g^{-1}.K^{-1}$ (mean ± range)
Mannitol alone (1%)	100	167.8 (±1.4)[#]	710.5 (±4.0)[#]
Glucose alone[†]	0	32[§]	n/a
Sucrose alone (1%)	0	66.6 (±0.3)	n/c[‡]
Lactose alone (1%)	0	100.3 (±0.5)	n/c[‡]
Trehalose alone (1%)	0	115.8 (±2.3)	0.93 (±0.23)
2% Mannitol + 0.25% Glucose	88.8	12.25 (±0.02)	1.15
2% Mannitol + 0.5% Glucose	80	12.61 (±0.20)	2.35
2% Mannitol + 1% Glucose	66.7	8.35 (±0.09)	2.31
1% Mannitol + 1% Glucose	50	11.88 (±0.03)	2.09
0.5% Mannitol + 1% Glucose	33	14.02 (n=1)[*]	1.74 (n=1)
2% Mannitol + 0.25% Sucrose	88.8	14.89 (±0.66)	n/c[‡]
2% Mannitol + 0.5% Sucrose	80	15.62 (±0.37)	n/c[‡]
2% Mannitol + 1% Sucrose	66.7	15.42 (±0.06)	n/c[‡]
1% Mannitol + 1% Sucrose	50	19.73 (±0.14)	2.14
0.5% Mannitol + 1% Sucrose	33	24.42 (±0.08)	2.04
2% Mannitol + 0.25% Lactose	88.8	16.03 (±0.06)	1.36
2% Mannitol + 0.5% Lactose	80	17.44 (±0.08)	2.35
2% Mannitol + 1% Lactose	66.7	21.88 (±0.03)	2.52
1% Mannitol + 1% Lactose	50	26.48 (±1.14)	1.73
0.5% Mannitol + 1% Lactose	33	n/a[†]	n/a[†]
2% Mannitol + 0.25% Trehalose	88.8	15.35 (±0.87)	1.59
2% Mannitol + 0.5% Trehalose	80	19.01 (±0.09)	2.24
2% Mannitol + 1% Trehalose	66.7	23.64 (±0.20)	2.29
1% Mannitol + 1% Trehalose	50	29.97 (n=1)[*]	2.17 (n=1)
0.5% Mannitol + 1% Trehalose	33	n/a[†]	n/a[†]

[#]Data for second melting endotherm. [†]Insufficient dried material for analysis. [§]From Franks *et al.* (1991)
[*]Insufficient product for duplicate analyses. [‡]Not calculable from thermograms

TABLE 3.8: *Glass-transition temperatures observed from reheating profiles of molten and quenched samples of lyophilised mannitol and co-solute mixtures*

A plot of glass-transition temperature versus % (w/w) mannitol for each mixture is given in Figure 3.5. In concurrence with the data obtained in the previous study, the quenched

mixtures exhibit low glass-transition temperatures compared to those of the individual components and the plot of T_g against proportion of mannitol (mol%) for each mixture describes a U-shape curve (key in Figure 3.5 shows amorphous additive in each mixture).

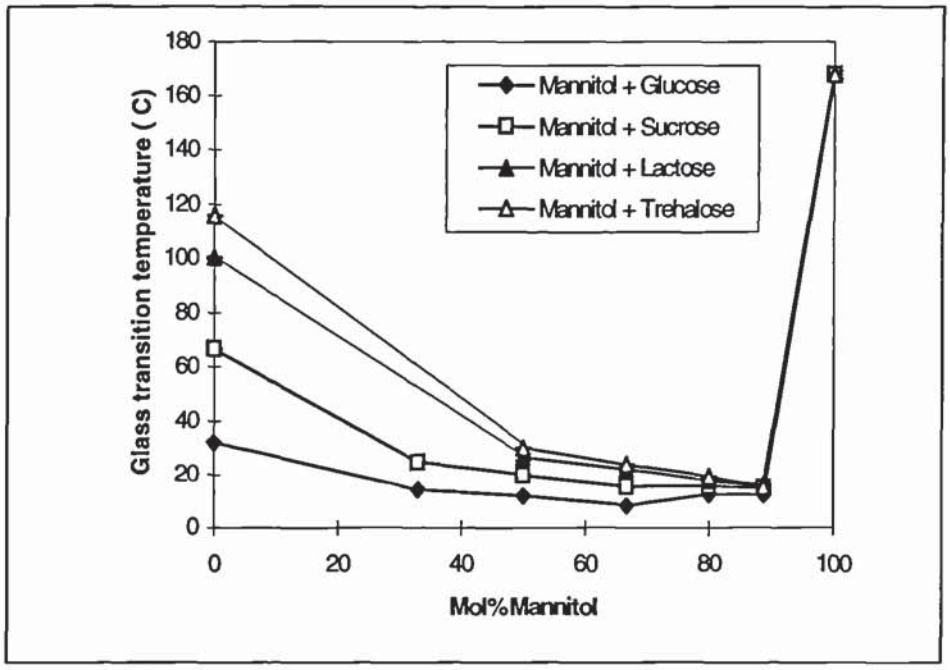


FIGURE 3.5: *Graph showing relationship between proportion of mannitol in mixture and glass transition temperature*

The above data indicate that, in parallel with previous studies using non-lyophilised materials (sections 3.3, 3.4), mixtures of excipients which typically display high collapse temperatures in the solid state may form glasses which display transitions at far lower temperatures than either of the individual components. This may have implications in terms of product shelf-stability, suggesting that storage at room temperature, even for lyophilised products containing these mixtures, is likely to lead to product collapse and possibly a significant loss of physical stability. It was surprising that the trend of T_g with respect to component proportions within mixtures did not follow a linear or v-shaped pattern, but that it was observed that even a combination of 11.2% (w/w) of glucose with 88.8% (w/w) mannitol was induced into a vitrified state which exhibited a glass-transition temperature of 12.25(±0.02)°C. These data suggest that the addition of even small proportions of secondary, amorphous excipients together with mannitol may produce glasses (or regions of glasses) with very low transition temperatures, leading to poor shelf-stability.

3.6.4 Conclusions

As stated above, the data in the present study seem to reinforce those obtained in the preceding studies detailed in this chapter. The most significant conclusion which may be drawn from these studies is that the use of amorphous excipients in combination with mannitol in lyophilised formulations, or in the solid state, may lead to the occurrence of vitreous regions which characteristically exhibit low glass-transition temperatures and will consequently display poor stability at ambient temperatures. It was not possible to ascertain the individual glass-transition temperature of amorphous mannitol alone, since the presence of mannitol alone in such a phase could not be isolated in stable form. However, the data in the present chapter are likely to be of more practical significance, since mannitol is unlikely to exist alone in such a state in pharmaceutically relevant preparations.

4. THE STABILISATION OF MODEL POLYMERIC PROTEINS BY FREEZE-DRYING

SUMMARY

The present chapter describes a number of investigations into the freeze-dry stability of two model polymeric proteins, L-asparaginase and lactate dehydrogenase (LDH). The process of freeze-drying is often used to stabilise proteins which prove to be unstable for long periods when stored as aqueous solutions. However, the process itself places stresses on proteins, and protective additives (lyoprotectants) are often employed in order to preserve the structure and biological activity of sensitive proteins during the lyophilisation process. The aim of this chapter was to elucidate the mechanism(s) by which such lyoprotectants interact with these model proteins at the molecular level. Studies were carried out to compare a range of saccharide additives as potential protective agents for L-asparaginase during freeze-drying. These studies revealed that each additive afforded similar levels of protection to L-asparaginase, which was dependent on additive concentration. It was noted that since all additives contained a similar number of hydroxyl groups *per* unit weight, the mechanism of proective interaction may implicate these hydroxyl groups. Molecular modelling enabled assessment of the pattern of available highly polar residues (HPR) present on the L-asparaginase molecule in both monomeric and tetrameric forms, which allowed predictions to be made as to the theoretical amounts of additive required to provide protection to L-asparaginase. These theoretical values gave a close approximation to those found experimentally to provide maximal levels of protection to L-asparaginase in the present work. A study to assess the success of PEG (MW 10kDa) as a lyoprotectant to L-asparaginase revealed that smaller amounts of PEG were required on a molar basis compared to the saccharides in the earlier study, to afford similar levels of protection to L-asparaginase. Preliminary DSC data suggested that it may be possible that one of the two PEG crystal types was reponsible for protective interaction with the protein; however, further studies would be necessary to confirm or disprove this theory. It was concluded that PEG may protectively interact with L-asparaginase *via* a different mechanism to the saccharides tested here, since each PEG molecule contains only one hydroxyl group but a large number of oxygen atoms which could form hydrogen bonds with L-asparaginase. A preliminary study was carried out to examine the freeze-thaw and freeze-dry stability of LDH in the presence of a number of additives. This was assessed on the basis of retained biological activity, following freezing, drying and storage at elevated temperature. The conditions adopted enabled differences in the protective abilities of the additive to be exposed. While all additives provided some protection to LDH during freezing, fewer afforded protection during drying and only trehalose and lactose were able to provide stabilisation to the protein during storage at 37°C in the dried state. These differences in observed protective abilities of the additives were attributed to molecular classification, reactivity and reducing properties.

4.1 INTRODUCTION

Many novel therapeutic agents currently emerging from molecular drug design and recombinant DNA technology are proteins and peptides (Adams, 1991; Pikal, 1990a,b; Arakawa *et al.*, 1993; Nail & Gatlin, 1993). The process of freeze-drying is often used to stabilise proteins which prove to be unstable for long periods when stored as aqueous solutions. However, the process itself places stresses on proteins (and other biologically sensitive materials) which are often not considered during the formulation design process. Freeze-drying may cause stability problems by inducing conformational instability in many proteins subjected to freezing and subsequent dehydration stresses (Crowe *et.al.*, 1990). To minimise damage to proteins, protective excipients (lyoprotectants) are invariably added to protein formulations. These additives must be pharmaceutically acceptable and compatible with the freeze-drying process. The use of such additives is well documented, both in reports which detail the freeze-dry stability of individual proteins, as well as in a number of review articles, such as those by Arakawa *et al.* (1993), Izutsu & Yoshioka (1995) and Skrabanja *et al.* (1994). Indeed, the extensive use of sugars as stabilising excipients for therapeutic agents has recently received attention by the national press (James, 1995). While the precise mechanism by which lyoprotectants function has not been fully characterised for all protein systems, it is generally accepted that protection is dependent on an interaction between protein and co-solute(s) *via* hydrogen bonding, with the persistence of an amorphous state in the frozen ice matrix necessary to effect protection (Arakawa *et al.*, 1993). Therefore, any interaction which does take place between a protein and a co-solute must necessarily occur before the freezing stage of the process; thus, the stability of proteins in solution and during freezing (and freeze-thawing) are important issues which should be considered when addressing the stabilisation of proteins by freeze-drying. An outline of the most relevant issues is provided in following sections.

4.1.1. Stabilisation of proteins in solution and during freeze thawing

Often, the very isolation and purification of a protein can be detrimental to its structure and biological activity. A number of factors can lead to the occurrence of damage to isolated protein molecules, such as high concentrations of macromolecules and low molecular weight compounds, temperature, pH, ionic strength and redox potential of the surrounding aqueous

environment. As a consequence, proteins may become modified, resulting in conformational changes and/or aggregation. A loss of its three-dimensional (native) conformation leads not only to a loss of biological activity for a protein, but also to increased susceptibility to further deleterious processes such as covalent or non-covalent aggregation (Manning *et al*, 1989; Volkin & Klibanov, 1989; Cleland *et al*, 1993). It has therefore become common practice to include stabilising additives in protein solutions, in order to preserve the native conformations of proteins during isolation and storage. Processes such as freeze-thawing and freeze-drying can also lead to protein damage, not least because they often tend to exacerbate the adverse environmental effects such as changes in pH and salt concentrations described above.

The review by Arakawa *et al.* (1993) provides reference to many studies carried out which showed that several types of additive such as sugars, amino acids, some amines and glycerol stabilised proteins against these factors in solution, but states that in many cases, the use of these additives was insufficient to stabilise proteins completely. It is also quite clear that the mechanisms of action of protective agents have only recently begun to be understood, even though many agents have been utilised pragmatically for some time to protect proteins during processing and storage. Indeed, glycerol and sugars have been used extensively for the protection and stabilisation of proteins in solution and during freeze-thawing; however, the mechanism of action of these protective agents was not fully understood until results of studies into the preferential interaction of proteins with solvent components were reported by Timasheff and co-workers (Timasheff & Inoue (1968); Arakawa & Timasheff (1985); Arakawa *et.al.* (1990a,b,c)) and by Lee & Lee (1981, 1987). Theoretical and experimental analysis has shown that it is the protein-solvent interaction which determines the effect of the co-solutes on the stability of the proteins, rather than direct interaction of proteins with the other solutes (see Arakawa *et.al.* (1991) for review). It has been observed experimentally that in the solution state, there is a deficiency of stabilising co-solute in the immediate vicinity of the protein, relative to that in the bulk solution (see Figure 4.1). Thus, the protein is said to be 'preferentially hydrated'; the co-solutes are consequently known as being 'preferentially excluded' from the surface of the protein. As Timasheff and colleagues explain, the presence of these co-solutes in a protein solution creates a thermodynamically unfavourable situation, since the preferential exclusion of the co-solute from the protein leads to an increase in the

chemical potential of each component (*i.e.* a chemical potential is created in keeping the protein(s) and co-solute(s) separated).

FIGURE 4.1: *Schematic illustration of preferential interactions of a protein with a stabilising co-solute (A) and with a destabilising co-solute, MPD (2-methyl-2,4-propanediol) (B) (from Arakawa et al, 1990a)*

Consequently, the native structures of monomers and the polymerised form of oligomeric proteins are stabilised, since denaturation or dissociation would lead to a greater area of contact between the protein and the solvent, therefore causing an increase in this thermodynamically unfavourable effect. In addition, this effect is concentration-dependent; at high concentrations, where random chance interactions between proteins and co-solutes will be more likely than in more dilute solutions, the process of excluding the co-solute from the surface of the protein is more destabilising to the system (*i.e.* entropically) than in a solution of low concentration, where the random chance of protein-co-solute interaction is lower.

A strong correlation between solution stabilisation and preferential interaction has been observed for many compounds. Those compounds which are strongly excluded from the protein surface tend to stabilise proteins against various stresses imposed on them in solution. However, there are some exceptions to this rule: for example PEG (polyethylene glycol) and MPD (2-methyl-2,4-pentanediol) (Arakawa *et.al.*, 1990b; Lee & Lee, 1981). It was particularly interesting to note from the above references that the behaviour of PEG as a potential protective agent was variable with temperature. PEG exhibits a high preference for exclusion from the surfaces of native proteins at room temperature. However, it is also known to be a weak protein destabiliser, and although it does not denature proteins at room temperature, it was observed to destabilise proteins at higher temperatures (Timasheff & Inoue, 1968; Lee & Lee, 1987). The reason for this apparently anomalous behaviour is that the preferential hydration of PEG (as well as other solutes such as $MnCl_2$ and MPD) is strongly dependent on environmental conditions such as pH, temperature and concentration; even though PEG is known to be readily soluble in aqueous media, it can also display some hydrophobic characteristics under certain environmental conditions. Under such conditions, PEG has a high affinity for the denatured state of proteins, as this state always displays several exposed hydrophobic groups (Arakawa *et.al.*, 1993). Thus its effect on the stability of proteins cannot be predicted simply from its preferential interactions with proteins in the native state; environmental factors also need to be taken into account.

The mechanism of preferential exclusion, known to stabilise proteins in aqueous solution, can also be applied to the protection of proteins during freezing and freeze-thawing (Carpenter *et.al.*, 1991). The most critical stress to which a protein is exposed during freeze-thawing is the formation of ice. Since the protein is excluded from the ice crystals during freezing, proteins are subjected to the physico-chemical changes occurring in the non-ice phase (see section 1.2). It is at this point, when most of the major physico-chemical changes occur in the environment surrounding the protein, where the protective agent is essential in preserving the native state of the protein. As ice is formed, the concentration of all solutes increases dramatically. If the solutes present in the non-ice fraction are destabilising, then this concentrating effect can lead to protein denaturation (Arakawa *et al.*, 1993). The effects of phenomena such as freeze-concentration on the protein must be minimised during freezing,

and the selection of additives which are able to protect the protein during this stage is fundamental to the preservation of the biological activity of the protein throughout freeze-thawing. Many groups of compounds have been found to be successful cryoprotective agents for proteins, including sugars, polyols, some amino acids, polymers and organic and inorganic salts (Arakawa *et al.*, 1993).

4.1.2. Stabilisation of Proteins during Dehydration

Whilst Izutsu & Yoshioka (1995) have recently suggested that most cryoprotectants also act as successful lyoprotectants, volatile cryoprotectants, such as DMSO or ethanol, are incompatible with the freeze-drying process, while polymers such as dextran may protect sensitive proteins only during freeze-thawing. A series of studies by Carpenter and co-workers (Carpenter & Crowe, 1988, 1989; Crowe *et al.*, 1990; Arakawa *et al.*, 1990a,b,c, 1991; Carpenter *et al.*, 1990, 1991, 1993; Prestrelski *et al.*, 1993a, 1993b) demonstrated that the stresses of freezing and dehydration placed on proteins during the lyophilisation process are essentially different, and that the requirements for protein protection during dehydration were much more specific than those for freezing alone. This observation was exemplified by the fact that many groups of compounds were shown to act as successful cryoprotectants for proteins, yet relatively few were found to be successful lyoprotectants (Arakawa *et al.*, 1993). In particular, proteins such as phospho-fructokinase (PFK) were observed to require a specific form of protection only afforded by disaccharides, in order for the loss of quaternary structure to be prevented during dehydration (Carpenter & Crowe, 1989). One suggestion for this specificity is the hypothesis that the spatial arrangement of the hydroxyl groups on the disaccharide molecules may have been important in the protective interaction, an observation made earlier for the mechanism of protein protection by co-solutes in aqueous solution (Gerlsma, 1968; Uedaira & Uedaira, 1980).

It appears that the disaccharides are among the most commonly used lyoprotectants found to date, as evidenced by numerous reports which detail their use as protective agents for alkaline phosphatase (Ford & Dawson, 1994; Ford & Allahiary, 1993), L-asparaginase (Hellman *et al.*, 1983; Adams & Irons, 1993), β-galactosidase (Izutsu *et al.*, 1991, 1993a,b, 1994a,b), catalase (Tanaka *et al.*, 1991), lactate dehydrogenase (Izutsu *et al.*, 1994b, 1995)

and numerous other enzymes (Pikal *et al.*, 1991; Prestrelski *et al.*, 1993a, 1993b; Carpenter *et al.*, 1993; Chang *et al.*, 1993; Constantino *et al.*, 1995; Vemuri *et al.*, 1994). However, it is apparent that direct comparisons are rarely made, either within groups of disaccharides, or of disaccharides with other groups of additives, in terms of relative abilities of such additives to afford freeze-dry protection to proteins. As such, articles describing isolated studies where stabilisation has been afforded to an individual protein during the lyophilisation process by an individual disaccharide can provide limited evidence as to the possible mechanism(s) of lyoprotection in general.

Evidence to support the hypothesis that protective interaction occurs through hydrogen bonding was provided by Carpenter & Crowe (1989). The fingerprint regions of the infra-red spectra of trehalose freeze-dried either alone or with proteins were compared, together with a spectrum for hydrated trehalose (Figure 4.2). It was noted that the presence of protein led to a pronounced decrease in absorbance in the entire region, major shifts in band position, and a loss of band splitting. The protein-induced spectral changes were titrated by freeze-drying the sugar with increasing amounts of either protein. Typical spectra for trehalose dried in the presence of either lysozyme or BSA were remarkably similar to that for hydrated trehalose, while all were very different to the spectrum of dried trehalose. Thus, it appeared that the proteins were serving the same role for dried trehalose as does water for the hydrated sugar.

The authors stated that it was implicit in the conclusion that proteins serve as water substitutes for dried carbohydrates, that the converse must also be true. This suggestion was then tested by the investigation of the influence of trehalose on the infra-red spectrum of lysozyme. Data from this study appeared to support the hypothesis and the authors also reported that similar results had been observed with lactose. It was concluded that an amorphous microstructure is necessary for successful protein protection, since it allows hydrogen bonding to occur between protein and protectant. However, it is also necessary to select excipients which are able to hydrogen bond with the protein in question, in order for protection to be offered to the native structure of the protein.

FIGURE 4.2: Infra-red spectra in the fingerprint region for trehalose alone (A), in the presence of 0.3g of lysozyme per gram of trehalose (B), in the presence of 0.1g of BSA per gram of trehalose (C) and for hydrated trehalose (D). The spectra for the dried samples have been corrected for differences in the amounts of trehalose present in the samples. The spectrum for the hydrated sugar has been normalised relative to the band at approximately 1000cm^{-1} (from Carpenter & Crowe, 1989).

4.1.3. The role of residual moisture in protein stability

Although the belief that exhaustive drying leads to greater dry-state stability probably holds true for many crystallising products, this is clearly not the case for many protein formulations. In its native (active) form, a protein has a hydration shell, where water molecules are associated with the protein molecule, thereby maintaining its native structure. The removal of this hydration shell by drying or freeze-drying is likely to perturb structure, damage native conformation and may increase the susceptibility of the protein to other components present in the formulation. Depending on the nature and concentration of the protein itself and other solutes present in the frozen matrix, protein-co-solute interactions could be either stabilising or damaging to the structure and biological activity of the protein. Early studies by Pauling (1945) and Green (1947) on the (warm) drying stability of proteins

led to the hypothesis that the so-called "highly polar residues" (HPR) within a protein could retain water during drying. Under such conditions, protein structure would be maintained, whereas 'overdrying' was shown to lead to loss of structure. Pauling classed five amino acid residues as being highly polar: aspartic acid, glutamic acid, serine, threonine and tyrosine, and the terminal carboxylic acid on each monomer chain.

More recently, the subject of the role of moisture in protein formulations has been reviewed in a number of articles (for example Hageman, 1988; Towns, 1995). Hsu *et.al.* (1991) present an excellent review of the role of moisture at the molecular level. The authors investigated the optimum levels of moisture in freeze-dried samples of two model proteins, tPA (tissue-type plasminogen activating factor) and met-hGH (methionyl human growth hormone). The amount of water adsorbed onto each protein was determined and approximated as a monolayer. The results were in good agreement with the theoretical values calculated from the number of strong polar groups in the molecules, without regard to the conformations of the proteins. The authors concluded that each protein may have a minimum moisture content which is necessary to shield the polar groups, and that 'overdrying' would lead to the exposure of these groups.

The effect of residual moisture content on the stability of tPA in lyophilised, excipient-free powder was also studied. Samples which were dried to a moisture level below that of the calculated monolayer exhibited opalescence on reconstitution, while those dried to either monolayer or multilayer water content tended to show a greater loss in biological stability upon storage under temperature stress conditions. The authors concluded from these studies that an optimum residual moisture content would be required, which balanced physical and biological stability. The authors' observations may also be applicable to other protein preparations.

It therefore seems evident that although the 'monolayer' level of moisture may be the optimal water content for the protection of the structure of a protein during processing, this amount of moisture in a sample could conversely reduce the storage stability of a protein over a period of time. However, it appears that drying a protein to an optimum moisture content is

not only difficult on a practical level, but in addition to this, it is not easy to be convinced that this technique could successfully be applied to the stabilisation of other proteins in different pharmaceutical formulations. Indeed, it evident from many other stabilisation studies, that almost any protein which has undergone investigation as a potential freeze-dried product has required stabilisation by cryoprotectant co-solutes in order to minimise freezing damage, and by the use of lyoprotectants in order that it may survive the stresses of drying. The necessary use of these excipients in protein formulations may also affect the level of moisture required for optimal stabilisation. As mentioned in section 3.1, amorphous regions present within a dried product will have a tendency to adsorb (or absorb) available moisture far more readily than crystalline regions within the same product. For example, moisture remaining in rubber or butyl lyophilisation vial stoppers following freeze-drying may migrate into the product. Since water exerts a plasticising effect on these regions within a product, it should always be taken into account that such regions are likely to have a far lower collapse temperature than the remainder of the product. Solutes within this portion of the 'dried' matrix may exhibit greater mobility through the product, which could lead to an increase in the possibility of degradative reactions.

Excipients which exhibit partially crystalline behaviour when lyophilised together with proteins often result in products which tend to be amorphous to a large extent. Equations which describe the thermal behaviour of such products during shelf storage are complex and contain many variables, while those which describe physical and chemical interactions between components within a low-moisture formulation are rare. Thermodynamic equations describing kinetics of degradation are better understood, although difficult to prove experimentally. The types of equation described above may be applicable to the stability of lyophilised protein formulations prepared and investigated in the present study, but in any case, preliminary studies on the degradation kinetics of the components used here would be necessary in order to determine the validity of such equations. The use of accelerated storage conditions was also an option available for the examination of the longer-term shelf stability of the lyophilised preparations detailed here. However, we are reminded by Cleland *et al.* (1993) that caution should be exercised in extrapolating stability data obtained under accelerated storage conditions to less extreme ones that are pharmaceutically relevant (37°C

for *in-vivo* and 4°C for refrigerated shelf storage), as such extrapolation is valid only if the kinetics clearly exhibit Arrhenius behaviour. Thus, once again, preliminary investigations would be necessary in order to assess the validity and suitability of using such equations in the current work. In addition, it is not only the physical stability of the dried product which needs to be considered, but also the numerous mechanisms by which a protein may degrade chemically, many of these reactions being catalysed by the presence of small amounts of moisture which lead to greater mobility of components in regions of the lyophilised cake.

Several reports detail the effects of residual water in protein products, which can lead to aggregation and loss of activity and structure for many sensitive proteins such as lysozyme (Fujita & Noda, 1979; Shah & Ludescher, 1993), L-asparaginase (Hellman *et al.*, 1983) and β-galactosidase (Yoshioka *et al.*, 1993) amongst others. In the case of cephalosporins, it was found that water absorbed by the amorphous form increased the number of decomposition products and greatly increased the net decomposition rate (Gatlin & DeLuca, 1980). It was also observed that poor long-term storage stability for many protein formulations in the food industry could often be attributed to a progressive degradation reaction in the presence of reducing sugars (Kramhöller *et al.*, 1993). This is known as the Maillard reaction, where the free carbonyl group in a reducing sugar can react with the amino-group of any number of peptide units which constitute a protein, causing a cleavage of peptide bonds. The early stages of this complex sequence of reactions have been shown to be catalysed by dry conditions; thus, lyophilised protein products are particularly susceptible to these reactions. As the later stages of the reaction often cause a feature known as 'browning' in such products, some workers have carried out investigations into the relationship between loss of protein activity and the extent of browning. A report by De Kok and Rosing (1994) showed that there was no direct link between the two parameters, and it was concluded that protein damage was caused in the early stages of the reaction, before browning was observed. Thus it is probable that the early stages of the Maillard reaction had caused a loss in activity of the protein studied in the report. This may also be applicable to other proteins, and serves to highlight the care which must be taken in the selection of excipients to be added to protein solutions prior to lyophilisation (some of which are added to increase the glass transition- and collapse- temperatures of the frozen matrix) in order to

enhance processing stability. For a comprehensive list of mechanisms of protein degradation, the reader is referred to Wang & Hanson (1989). It may be seen from this reference that long-term stability issues for proteins are both numerous and complex; as the remit of the present work was essentially to examine freeze-drying characteristics of model proteins, this report shall concentrate on the stability of the model proteins during and immediately following freeze-drying.

4.1.4 Aims of this Chapter

The overall objective of the work described in this chapter was to investigate the freeze-dry stability of two model enzymes, L-asparaginase and lactate dehydrogenase (LDH) and to examine the effects of a range of potentially stabilising additives (*e.g.* amorphous disaccharides such as trehalose) and destabilising additives (*e.g.* crystallising solutes such as mannitol) on enzyme structure and biological activity immediately following lyophilisation. As an extension to the studies described in the preceding chapter, the thermal characteristics of a number of protein solutions and lyophilised products would also be carried out using DSC and FDM, in order that processing behaviour for such formulations may be correlated with biological activity of the lyophilised product. In addition, it was anticipated that molecular modelling would also be employed in order to provide possible evidence of the nature of stabilising and destabilising interactions between protein and additive at the molecular level.

4.2 A Study of the Effects of Trehalose and Lactose on the Quaternary Structure of L-Asparaginase during Freeze-Drying

4.2.1 Background

L-Asparaginase is an enzyme used therapeutically in the treatment of acute lymphoblastic leukaemia. In the active form, the enzyme exists as a tetramer of molecular weight approximately 135 kDa. In aqueous solution, the native enzyme has been shown to be stable to chilling and freeze-thawing (Marlborough *et al.*, 1975). However, freeze-drying the active enzyme in the absence of excipients results in dissociation of the tetramer into four identical inactive monomers (each of molecular weight approximately 34 kDa). It has been reported that the addition of a range of compounds to solutions of L-asparaginase prior to freeze-drying provided stabilisation to the quaternary structure of the enzyme during the process (Adams & Irons, 1993). The extensive use of lactose as a bulking agent in tabletting and in freeze-dried formulations means that it is probably the most commonly used excipient in drug formulation. Trehalose, however, whilst not currently widely-used in pharmaceutical formulations, has been shown to be one of the most successful protectants in plant dehydration and anhydrobiosis (Crowe *et al.*, 1990). In addition, it is relatively unreactive and a non-reducing sugar, and is thereby not capable of inducing Maillard reactions, which makes this the additive of choice for proteins, live micro-organisms and bioproducts (Levine & Slade, 1988; Colaco *et al.*, 1994). The aim of this study was to lyophilise solutions containing L-asparaginase with trehalose or lactose and to examine the quaternary structure of the resulting product on rehydration, using FPLC.

4.2.2 Materials and methods

4.2.2.1 Materials

L-Asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 24.3 mg/ml and activity 15,360 iu/ml (1iu is equivalent to the amount of enzyme required to reduce one millimole of the substrate L-asparagine *per* minute at 37°C), by CAMR, Porton Down, Wiltshire, UK. All excipients were purchased from Sigma Chemical Co., Poole, Dorset, UK.

4.2.2.2 Freeze-Drying of Solutions

Aliquots (1.00ml) of diluted aqueous solutions of L-asparaginase (1.45mg/ml) in the presence or absence of lactose or trehalose (0.25 to 1.00mg/ml), were dispensed into glass lyophilisation vials and frozen at -70°C for 60 minutes. After freezing, the samples were transferred to an Edwards Modulyo freeze drier (see section 2.1) and dried at a chamber pressure of ~0.2 Torr for 4 hours at a shelf temperature of 30°C, followed by a further 12 hours at the same chamber pressure with no shelf heating applied. Dried products were sealed under vacuum and stored at 4°C.

4.2.2.3 FPLC analysis of quaternary structure of L-asparaginase

Lyophilised products were reconstituted to original volume (1.00ml per vial) using Sørensen's Glycine II buffer (100mM glycine + 100mM NaCl, adjusted to pH10.0 using 10M NaOH), which had previously been shown to stabilise monomer and tetramer in the reconstituted mixture (Marlborough *et al.*, 1975) and the developed FPLC running protocol followed, as detailed in section 2.7.

4.2.3 Results & Discussion

Calculation of the required amounts of trehalose and lactose to be added to the solution of L-asparaginase to give desired levels of protection to the tetramer were based on the studies by Pauling (1945), Green (1947) and Hsu *et al*, (1991) (see section 4.1.3). The monomeric sequence of *Erwinia chrysanthemi* L-asparaginase (E.C.3.5.1.1) was obtained using the Genbank Nucleic Acid database, which was accessed *via* Daresbury Seqnet. The sequence obtained (Figure 4.3) was calculated to contain 95 highly polar residues, as categorised by Pauling (1945). These consisted of 21 aspartic acid (D) units, 15 glutamic acid (E) units, 20 serine (S) units, 26 threonine (T) units, 12 tyrosine (Y) units and one terminal carboxylic acid group.

Since the native tetrameric enzyme consists of four identical monomer sub-units, the tetramer would then contain (4x95=) 380 of the highly polar units described above. Without regard to the tertiary structure (folding) of the protein, and on the arbitrary basis that one molecule of water were required for each polar residue on the protein, 380 molecules of water were

predicted to be associated with each tetramer molecule. Hsu *et al.* (1991) had shown experimentally that optimum levels of residual moisture present in lyophilised met-hGH and tPA products immediately following the freeze-drying process reflected the theoretical values which had been calculated on this basis (*i.e.* without regard to folding). As other workers had previously reported that saccharide additives which stabilise proteins during the lyophilisation process act *via* a mechanism of hydrogen bonding, and that they replace water in the dried matrix (see Arakawa *et al.* (1993), for review), it was decided that the concentrations of trehalose added in the present study should reflect the levels of water retention calculated to be required during the process.

(H_2N)-MERWFKSLFVLVLFFVFTASAADKLPNIVILATGGTIAGSAA
TGTQTTGYKAGALGVDTLINAVPEVKKLANVKGEQFSNMASENM
TGDVVLKLSQRVNELLARDDVDGVVITHGTDTVEESAYFLHLTV
KSDKPVVFVAAMRPATAISADGPMNLLEAVRVAGDKQSRGRGVM
VVLNDRIGSARYITKTNASTLDTFKANEEGYLGVIIGNRIYYQNRI
DKLHTTRSVFDVRGLTSLPKVDILYGYQDDPEYLYDAAIQHGVKG
IVYAGMGAGSVSVRGIAGMRKAMEKGVVVIRSTRTGNGIVPPDEE
LPGLVSDSLNPAHARILLMLALTRTSDPKVIQEYFHTY-*(CO_2H)*

FIGURE 4.3: *Amino-acid sequence of monomeric* L-asparaginase *from* Erwinia chrysanthemi *(highly polar residues in bold type). Key to abbreviations for residues is given in Appendix IV.*

FPLC provided adequate and reliable separation of tetramer and monomer components in each mixture, thus enabling a reasonable estimate to be made of the relative proportion of each component present. The relative retention ratios of the tetramer and monomer were calculated to be 1.75 (±0.03) and 1.95 (±0.025) for the tetramer and monomer, respectively, compared to a void volume marker of blue dextran (MW~2,000,000). The examination of monomer peak height indicates that even at the lowest concentration used (0.25g *per* gram of L-asparaginase), trehalose was apparently able to provide 100% protection to the native tetrameric form of the enzyme, since no evidence of a monomer peak was apparent on the chart. However, the major limitation with the method employed here is that the data

produced could be used only as a 'semi-quantitative' measure of the proportions of the monomeric and tetrameric components present in the rehydrated solution, since the molar absorptivities (extinction coefficients) for the monomer and tetramer could not be determined individually.

From the FPLC data obtained, it appeared that a substantially smaller amount of trehalose was required to provide total stabilisation to the tetrameric form of the L-asparaginase present in the initial solution than was intially estimated. This may perhaps be explained by considering the structure of the protein. In the folded (native) state, conceptually most of the polar residues of the protein may remain in close proximity to the molecules of water (which may or may not be those of the hydration shell of the protein) which are essential in the maintenance of that state, since most of these residues are likely to be on the outside of the protein in the first instance. However, there may still be some residues which become removed from the outside of the structure due to the folding process itself, which may prevent such residues from interacting with the water molecules present. Thus, not all of the polar residues which were initially calculated as being present on the L-asparaginase molecule may have been capable of interacting with the water molecules even in aqueous solution. In addition to this, it may be incorrect to assume that trehalose is able (or, indeed, required) to substitute for, or share, each molecule of water which may be interacting with the polar residues of the native protein in solution or as the solution freezes. Indeed, Tanaka *et al.* (1991) found, in a study of the effects of saccharide and dextran additives on the freeze-dry stability of catalase, that glucose, maltose and maltotriose showed a similar protective effect compared on the basis of their weights, and hypothesised that a glucoside group of those saccharides occupied about 5 hydrogen-bonding sites on a catalase molecule at the maximum protection. The authors also concluded that saccharides with longer glucoside chains had less protection effect, depending on their molecular weights, and judged from the data produced in the study that saccharides were bound to catalase as a monomolecular layer and that this layer protected catalase from being denatured instead of the hydration monolayer. The data from the present study suggest that each saccharide molecule may have been able to interact with several HPR on the protein molecule, but further studies would be necessary to confirm this.

4.2.5 Conclusions

Successful separation of tetrameric and monomeric forms of L-asparaginase was achieved by the FPLC method developed for this purpose. The results from FPLC showed that much lower levels of saccharide were required compared to the calculated concentrations in order to provide apparently full freeze-dry protection to the tetrameric form of L-asparaginase. However, it was concluded that further studies would be required, employing a wider range of analytical techniques, in order that a more detailed knowledge of the mechanism of action of the disaccharides tested could be gained at the molecular level. It was later found that the monomer sequence for L-asparaginase used in the present study differed from that used in a later study described in the following section (4.3) and it was believed that since the sequence used in the later study was from a more recent source, this was likely to be a more reliable structure. Therefore, it was concluded that the assumptions made from the sequence used in the present study may have been incorrect. However, the experimental data produced in the present study were still believed to be reliable and the theoretical HPR calculations performed in the present study were not far removed from those calculated using the more recent structure. Thus, the current study was seen to be a legitimate precursor to the study described in the following section.

4.3 EXAMINATION OF THE LYOPROTECTIVE EFFECTS OF A RANGE OF SACCHARIDE ADDITIVES ON THE STRUCTURE AND ENZYME ACTIVITY OF L-ASPARAGINASE DURING FREEZE-DRYING

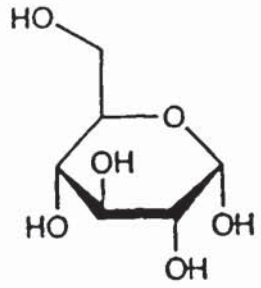
4.3.1 Background

The study detailed in the previous section illustrated the stabilising effects of the disaccharides, lactose and trehalose, on the quaternary structure of the enzyme L-asparaginase during lyophilisation. The subsequent development of a successful method of analysis for the biological activity of L-asparaginase (see section 2.9) enabled the stability of the enzyme to be monitored on the basis of activity as well as in terms of quaternary structure as detailed in the previous study.

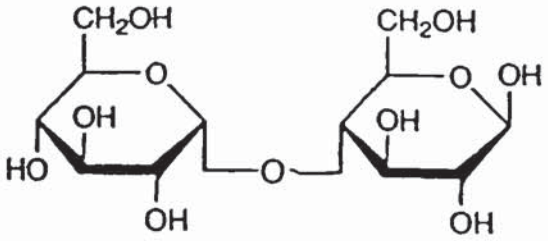
One of the purposes of this study was to examine the stability of the enzyme L-asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) when freeze-dried with a range of potentially protective additives. To test the hypothesis that the spatial arrangement of hydroxyl groups around saccharide molecules may be significant in the mechanism of protective interaction, disaccharides differing in hydroxyl group positioning (axial or equatorial) were used as model protectants in this study, as shown in Table 4.1, for which the chemical structures are shown in Figure 4.4.

Additive	Description	Typical Frozen Matrix
Trehalose	Disaccharide	Amorphous
Lactose	Disaccharide*	Amorphous
Maltose	Disaccharide*	Amorphous
Sucrose	Disaccharide	Amorphous
Glucose	Monosaccharide*	Amorphous
Mannitol	Monosaccharide Alcohol	Crystalline

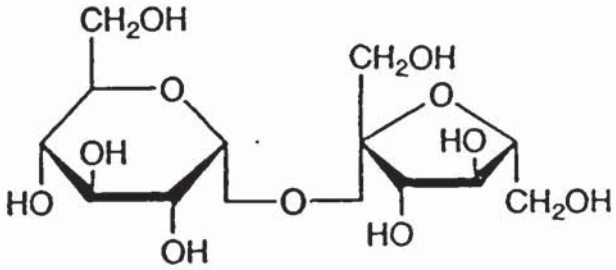
TABLE 4.1: *Classification and typical freezing behaviour of additives used in the present study (*denotes reducing sugar)*



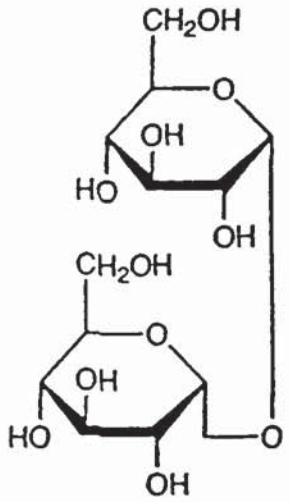
α-D-Glucose



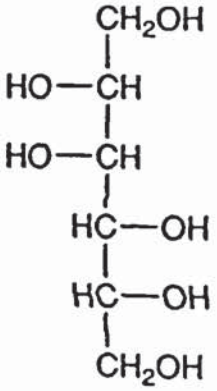
Maltose



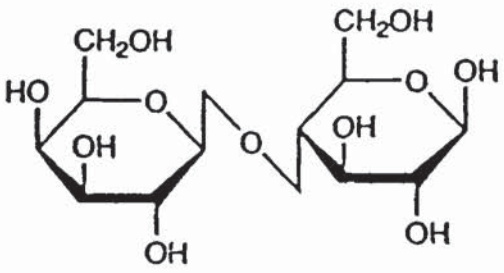
Sucrose



Trehalose



Mannitol



Lactose

FIGURE 4.4: Molecular structures of the excipients used in this study

Mannitol was included in the experimental series, although this sugar alcohol had been observed to provide little protection to tetrameric L-asparaginase during freeze-drying when used at 2.0% (w/w), since this excipient typically crystallises during freezing (Hellman *et al.*, 1983). In addition, Izutsu *et al.* (1993a, 1994c) reported that the ability of mannitol and other crystallising excipients to afford protection to a number of enzymes during freeze-drying was observed to decrease as the degree of crystallinity increased at elevated concentrations, although the authors used concentrations of mannitol which were significantly higher than those of the proteins studied.

4.3.2 Materials and Methods

4.3.2.1 Materials

L-Asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 24.3 mg/ml and activity 15,360 iu/ml, by CAMR, Porton Down, Wiltshire, UK. All excipients were purchased from Sigma Chemical Co., Poole, Dorset, UK.

4.3.2.2 Freeze-drying

Aliquots (1.00ml) of diluted aqueous solutions of L-asparaginase (1.45mg/ml) containing excipients at a range of concentrations (saccharides 0.05-1.00mg/ml; PEG 0.5-10mg/ml) were dispensed into glass lyophilisation vials and frozen at -70°C for 60 minutes. After freezing, the samples were transferred to an Edwards Modulyo freeze drier (see section 2.1) and dried at a chamber pressure of ~0.2 Torr for 4 hours at shelf temperature ~30°C, followed by a further 12 hours at the same chamber pressure with no shelf heating applied. Dried products were sealed under vacuum and stored at 4°C. For the enzyme activity assay and for FPLC analysis, dried samples were reconstituted to original volume with Sørensen's Glycine II buffer (0.1M, pH 10.0), a buffer shown to stabilise any inactive monomer present (Marlborough *et al.*, 1975).

4.3.2.3 Moisture Content Determination

Moisture contents of a representative batch of lyophilised cakes were determined by thermogravimetric analysis (TGA), as detailed in section 2.6. Samples of freeze-dried product (2-3mg) were heated to 130°C at a constant rate of 10°C/min, in a Perkin-Elmer

TGS-2 thermogravimetric analyser, linked to a Perkin-Elmer TADS 3600 thermal analysis data station with related software. Sample weight was plotted as a function of temperature, and moisture content determined using system software.

4.3.2.4 Measurement of retained enzyme activity of L-Asparaginase

The biological activity of L-asparaginase was determined by the rate of turnover of the substrate L-aspartyl-β-hydroxamic acid (AHA), using a method based on that first reported by Frohwein *et al.* (1971), as described in section 2.9. Calibration standards were prepared by dilution of original L-asparaginase/additive solutions and the retained activity of L-asparaginase in reconstituted samples expressed as a percentage of original activity.

4.3.2.5 FPLC method of separation of monomer and tetramer

Reconstituted solutions were introduced into two Sepharose HR 10/30 columns (Pharmacia, Sweden) connected in series, as described in section 2.7. Sørensen's Glycine II buffer (0.1M, pH 10.0) was used as eluent (flow rate 0.40ml/min). Column eluate was monitored by a UV/visible flow-through detector at wavelength 280nm, and absorbance plotted against time.

4.3.2.6 FT-IR Spectrophotometric Analysis of dried products

FT-IR spectrophotometry was carried out on KBr discs of freeze-dried samples, using a Mattson Fourier-Transform Infrared spectrophotometer (Mattson Instruments, UK) and related software, as described in section 2.16.

4.3.2.7 Molecular modelling of L-asparaginase

Molecular modelling of *Erwinia* L-asparaginase was carried out by Dr Melanie Duffield at CAMR, Porton Down, following the publication of the crystal structure of the protein by Miller *et al.* (1993).

4.3.3 Results and Discussion

Following drying, sample moisture contents were found to be typically between 3% and 5% (w/w), as determined by TGA. Water can plasticise amorphous systems, lowering the glass transition temperature and the collapse temperature of a dried product (Levine & Slade, 1987). Since high levels of residual moisture in freeze-dried formulations can lead to reduced

shelf stability, the results reported here consequently describe the protective effects of the excipients assessed on the basis of enzyme activity and quaternary protein structure immediately following freeze-drying. It was found in a previous study (Carpenter & Crowe, 1989) that the addition of increasing amounts of trehalose to a protein did not necessarily cause an increase in the residual levels of moisture following lyophilisation and thus the moisture content of the lyophilised product did not correlate with the data describing the extent of retained biological activity of the protein after drying. It was this observation which in part led to the development of the water-replacement hypothesis.

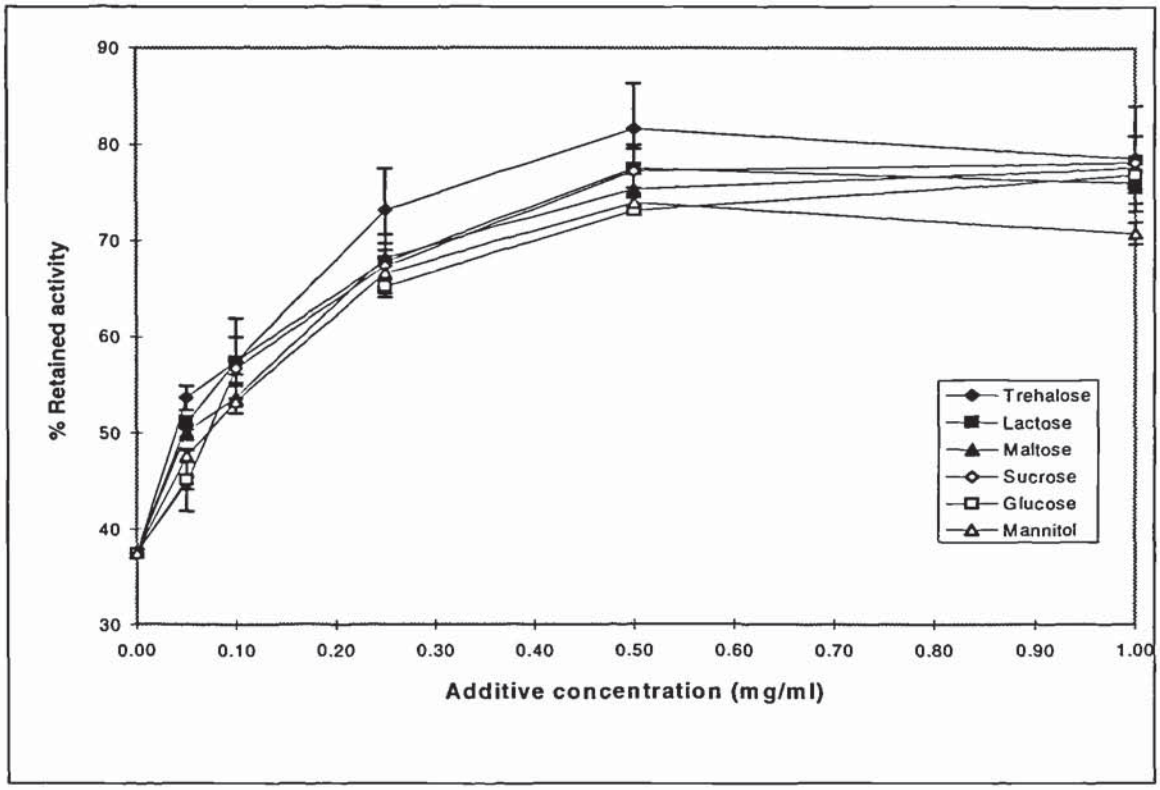


FIGURE 4.5: *Effect of saccharides on the retention of enzyme activity of L-asparaginase immediately following freeze-drying, as determined using the AHA assay developed from Frohwein et al. (1971) (n=3, mean ±s.d).*

The data from the activity assay showed that each disaccharide appeared to confer similar levels of freeze-dry stability on L-asparaginase, dependent on the concentration of sugar used (Figure 4.5). Glucose and mannitol appeared to be equally as effective as the disaccharides at all concentrations used, in contrast to stability data reported for PFK (Carpenter & Crowe, 1989), which was stabilised only by disaccharides. The additives used here were compared on a weight/weight basis for practical simplicity and as a formulation exercise; however, for

the purposes of examination of the potential significance of hydroxyl groups on the additive molecules, the additives should be compared on the basis of hydroxyl group frequency. Interestingly, the weight/weight comparison of the additives also corresponds to their comparison on the basis of hydroxyl group frequency, since disaccharides and monosaccharides contain a similar number of hydroxyl groups *per* unit weight of additive (monosaccharides contain 4 groups (mannitol contains 5) and have a molecular weight of 180.3, while disaccharides contain 8 groups and have a molecular weight of 342.3). Thus, the comparison of the additives on a w/w basis here was also seen to be legitimate for the purposes of studying protein-additive interactions at the molecular level with respect to the hydroxyl groups.

FPLC analysis of quaternary protein structure gave retention ratios (V_e/V_0) for the monomer and tetramer of 1.94 (±0.02) and 1.76 (±0.02), respectively. The data from the enzyme activity assay were reflected by the FPLC results, which showed that for any given additive concentration, the proportion of tetramer retained after dehydration was similar, irrespective of the additive used (Figure 4.6) and that higher concentrations of additive led to increased levels of tetramer retention immediately following freeze-drying. Appendix VIII clearly shows the similarity between the different additives in terms of ability to preserve the quaternary structure of L-asparaginase.

FT-IR spectra provided evidence of possible hydrogen bonding between L-asparaginase and trehalose (Figure 4.7), an observation which has been previously reported for trehalose with other proteins (Carpenter & Crowe, 1989). For the enzyme freeze-dried alone (A), where little intermolecular hydrogen bonding would be expected, the amide (II) band showed a maximum at 1538cm^{-1}. The shifting of this band in spectra (B) and (C) to around 1548cm^{-1} suggests that levels of hydrogen bonding between L-asparaginase and trehalose when freeze-dried together (B) were similar to those present in the hydrated L-asparaginase itself (C). However, from the data presented here, which parallel those for lysozyme and trehalose (Carpenter & Crowe, 1988), it is difficult to determine whether protective effects are due to the presence of residual water in the dried product, or to a direct interaction between protein and additive.

The water replacement hypothesis suggests that a lyoprotectant mimicks the water in the hydration shell of the protein molecule (Arakawa *et al.*, 1993). Since water molecules form hydrogen bonds, it is possible that the interactions between proteins and co-solute molecules occur *via* hydrogen bonding, and that successful lyoprotectants should be chosen on this basis. If this were the sole criterion which needed to be fulfilled, then it is difficult to understand why other compounds capable of forming hydrogen bonds with amino residues of proteins (including monosaccharides) were not equally effective lyoprotectants in the case of PFK. It seems possible that the water-replacement hypothesis may help partially explain lyoprotectant activity, but that simple mimicking of water in the hydration shell may be insufficient for successful stabilisation of a protein during the removal of the hydration shell itself. Even when a compound is able to form hydrogen bonds with other molecules, it may also have to fulfil further criteria in order to be able to confer stability on a protein during lyophilisation. Such additional criteria could include steric factors and surface charge profiles, and thereby may differ for each protein studied. It is also possible that cryoprotectant and lyoprotectant additives do not function by mimicking water molecules and their interactions with proteins, but operate by a simpler "water-sharing" mechanism. In such cases, the protectant molecule might act by holding water molecules (possibly those initially present in the hydration shell) close to the protein in such a way as to render the water molecules difficult to remove under normal freeze-drying conditions. Steric criteria and other factors particular to each protein may also have to be satisfied.

Since quaternary, tertiary, secondary and primary structure varies markedly from protein to protein, it is possible that different protective criteria need to be satisfied when stabilising different proteins. Consequently, when attempting to develop general guidelines for screening potential protective compounds, it is possible that there are more examples which are exceptions to, than those conforming to, a general pattern. Nevertheless, there are some factors which are applicable to protein stability during freeze-drying. In particular, the role of residual water, its interaction with the protein molecule and retention in the dry matrix are all crucial to the success of the freeze-drying process (Franks, 1982). It appears from the present data that such interactions in the presence of additives investigated were stabilising, when compared to those in the absence of additive.

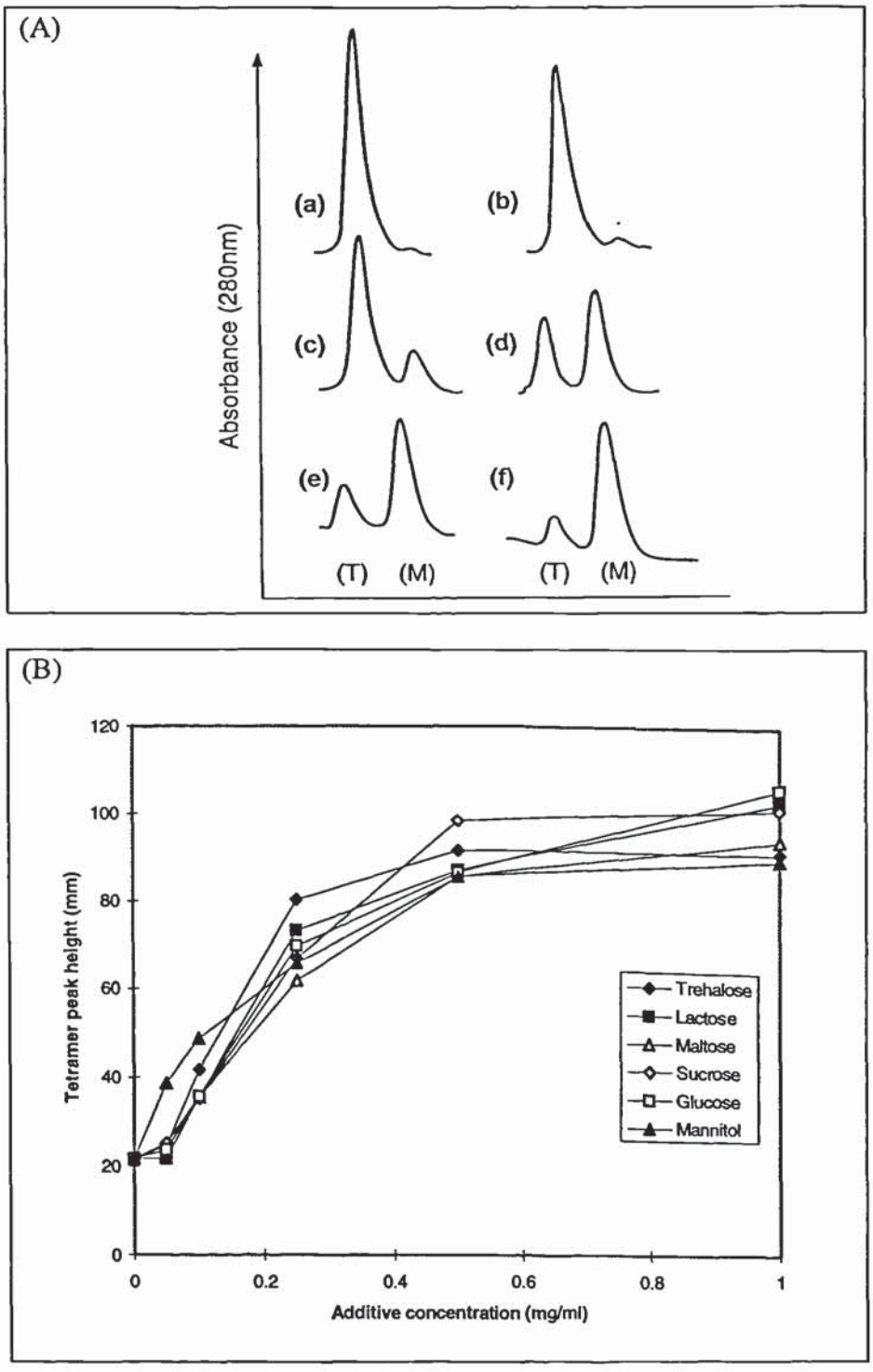


FIGURE 4.6: FPLC profiles (A) for reconstituted solutions of L-asparaginase (1.45mg/ml) which had been lyophilised in the presence of lactose at concentrations of: (a) 1.0, (b) 0.50, (c) 0.25, (d) 0.10 and (e) 0.05mg/ml and in the absence of additive (f). Profiles provided a semi-quantitative assessment of the relative proportions of (active) tetramer (T) and (inactive) monomer (M) present in reconstituted solutions. A plot of tetramer peak height against additive concentration (B) demonstrates the similarity of the protective abilities of the saccharides used in this study.

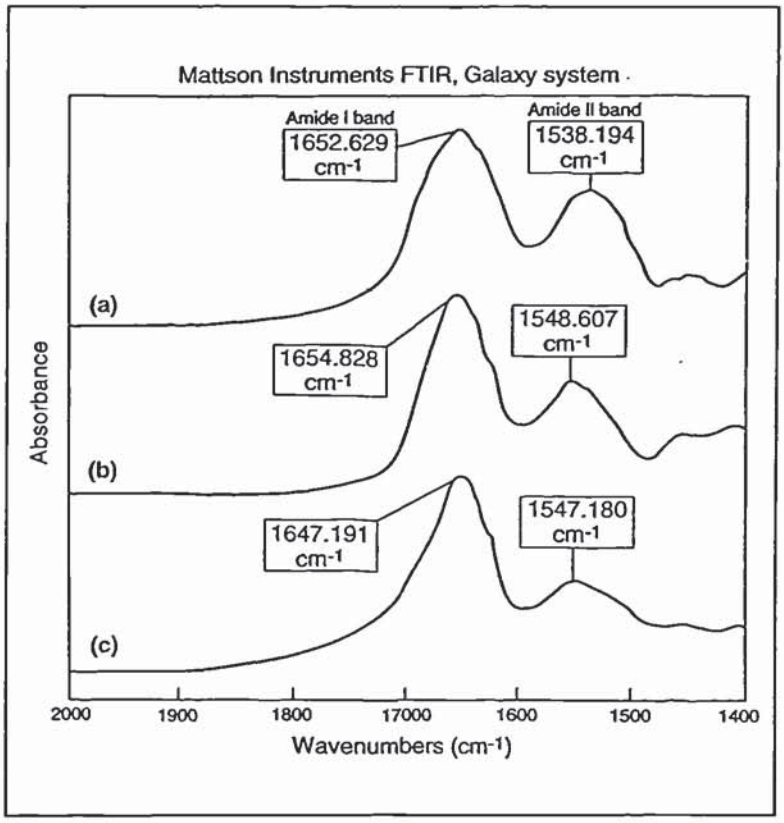


FIGURE 4.7: Amide band region for dried samples, as determined by FT-IR spectroscopy. Spectra shown are of L-asparaginase freeze-dried alone (a), freeze-dried in the presence of trehalose (1.0mg/ml) (b) and hydrated L-asparaginase (c). All spectra have been normalised with respect to the amide I bands.

4.3.3.1 Molecular modelling of L-asparaginase

The three-dimensional structure of L-asparaginase was carried out by Dr Melanie Duffield at CAMR, using molecular modelling software. The structures of the L-asparaginase monomer and tetramer, together with a prediction of exposed HPR, are shown in Appendices V to VII. Appendix V shows the L-asparaginase tetramer as a ribbon, with each monomer subunit highlighted in a different colour. Appendix VI shows the L-asparaginase tetramer, with one monomer sub-unit highlighted in pink and the HPR exposed in unassociated form depicted in green. The remaining three sub-units are depicted as single strands. Appendix VII shows the molecule in a similar manner to Appendix VI, but highlights the HPR exposed in the associated (tetramer) form, as opposed to the unassociated form.

The calculated distribution of HPR in the monomeric and tetrameric forms are given in Table 4.2 below.

RESIDUE	**Total number of HPR exposed in unassociated form (*per* monomer)**	**Number of HPR remaining exposed in associated form (*per* monomer)**	**Number of HPR becoming hidden upon association (*per* monomer)**
Aspartic acid (D)	18	11	7
Glutamic acid (E)	14	8	6
Serine (S)	13	9	4
Threonine (T)	20	11	9
Tyrosine (Y)	9	3	6
∴Total *per* monomer	74	42	32
∴Total *per* tetramer	296	168	128

TABLE 4.2: *Pattern of HPR distribution in* Erwinia *L-asparaginase*

On the arbitrary basis that one molecule of additive is required for each HPR exposed on the surface of the L-asparaginase monomer, it was calculated that for the concentration of protein employed in the present study (1.45mg/ml), the concentrations of additive required to interact with all exposed HPR were 1.096mg/ml disaccharide (MW 342.3) and 0.577mg/ml monosaccharide (MW 180.2). Similarly, on the assumption that only HPR exposed on the surface of the tetramer would protectively interact with additive, it was calculated that under the conditions of the present study, 0.622mg/ml disaccharide and 0.328mg/ml monosaccharide should be required to achieve full interaction. In either case, the range of concentrations of additive found to be required experimentally in the present study for the lyoprotection of L-asparaginase suggested that the amounts of additive required to provide full protection to the protein during drying would be in reasonable agreement with these theoretical concentrations. Any discrepancy between theoretical and experimental values may be attributed to a number of factors. Firstly, the HPR defined above may not be the only residues which require the presence of water during drying, since the basis on which residues

were defined as "highly polar" was somewhat arbitrary. However, it is also possible that not all of the HPR defined above may require the presence of water during drying. This might in turn be dependent upon further factors such as neighbouring residues to the HPR, or additives present in a protein solution, particularly where the protein has been isolated from an organism and trace levels of compounds such as glucose may be present. Secondly, it is possible that some of the residues at the monomer-monomer interface (which may or may not fall into the category of HPR as defined above) may have also required the presence of water in order to maintain the quaternary structure of the protein. The elucidation of the precise interactions at the molecular level might perhaps be facilitated by the use of more sensitive analytical techniques, such as microcalorimetry.

4.3.4 Conclusions

From the experimental data produced in this study, it may be concluded that each of the additives investigated here appeared to provide similar levels of freeze-drying protection to L-asparaginase at all (additive) concentrations tested. This similarity also holds true when the additives are assessed on the basis of the number of free hydroxyl groups *per* mole of additive, suggesting that protective interaction between L-asparaginase and additive in this instance might imply a direct involvement of these hydroxyl groups; however, the relative availabilities of the various hydroxyl groups on each molecule for such interaction have not been quantified, and so further studies would be necessary to test this hypothesis. It was interesting to note that the amounts of additive required to provide the maximum levels of protein preservation observed in the current study (around 75%) compared well with the theoretical amounts of additive calculated to be required using the molecular modelling approach together with the HPR theory. For further studies, sensitive analytical techniques such as solution microcalorimetry may be employed to provide quantitative real-time data for thermal events which occur on the titration of increasing amounts of excipient with protein and may thus allow the fuller elucidation of protein-co-solute interactions at the molecular level.

4.4 Examination of the Effects of Poly(ethylene glycol) (PEG) on the Freeze-dry Stability of L-Asparaginase

4.4.1 Background

The previous study examined the effects of excipients on the freeze-dry stability of the model protein, L-asparaginase. The aim of the present study was to examine the effect of using poly(ethylene glycol) (PEG) as an excipient on the stability of L-asparaginase during lyophilisation. PEG has been one of the most successful cryoprotectants found to date, displaying the ability to stabilise numerous proteins during freeze-thawing and proving more successful than saccharides such as lactose or sucrose (Arakawa *et al.*, 1993). The reason for its success as a cryoprotectant is believed to be its willingness to remain preferentially excluded from the surface of a protein at low temperatures in concordance with the "water replacement hypothesis" (Carpenter *et al.*, 1994).

4.4.2. Materials & Methods

4.4.2.1 Materials

L-Asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 24.3 $mg.ml^{-1}$ and activity 15,360 $iu.ml^{-1}$, by CAMR, Porton Down, Wiltshire, UK. PEG (number average of molecular weight fraction 10,000 Daltons) was purchased from Sigma Chemical Co., Poole, Dorset, UK.

4.4.2.2 Freeze-drying microscopy of PEG + L-asparaginase solution

Freeze-drying microscopy was performed on a solution containing $1.45mg.ml^{-1}$ L-asparaginase and $10mg.ml^{-1}$ PEG, following the procedure described in section 2.4.

4.4.2.3 Freeze-drying

Aliquots (1.00ml) of diluted aqueous solutions of L-asparaginase (1.45mg/ml or 12.2mg/ml) and PEG (0.5-10mg/ml) were dispensed into glass lyophilisation vials and freeze-dried under the conditions described in section 4.3.2.2.

4.4.2.4 Moisture content determination

Moisture contents of a representative batch of lyophilised cakes were determined by thermogravimetric analysis (TGA), as detailed in section 2.6., under the conditions described in section 4.3.2.3.

4.4.2.5 Measurement of retained enzyme activity of L-asparaginase

The biological activity of L-asparaginase was determined by the rate of turnover of the substrate L-aspartyl-β-hydroxamic acid (AHA), using a method based on that first reported by Frohwein *et al.* (1971), as described in section 2.9. Calibration standards were prepared by dilution of original L-asparaginase/additive solutions (pre-freeze-drying) and retained activity of L-asparaginase in reconstituted samples expressed as a percentage of original activity. Preliminary tests indicated that the presence of PEG in the rehydrated samples had a minor influence on the rate of turnover of the substrate, and so a set of calibration standards, from 100% to 10% were prepared for each given concentration of PEG used in initial solutions (prior to lyophilisation). Each individual set of standards were analysed for activity in the same microassay plates. The correlation coefficients (r^2) for the calibration plots were between 0.991 and 0.997 for products containing the lower concentration of protein (1.45mg/ml) and between 0.981 and 0.986 for those containing the higher concentration (12.2mg/ml).

4.4.2.6 Thermal analysis of lyophilised products by DSC

Thermal analysis of samples (5-10mg) of the dried products was carried out as described in section 2.5 of this report.

4.4.3 Results & Discussion

Analysis of the solution containing 1.45mg.ml^{-1} L-asparaginase and 10mg.ml^{-1} PEG (10kDa) by FDM demonstrated that the mass dried with collapse of structure until cooled to below approximately -34°C, after which it continued to dry with retention of structure until warmed to approximately -15°C. Thus, the collapse temperature was taken to be approximately -34°C. For many protein systems, especially dilute solutions, T_g' cannot be determined by DSC (Chang & Randall, 1992; Pikal, 1994; Shalaev *et al.*, 1996). Therefore, in the present

study, FDM was employed to determine the collapse temperature of the frozen mass, which is an equally useful parameter as T_g' for the selection of processing conditions.

Sampling and analysis of a representative batch of lyophilised product showed that, in parallel with the previous study, moisture contents were typically between 3% and 5% (w/w). Data from the enzyme activity assay indicated that PEG was able to stabilise the active tetrameric form of L-asparaginase (Figure 4.8), although higher concentrations of PEG were required to achieve similar levels of protection compared to the disaccharides studied in section 4.3. However, when assessed on a molar basis, a far lower concentration of PEG was required in order to achieve similar levels of protection as the saccharides tested previously. Indeed, the addition of PEG at just 1mg/ml to the solution of L-asparaginase prior to lyophilisation resulted in activity levels of greater than 60% retained in the freeze-dried product, as shown in Figure 4.8. Calculation suggests that four or five times the mass of PEG was necessary compared to that of saccharide, in order to achieve such a level of retained enzyme activity.

One suggested mechanism by which PEG is believed to stabilise proteins in solution and in the frozen state is not by hydrogen bonding, but rather by a mechanism known as preferential exclusion, as in the case of saccharide additives (see section 4.3). According to Lee & Lee (1987), preferential exclusion is likely to be due to the repulsion of PEG by charges on the surface of a protein, leading to an exclusion of PEG from the protein domain, the creation of an exclusion zone around the immediate surface of the protein which contains only solvent molecules, and a condensation of the protein molecules in solution, as described previously by Arakawa & Timasheff (1985). A later article by Arakawa *et al.* (1990a) provides the reader with a diagrammatic representation of this mechanism. Carpenter *et al.* (1994) explain the relative success of PEG as a cryoprotectant (*i.e.* during freeze-thawing only) compared to saccharides in terms of relative preference for exclusion from, as opposed to binding to, protein domains.

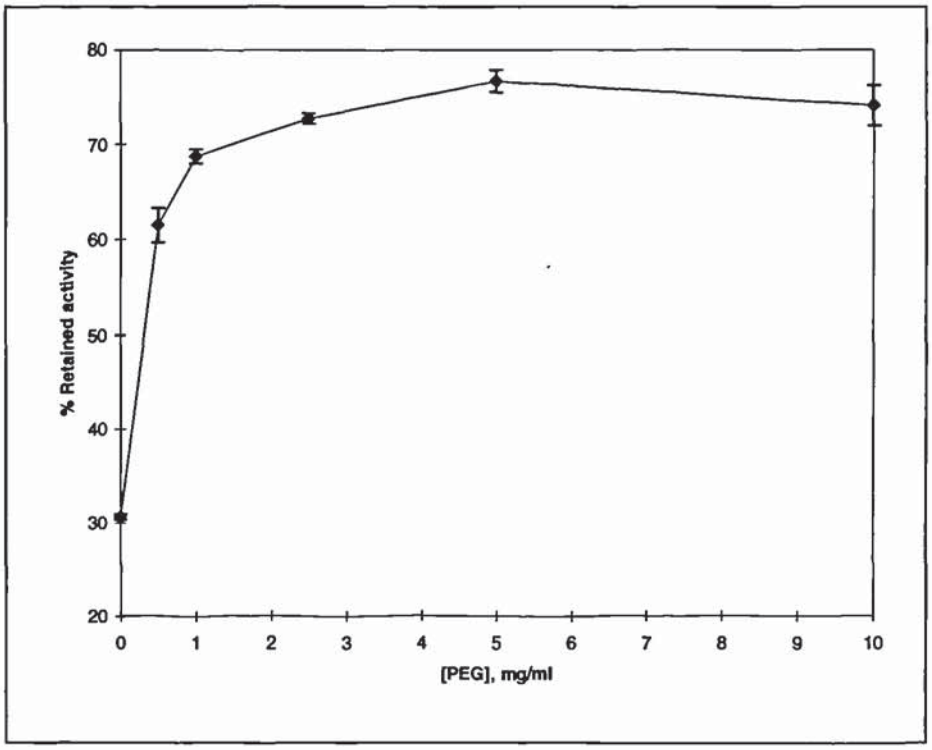


FIGURE 4.8: Effect of PEG concentration on the retention of enzyme activity of L-asparaginase immediately following freeze-drying, as determined using the AHA assay developed from Frohwein et al. (1971).

The authors state that for the model protein chymotrypsinogen, the increase in chemical potential of the protein in the presence of either of two different molecular weights of PEG (400 or 6000) was greater than that noted in the presence of sucrose, even though PEG was excluded to a lesser degree on a molar basis. When the authors compared the two PEG molecules, it was found that the larger the PEG, the less it was excluded on a molar basis, but the more it increased the chemical potential of the protein. Indeed, the values for the chemical potential of chymotrypsinogen (in kcal. (mole of protein)$^{-1}$. (mole of solute)$^{-1}$) were reported to be: 5.7 in the presence of sucrose, 16.6 in the presence of PEG 400 and 297.6 in the presence of PEG 6000, thus demonstrating that PEG 6000 was more than fifty times as potent a cryoprotectant than sucrose on a molar basis. However, the 'self-interaction' parameter of PEG was also reported to increase with molecular weight, thus leading to increasingly non-ideal conditions. Interestingly, at higher temperatures, sugars have been shown to increase the melting temperature of proteins, whereas PEG causes T_m to decrease (Lee & Lee, 1987). Since the degree of stabilisation correlates directly with the increase in

protein chemical potential in the presence of a solute, it is perhaps not surprising that PEG was found to be more effective than sugars in stabilising proteins during freezing.

Carpenter *et al.* (1994) concluded that the cryoprotection of proteins by PEG is due to preferential exclusion of PEG from the surface of the protein, which is in turn due to steric hindrance of the interaction of PEG with the protein. It may be possible that the authors' conclusions may be applicable to protein stabilisation during lyophilisation, which must occur during the initial stages of the process, *i.e.* during freezing. It should be remembered that the mathematical equations describing preferential exclusion become invalid upon removal of the solvent from the system during the drying process (Arakawa *et al.*, 1993) and thus caution should be exercised in extrapolating conclusions from simple protein cryoprotection studies to the more specific area of protein lyoprotection.

PEG is also known to be a hydrogen bond acceptor and thus it is possible that some element of hydrogen bonding had occurred between L-asparaginase and PEG in the present study. It has been suggested that hydrogen bonding is a necessary element of the protein protection process (Carpenter & Crowe, 1989; see section 4.1), although the data produced using FT-IR by the authors to illustrate this phenomenon may not have provided conclusive evidence of direct hydrogen bonding between protein and additive (see section 4.1). Further analyses would be necessary to evaluate the extent to which this may occur and the point in the lyophilisation process at which such an interaction is initiated: a sensitive thermo-analytical technique such as isothermal microcalorimetry may allow such parameters to be investigated, including detailed studies of the solution prior to lyophilisation.

On heating lyophilised samples at a rate of 5°C/minute to 100°C, only one major thermal event was noted for each sample. However, with decreasing proportions of PEG (relative to L-asparaginase), this event became more complex and the endotherm peak displayed an increasingly large shoulder, as demonstrated in Figure 4.9. Data from the DSC thermograms are summarised in Table 4.3.

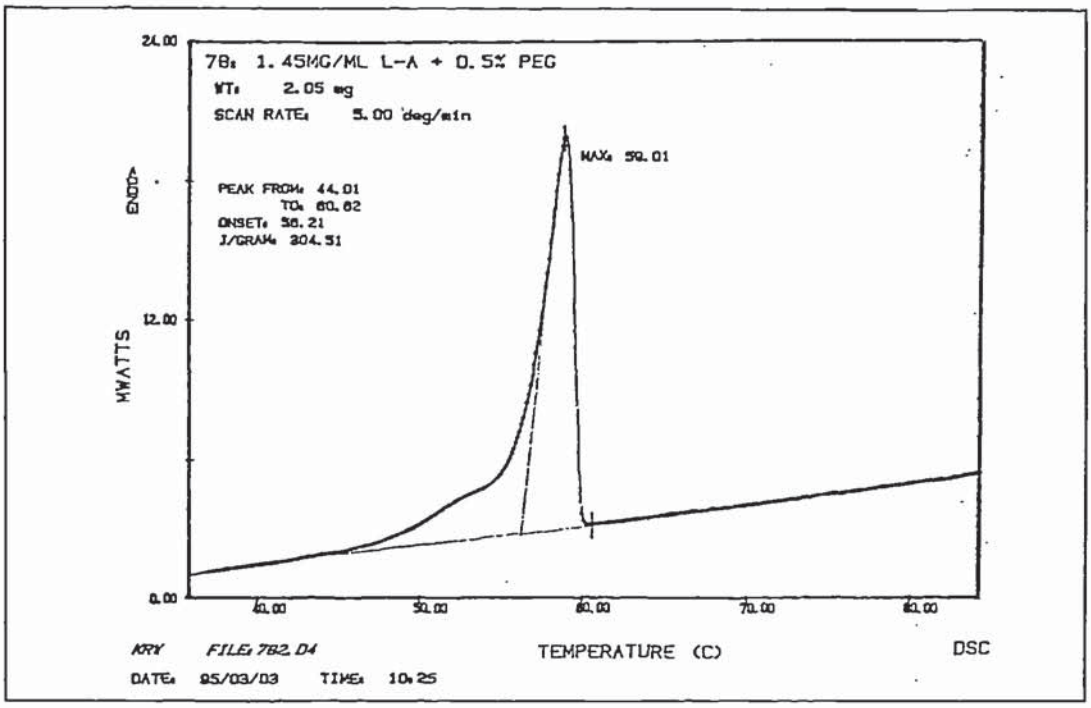


FIGURE 4.9: DSC thermogram of lyophilised L-asparaginase (initial concentration 1.45mg/ml) and PEG 10kDa (initial concentration 0.5% w/w)

Table 4.3 shows the relationship between retained activity of L-asparaginase (tetramer retention) and the total energy of the endotherms on the thermograms. Values have been converted from the original units (J/g) to give peak energies in terms of Joules *per* gram of PEG present, for comparative purposes.

[L-Asparaginase] (mg/ml)	[PEG] (%, w/w)	T_{max} of melting endotherm (°C)	Peak energy (J *per* gram of PEG)
0	1.0	61.0	430
1.45	1.0	59.4	421
1.45	0.50	59.0	393
1.45	0.25	58.7	373
1.45	0.10	56.4	344
1.45	0.05	56.0	184*
5	4.0	60.4	443
5	2.0	59.4	426
5	1.0	56.6	408

TABLE 4.3: Relationship between peak energy and PEG concentration in lyophilised products prepared in the present study (n=1) (=less than 1mg of sample available for analysis)*

Three trends seem apparent from the data presented in Table 4.3. A decrease in the proportion of PEG appeared to cause a decrease in the peak maximum temperature of the event, a decrease in the total energy of the event (even when normalised to give energy *per* gram of PEG) and an increase in the magnitude of the peak shoulder. However, it should be noted that the thermal analysis was not carried out in duplicate. In addition, for the lyophilised sample produced from a solution of 1.45mg/ml L-asparaginase and 0.05% (w/w) PEG, only a small amount of dried sample was available for analysis, and so the data for this particular sample may not be reliable. The shoulder might be attributed to one of the discrete crystal forms of PEG, or possibly to the increasing incidence of L-asparaginase aggregation in response to decreasing amounts of PEG *per* protein unit.

It is conceivable that either non-crystalline PEG, or one of the discrete types of PEG crystal forms, may have preferentially interacted with L-asparaginase, although due to the complex crystallising behaviour of PEG, which is condition-dependent for many weight fractions (Ford & Timmins, 1989), a fuller explanation for the apparent success of PEG as a lyoprotectant for L-asparaginase here is likely to require crystallographic studies in addition to the thermal analysis described above.

4.4.5 Conclusions

PEG (10kDa) was observed to provide freeze-dry protection to L-asparaginase in the present study at lower concentrations than those required of the saccharides used in the previous study (section 4.4). The increased ability of PEG, compared to the saccharides, to offer lyoprotection to L-asparaginase was believed to be due to the greater extent of preferential exclusion of PEG from the surface of the protein, compared to the sugars. This observation parallels the data reported for the cryoprotection of chymotrypsinogen by PEG and sucrose (Carpenter *et al.*, 1994), in which the authors' conclusions with regard to the cryoprotection of proteins may also be applicable to lyoprotection.

4.5 A STUDY OF THE INTRINSIC FREEZE-DRY STABILITY OF L-ASPARAGINASE

4.5.1 Background

Results of preliminary studies have suggested that the extent of structural loss of the native tetramer of L-asparaginase during lyophilisation in the absence of protective excipients may vary with the concentration of the protein in the solution prior to freeze-drying (Adams, unpublished data). In particular, it was noticed that higher concentrations of protein apparently led to an increased proportion of retained structure and activity. The purpose of this study was to carry out a quantitative investigation into this phenomenon with respect to the enzyme activity of L-asparaginase, using the activity assay developed for the studies detailed in the previous experiments in the present report.

4.5.2 Materials and methods

4.5.2.1 Materials

L-Asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 23.6 mg/ml and activity 15,360 iu/ml, by CAMR, Porton Down, Wiltshire, UK.

4.5.2.2 Freeze-drying of solutions

L-Asparaginase stock solution was serially diluted with sterile distilled water to give concentrations of 11.8, 5.90, 2.95, 1.475 and 0.7375mg/ml. Aliquots (0.50ml) of these solutions were freeze-dried using an Edwards Modulyo freeze-dryer, according to the procedure given in section 2.2.

4.5.2.3 Analysis of retained enzyme activity following lyophilisation

The enzyme activity of L-asparaginase following lyophilisation was assayed using the AHA method described in section 2.9.

4.5.3 Results and discussion

The activity data for L-asparaginase following lyophilisation and rehydration are given in Table 4.4. Lyophilised products of solutions with concentrations of 1.45mg/ml and 0.7375mg/ml yielded collapsed products which gave poorly-reproducible activity data, possibly due to the difficulties experienced in rehydration.

Initial [L-asparaginase], mg/ml	% Retained activity (±s.d., n=3)
2.95	15.63 (±2.06)
5.90	42.33 (±7.49)
11.8	70.20 (±4.91)
23.6	78.40 (±5.10)

TABLE 4.4: Effect of initial L-asparaginase concentration on retained activity following lyophilisation, determined using the AHA assay developed from Frohwein et al. (1971)

A plot of retained activity against initial protein concentration is given in Figure 4.10. It was noted that the percentage retained activity increased in accordance with the initial protein concentration, as observed previously by Adams (unpublished data). In addition, D'Andrea *et al.* (1996) observed a protein concentration-dependence for ascorbate oxidase during freeze-drying in the presence and absence of lyoprotectants. Carpenter *et al.* (1994) reported a similar observation for LDH, stating that increased recovery could be realised by simply increasing the initial concentration of the enzyme. The authors reported that the addition of PVP or BSA, or increasing protein concentration, should shift the equilibrium between molecular forms of the enzymes (tetramers, dimers, monomers) towards the fully polymerised form. In the same review chapter, the authors focussed on the use of PEG to stabilise proteins (see also section 4.4 of the present chapter) and stated that due to steric hindrance, bulky polymers are preferentially excluded fom the surface of proteins in aqueous solution and that this interaction thermodynamically stabilises the polymerised form of multimeric enzymes. They suggested that the same argument may be used to explain the enhanced stability noted at increasing protein concentrations, if one views the remainder of

the protein population as being preferentially excluded from a given individual protein molecule, and that in addition, mass action will favour polymerisation at increasing enzyme concentration. Thus, as stated in the previous study (section 4.4), the presence of an increased number of protein molecules within a given volume of solution will lead to an increase in the chemical potential of any given protein molecule, thereby resulting in enhanced protein stability during drying. However, this theory might lead to the expectation that retained activity would increase in a linear fashion with initial protein concentration, and the data produced in the present study shows that this is clearly not the case here. It is possible that at higher protein concentrations, proportionally more degradation may occur during processing, which may be a zero order reaction and therefore independent of the original concentration and thus might explain the parabolic shape of the curve in Figure 4.10.

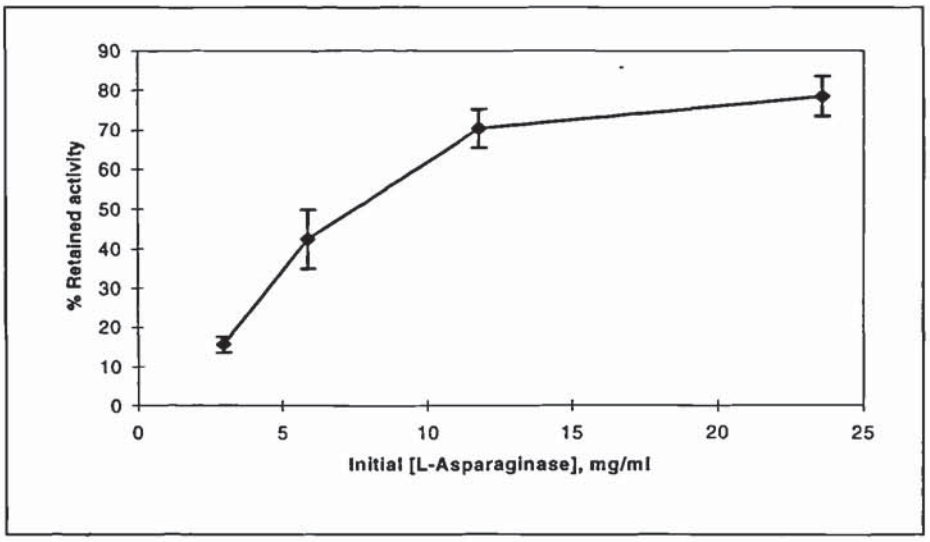


FIGURE 4.10: Graph to demonstrate the relationship between initial protein concentration and retained biological activity following freeze-drying, determined using the AHA assay developed from Frohwein et al. (1971)

4.5.4. Conclusions

The present data confirm the preliminary findings by Adams (unpublished data) and provided a quantitative measure of the extent of intrinsic stability of L-asparaginase in terms of enzyme activity. Intrinsic stability was also observed to increase with L-asparaginase concentration and this phenomenon was believed to be due to an increase in the chemical potential of L-asparaginase resulting from the preferential exclusion of increased numbers of protein molecules present at increased concentrations.

4.6 THERMAL ANALYSIS OF LYOPHILISED L-ASPARAGINASE-MANNITOL MIXTURES

4.6.1 Background

It was observed in a previous study (section 4.4) that mannitol acted as a lyoprotectant for L-asparaginase during freeze-drying. Since the mechanism of protein protection is believed to rely on the persistence of an amorphous matrix, it is possible that mannitol, which typically crystallises during freezing or drying, had persisted in the amorphous (or partially amorphous) state when freeze-dried in combination with L-asparaginase. The aim of the present study was to prepare a range of lyophilised L-asparaginase-mannitol mixtures in order to determine whether their thermal characteristics could be quantified using differential scanning calorimetry.

4.6.2 Materials and methods

4.6.2.1 Materials

L-Asparaginase (E.C.3.5.1.1, from *Erwinia carotovora*) was supplied as an aqueous solution of concentration 23.6 mg/ml and activity 15,360 iu/ml, by CAMR, Porton Down, Wiltshire, UK. AnalaR grade mannitol was supplied by BDH (UK) Ltd.

4.6.2.2 Freeze-drying microscopy (FDM) of a L-asparaginase and mannitol solution

FDM was carried out on a sample mixture, in order to enable selection of suitable freeze-drying conditions for the solutions. Analysis was carried out as described in section 2.4 of the present report. It has been reported that DSC is not sufficiently sensitive to provide a glass transition temperature (T_g') measurement for protein solutions (Chang & Randall, 1992; Pikal, 1994) or that solutions of such entities require greater than 20-25% (w/v) solute content to provide acceptable thermal data (Shalaev *et al.*, 1996). Indeed, Pikal (1994) recommends that FDM be used for protein solutions in order to provide such data when DSC fails to do so.

4.6.2.3 Lyophilisation of aqueous solutions

Lyophilisation of 0.5ml aliquots of solution in 3ml freeze-drying vials was carried out in accordance with the procedure given in section 2.1.

4.6.2.4 Thermal analysis of lyophilised products

Freeze-dried products were analysed by DSC, using the method given in section 2.5.2. Samples were subjected to a heating program using the Perkin-Elmer DSC-4 apparatus described in section 2.5.1. of the present report.

4.6.3 Results and discussion

The data obtained from the DSC thermograms of lyophilised products may be divided into two groups, reflecting two distinct patterns of thermal behaviour. The first group of data, presented in Table 4.5, describes the thermal characteristics of freeze-dried products containing mannitol, either alone or in combination with low concentrations (1.45mg/ml) of L-asparaginase. It was observed that these products tended to exhibit a melting endotherm typical of mannitol alone and in most cases, a secondary thermal event was noted (~60°C) in addition to the melting endotherm. In each case, a second heating profile revealed the loss of this secondary event, yielding only a melting endotherm. Table 4.5 shows the melting point and secondary event data, for which the peak energies have been normalised with respect to the quantity of mannitol present in each mixture.

The secondary event at approximately 60°C was initially believed to have been due to an impurity present in the mannitol, since it occurred for products containing mannitol alone, where full crystallisation would have typically been expected. However, it was observed that this event occurred even when different batches of material were used, and that the energy associated with this event showed no correlation with the amount of material present in a given product. It was therefore concluded that the event was unlikely to be the result of an impurity. One theory which may explain this event is that a proportion of mannitol persisted in the amorphous state during drying. This correlates with the suggestion that mannitol had remained in the amorphous phase in a previous study (section 4.3) where it had been seen to act as a lyoprotectant for L-asparaginase during freeze-drying.

[L-asparaginase] (mg/ml)	Mannitol (%, w/w)	Melting endotherm		Secondary event	
		T_{max} (°C)	ΔH_m (J/g)	T_{max} (°C)	ΔH_m (J/g)
0	2.0	169.4 (±1.4)	697 (±33)	n/c	n/c
0	1.0	169.1 (±0.2)	682 (±6)	61.1 (±0.7)	36.1 (±9.5)
0	0.5	168.7 (±0.2)	695 (±30)	59.2 (±0.1)	42.1 (±0.8)
0	0.25	168.0 (±0.3)	711 (±12)	59.5 (±0.6)	19.4 (±1.7)
1.45	1.0	168.0 (±0.8)	693 (±10)	61.4 (±0.4)	102.8 (±8.5)
1.45	0.5	167.4 (±0.6)	672 (±6)	63.1 (±0.5)	64.9 (±18.5)
1.45	0.25	166.5 (±0.4)	660 (±6)	65.8 (±0.4)	47.8 (±2.3)

TABLE 4.5: Data from DSC thermograms for lyophilised samples containing mannitol alone or in combination with low concentration (1.45mg/ml) L-asparaginase. All energy data have been normalised to represent Joules per *gram of mannitol present in sample (n/c= not calculable from thermogram).*

The temperature of the secondary event was observed to decrease and its associated energy to increase with increasing L-asparaginase concentration. The decrease in temperature mirrored the decrease in melting temperature, which suggested that L-asparaginase had acted as an increasing 'impurity' to mannitol as the protein concentration was increased. It was interesting to note the increase in energy associated with the secondary event with increasing protein concentration (even when normalised to the amount of mannitol present), which suggested that increasingly higher proportions of mannitol were induced into this secondary, possibly amorphous, state by the addition of increasing amounts of protein. It was not possible to conclude whether the event around 60°C represented a softening of a glassy region or a molecular rearrangement, but the former is the favoured suggestion, since a rearrangement such as crystallisation would have been represented by an exothermic event on the DSC thermogram.

The second group of data is presented in Table 4.6 which records preliminary data describing the thermal behaviour of mannitol when freeze-dried with high concentrations of L-asparaginase. It may be seen that the pattern of thermal behaviour of these products was quite distinct from that displayed by the products described above (Table 4.5). All products

in the second group displayed two endotherms on initial heating, yet no event was observed in the region of 60°C. The exceptions in this group were those products freeze-dried from solutions containing 12.2mg/ml L-asparaginase and 0.5% or 0.25% mannitol, which exhibited no identifiable mannitol characteristics. In cases where products did exhibit quantifiable thermal characteristics, it was apparent that no obvious trend existed between the events observed on either initial or second heating profiles and the composition of mixtures. However, it was interesting to note that all products displayed different thermal characteristics on reheating than they did on initial heating. This suggests that all products within this group may have been in a metastable state following lyophilisation, which yielded a more thermally stable state upon heating and quenching. However, these data (Table 4.6) were preliminary observations. and further studies would be necessary to corroborate the present findings.

[L-asparaginase] (mg/ml)	Mannitol (%, w/w)	Endotherms on initial heating profile [T_{max}(°C); ΔH_m (J/g)]	Endotherms on reheat profile [T_{max}(°C); ΔH_m (J/g)]
5.0	0.5	165.9; 392 155.8; 304	167.6; 608
5.0	0.25	155.4; 254 164.6; 47	60.6; 8.4 155.0; 26 164.6; 164
5.0	0.125	154.9; 14.4 164.7; 36.6	none
12.2	1.0	156.4; 107 141.5; 266	155.1; 211 141.3; 124
12.2	0.5	165.1; n/c	165.3; n/c
12.2	0.25	none	none

TABLE 4.6: *Data from preliminary thermograms for lyophilised samples containing mannitol in combination with high concentrations of L-asparaginase. All energy data have been normalised to represent Joules* per *gram of mannitol present in sample (n/c= not calculable from thermogram).*

4.6.4 Conclusions

Differential scanning calorimetry (DSC) was employed to provide assessment of the thermal characteristics of most of the mannitol/L-asparaginase mixtures analysed in the present study. On freeze-drying mannitol either alone or in combination with low concentrations of L-asparaginase, a secondary thermal event was observed in addition to the typical melting endotherm of mannitol; it was not possible to conclude from the present data whether this event was attributable to the presence of a metastable state of mannitol induced by the presence of the protein, although this is the favoured suggestion. For lyophilised products containing mannitol in combination with high concentrations of L-asparaginase, the presence of the protein appeared to cause a decrease in the melting temperature of the mannitol and led to the occurrence of secondary thermal events which were typically 10-20°C lower than the observed melting endotherm. The temperature change or energy of the melt or the secondary events did not appear to correlate with the amounts of either mannitol or protein present in the mixtures analysed. This may be indicative of the non-unique nature of glasses, which are dependent on the thermal history of samples; however, the data produced in the present study represent preliminary findings and further studies would be necessary to allow more concrete conclusions to be reached.

4.7 Investigation of the effects of additives on the freeze-dry stability of Lactate Dehydrogenase (LDH)

4.7.1 Background

The objective of this study was to assess the effect of additives on the stability of LDH during freeze-drying. LDH is similar in structure and molecular weight to L-asparaginase, and is a tetramer of molecular weight of approximately 140kDa (*cf.* 135kDa for L-asparaginase) composed from four monomer subunits. While L-asparaginase monomers are identical to each other in all aspects, the LDH subunits may be of heart or skeletal muscle origin (denoted as 'H' and 'M', respectively). However, since 'H' and 'M' subunits are identical, the overall structure of the LDH tetramer remains unaffected by the type of subunits which constitute it. There are five possible combinations of 'H' and 'M' subunits to give a tetramer, as demonstrated thus:

HHHH HHHM HHMM HMMM MMMM

(Note: Since the three-dimensional shape of the tetramer is rotationally symmetrical, the order in which 'H' and 'M' are written in the description of the tetramer is not significant. Thus, HMMH is identical to MHMH, *etc.*)

It has previously been observed that LDH loses biological activity following freeze-drying (Carpenter *et al.*, 1994). Indeed, even freeze-thawing has been shown to cause a partial loss in quaternary structure. The aim of the present study was firstly to assess whether this observation could be confirmed and quantified, and secondly, to compare the relative successes of a range of excipients in preventing activity loss during freezing, drying and on storage in the lyophilised state.

4.7.2 Materials and methods

4.7.2.1 Materials

LDH (lactic dehydrogenase) was obtained from Sigma Chem. Co, Poole. UK., as 1.00ml of a crystalline suspension in ammonium sulphate solution, with an activity of 960 units *per* mg of protein and a concentration of 11mg/ml. All additives were obtained from Sigma Chem. Co., UK., or BDH, UK., or Fisons, UK., and were of analytical grade or equivalent.

4.7.2.2 Preparation and lyophilisation of LDH solutions

The LDH suspension was placed in a dialysis bag and dialysed overnight against 5 litres of double-distilled water at 4°C, in order to remove the ammonium sulphate. The resulting suspension, which had become almost clear during dialysis, had increased in volume to 2.174ml. This volume was divided between two 1.5ml Eppendorf tubes and spun using a bench centrifuge at 13,000rpm for 30 minutes, in order to remove any particulates. A sample of resulting solution (50µl) was analysed for biological activity using the Sigma assay kit as described in section 2.11. The remainder of the solution (2.124ml) was diluted to 25.20ml with double distilled water and split into five equal parts (5.04ml). To four of these parts, 0.100g of excipient (trehalose, lactose, mannitol or dextran (MW70kDa)) was added, giving 2% (w/w) additive. To the remaining 5ml, no excipient was added. Each 5ml solution was split further into five freeze-drying vials, (in 1.00ml aliquots). Four of each five vials were lyophilised using an Edwards Modulyo freeze-dryer as described in section 2.1 of this report (0.2mbar, shelf heating 4 hrs), and the remaining vial from each group stored at 4°C for comparison.

4.7.2.3 Analysis of retained biological activity

Lyophilised samples were reconstituted with 1.00ml PBS (pH7.4) and analysed for activity using the Sigma LDH assay kit described in section 2.11 of this report. It was necessary to dilute samples with PBS prior to analysis (1 volume sample + 99 volumes PBS), in order to reduce the enzyme activity to within the range of sensitivity of the assay. In addition, original solutions were analysed using the same assay, for comparative purposes, and the activity of the reconstituted samples expressed as a percentage of the original activity.

4.7.3 Results and discussion

It was noted that following dialysis, the LDH solution had lost much of its activity, compared to the original activity given by the supplier (47.7u/ml following dialysis, compared to 10,560u/ml given as original activity). The freeze-dried cakes all demonstrated good appearance apart from ones where no additive had been used, as the bulk of these samples was very low. Full reconstitution of all samples was observed to occur within 10 seconds of the addition of PBS. Table 4.7 shows the activity data for LDH upon reconstitution, measured using the Sigma kit assay and expressed as a percentage of the activity immediately prior to freeze-drying. Figure 4.11 shows a graphic representation of these data.

Additive	Original activity (units/ml)	% ACTIVITY REMAINING		
		after Freeze-Thawing	after Freeze-Drying	after 1 week at 37°C
None	47.69 (±2.23)	91.44 (±0.87)	83.94 (±1.20)	79.15 (±0.77)
Mannitol	58.46 (±0.71)	75.42 (±0.98)	72.78 (±0.15)	24.47 (±0.11)
Dextran	55.90 (±0.93)	95.87 (±0.66)	85.56 (±0.89)	80.50 (±0.74)
Lactose	57.21 (±0.72)	102.3 (±0.90)	113.8 (±2.1)	83.80 (±0.61)
Trehalose	60.91 (±1.71)	100.2 (±0.75)	99.62 (±2.76)	90.56 (±0.53)

TABLE 4.7: Effect of excipients on the retained biological activity of LDH following freeze-thawing, freeze-drying and storage in the lyophilised state for one week at 37°C

It may be seen from the data in Table 4.7 that under the conditions employed in the present study, LDH alone was relatively stable to freezing, drying and subsequent storage at 37°C. It was interesting to note that dextran, lactose and trehalose provided some degree of stabilisation to LDH during freezing and drying, yet mannitol exerted a destabilising effect relative to lyophilised LDH samples where no additive was used.

It appears from the present data that the destabilising effect of mannitol occurred both during freezing and during storage in the lyophilised state, yet the activity following drying in the presence of mannitol was not appreciably lower than that following freezing, which suggests that the mannitol may have crystallised during freezing, which then rendered it unable to

provide stabilisation or further damage to the LDH during drying. This behaviour is unusual for mannitol, which is more typically observed to crystallise only on warming of the frozen mass or during the initial stages of primary drying (see chapter 3). Thus, it was surprising that the LDH activity remained similar following drying as it had been after freeze-thawing. On storage of the lyophilised product for 7 days at 37°C, the LDH activity was observed to decrease substantially in the presence of mannitol, indicating that a damaging reaction had taken place in these vials, which was catalysed by the presence of mannitol, or involved mannitol directly.

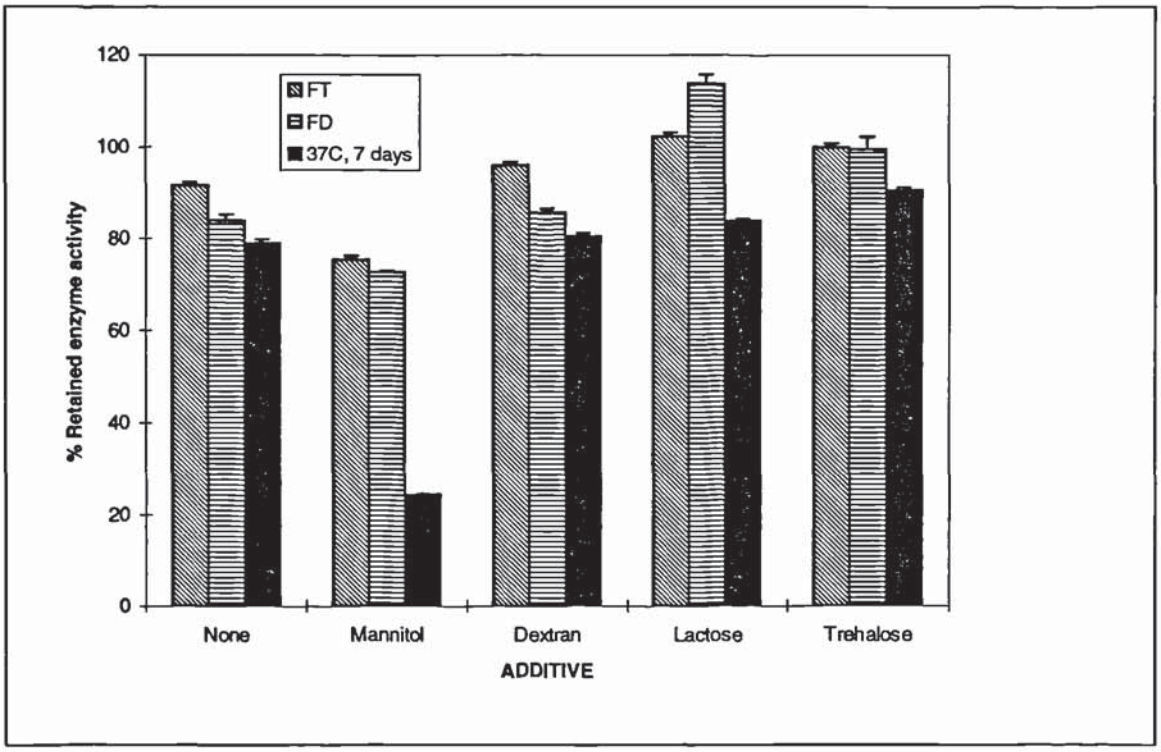


FIGURE 4.11: Bar graph to demonstrate the relationship between excipient and retained biological activity of LDH following freeze-thawing, freeze-drying and storage in the lyophilised state at 37°C for one week (n=3)

Data for samples containing dextran indicated that this polymer appeared to offer little protection to LDH. Following freeze-thawing, LDH activities were only marginally higher than those for samples containing no additive. Similarly, during drying, the addition of dextran was seen to provide apparently no protection to LDH. The product was observed

not to degrade substantially during storage in the lyophilised state, suggesting that although dextran was not destabilising to the protein, it was unable to provide stabilisation.

Lactose was seen to provide full stabilisation to LDH during both freezing and drying, yet some activity loss was observed following storage in the lyophilised state. This activity loss may have been due to the fact that lactose is a reducing sugar and thus may have undergone a degradative reaction with the protein, possibly a Maillard reaction.

Finally, the data indicated that trehalose was able to provide full stabilisation to LDH during freezing and drying and maintained over 90% of LDH activity during storage in the lyophilised state at 37°C for 7 days. Trehalose was thus seen to exhibit all the virtues of lactose in terms of stabilisation during processing, but in addition was able to provide better stabilisation on a longer-term basis. This extra degree of stabilising ability was attributed to the non-reducing nature and relatively inert nature of trehalose (see section 4.3), which makes this sugar an increasingly popular choice of excipient to be included in protein formulations (Colaco *et al.*, 1994).

4.7.4. Conclusions

The use of the model protein LDH highlighted differences in the protective abilities of a range of additives in terms of protein stability even under the three simple selected procedures, *i.e.* freeze-thawing, freeze-drying and storage in the lyophilised state under elevated temperatures. These differences were attributed to the molecular classification, crystallising behaviour and reducing or non-reducing nature of each additive.

5. THE USE OF FREEZE-DRYING IN THE PREPARATION AND STABILISATION OF STEALTH LIPOSOMES

SUMMARY

The aim of this phase of the project was to examine the effects of freeze-drying and the use of lyoprotectant additives on the preparation of novel stealth liposomes and on the storage of these liposome preparations, both in suspension and in the dried state. Preparations were characterised by examination of size, surface charge and drug loading. Stability of vesicles in aqueous suspension was assessed by examination of release profiles of marker compounds; stability in the dried state was assessed by thermal analysis, together with the study of release of marker compounds in subsequently rehydrated preparations. The effects of adding potentially protective and damaging excipients to the preparations prior to lyophilisation were investigated by the use of the same methods. It was generally observed that neither the potentially protective nor the potentially damaging excipients appeared to markedly affect either liposome formation, drug encapsulation or post-formation stability. The feasibility of drug encapsulation into stealth liposomes using the method of simultaneous loading-rehydration was also investigated. It was found that the model drug compound polymyxin B (PXB) could be encapsulated into stealth liposomes using this method, but that the efficiency of loading was lower than when a conventional method of preparation was employed.

5.1. INTRODUCTION

5.1.1. The use of liposomes as drug carriers

Liposomes were first described in 1965 by Alec Bangham and colleagues, who reported that 'the diffusion of univalent cations and anions out of spontaneously formed liquid crystals of lecithin is remarkably similar to the diffusion of such ions across biological membranes' (Bangham *et al.*, 1965). These liquid crystals were formed when an aqueous solution was added to a dried lipid film and were shown to be closed membrane structures which exhibited semi-permeability and osmotic properties. These structures were later termed 'smectic mesospheres', before the term 'liposome' was eventually adopted. The ability of liposomes to entrap solutes to which they were selectively permeable made the system an excellent model for cell membranes and led to an intensification of studies on cell-membrane biophysics, structure and function. The ability of liposomes to entrap and release ions formed the basis of the liposome drug-carrier concept that was proposed by Gregoriadis and Ryman (1972), in which liposomes were used as drug delivery vehicles. Subsequent work by Gregoriadis and colleagues established the feasibility of using the system with enzymes, anticancer and antimicrobial drugs (Gregoriadis, 1976a, 1976b), and also of the ability of liposomes to potentiate immune responses to entrapped antigens (Allison & Gregoriadis, 1974). Since then, many workers have investigated the possible use of liposomes as a suitable system for the delivery and targetting of many therapeutic drugs (Torchillin, 1985; Gregoriadis, 1988) and vaccines (Gregoriadis, 1990). These investigations, together with the extensive studies which have been carried out into the behaviour of liposomes and their control within biological environments, as well as the tremendous advances in liposome technology (reviewed in Gregoriadis, 1993), have culminated in the recent development of a number of liposome-based formulations that have been licenced for use in humans (Gregoriadis, 1995). Such remarkable progress remains unsurpassed by the numerous other drug delivery systems currently being investigated, including that of monoclonal antibodies, which have the potential to provide incredibly specific targetting. The apparent success of liposomes may be attributed to the innocuous nature of the liposomal components (many of which are non-toxic, non-immunogenic, biodegradable lipids) and to the structural versatility of the system.

It was recognised that liposomal formulations of therapeutic agents could be modified in numerous ways in order to tailor the formulation to conform to specific needs. There is a vast array of lipids available to the formulation scientist, and the ever-increasing number of preparative methods and manufacturing technologies have given rise to several different types of liposome, varying in size, structure, surface charge and bilayer rigidity, amongst other factors (see section 5.2). It is perhaps not surprising that the liposome was believed to be the revolutionary delivery system which could improve the efficacy of a large number of drugs. Indeed, it had been demonstrated that many types of compounds could be encapsulated into liposomes, both hydrophilic and lipophilic molecules, those of high molecular weight as well as smaller molecules. However, following initial *in-vivo* work, several potential problems became apparent. Even though it was encouraging that mainly high molecular weight, lipophilic compounds could be entrapped and that these took on the pharmacokinetics of the carrier system, it was also soon found that low molecular weight, water-soluble drugs leaked extensively into the circulating blood. Also, endocytosis of liposomes (and their contents) by the tissues of the reticulo-endothelial system (RES) was a common observation (Gregoriadis, 1993). In addition, many researchers were attaining low drug entrapment values, as well as vesicle size heterogeneity and poor reproducibility and instability of formulations. These *in vivo* studies highlighted the significance of the RES and pharmacokinetic factors which could effectively be seen as being the factors which determined the success or failure of any particular liposomal formulation on the practical level. Thus, it was also appreciated that the design process for a liposomal delivery system for a target compound had to take into account not only the regular physico-chemical factors such as lipid stability, drug stability and drug-lipid interactions, but also had to incorporate measures to enable the liposomes to reach their intended site of action before releasing the drug at the desired rate. The advances in liposome manufacturing and analytical technologies which occurred chiefly in the 1980s enabled scientists to prepare liposomes with much greater efficiency, more reliably and with better reproducibility. In this way, many of the previous problems and pitfalls associated with liposome preparation were effectively eliminated. The vast amount of research carried out in the last quarter of a century has not only yielded many types of novel liposome-based drug formulations, but also a wealth of information on a myriad of related issues, such as the mammalian pharmacokinetics and

pharmacodynamics of drug carriers, and a better understanding of how anatomical and physiological barriers to novel drug delivery systems may be overcome (Gregoriadis, 1993). Although liposomes appear to have attracted the attention of both the food and cosmetic industries in recent years, several large pharmaceutical companies such as Ciba-Geigy, Upjohn, Becton-Dickinson, Bristol-Myers Squibb and specialised companies like Liposome Technology, Vestar and The Liposome Company are currently also active in manufacturing liposome-based products (Watwe & Bellare, 1995). Two relatively recent advances in liposome technology which are relevant to the present study are the development of long-circulating liposomes and the concept of simultaneous loading/rehydration. Section 5.1.3 provides an overview of these areas.

5.1.2 Liposome structure and assembly

The spontaneous assembly process of phospholipids into liposomes occurs when relatively insoluble lipid components arrange themselves into stable, concentric bilayers around an internal aqueous compartment (Fielding, 1991), as shown in Figure 5.1. The central aqueous core can range in diameter from 20nm to several micrometers, and liposomes can contain from one to tens of concentric bilayers, each creating its own aqueous compartment.

FIGURE 5.1: *Diagram depicting the arrangement of phospholipid molecules around an aqueous core to form a unilamellar liposome (from Alpar, 1989)*

The conformation of the bilayer is such that the polar headgroups of the individual phospholipids are in contact with water molecules on either side of the structure, while apolar hydrocarbon tails form a thin, hydrophobic phase between these polar regions. It has been demonstrated that covalent bonds are not formed during the assembly process, but the summation of hydrophobic interactions between the hydrocarbon chains of the membrane interior leads to high stability (Martin, 1990). The hydrophobic effect is a consequence of the large free energy change between water and hydrophobic environments and is the driving force behind the assembly process (Cevc, 1993). Lipids aggregate to form bilayers which exclude as much water as possible from the hydrocarbon core in order to achieve the lowest free energy level and therefore the highest stability state for the aggregate. The extremely low critical micelle concentration (4.6×10^{-10}M) of phosphatidylcholines (lecithins) is further evidence of the preference which these molecules have for the hydrophobic environment of a micelle or bilayer core (Riaz *et al*, 1989), which renders them particularly suitable for application as liposomal preparations.

The overall structure of the liposome will not only depend on the choice of lipid(s) used, the ratio of the combination, the total lipid concentration and the type (and concentration) of any drug (and other co-solutes) present, but in addition, the choice of preparative method will be instrumental in determining the way in which the liposomes assemble. Although some general principles apply to liposome formulation, it is accepted that a specific formulation needs to be optimised for the particular drug in question (Fielding, 1991). A review article by Lasic (1988) seeks to provide the reader with a thorough understanding of the mechanism of vesicle formation.

5.1.2.1 Liposome classification

The liposomes which Bangham first described are now referred to as multilamellar vesicles (MLV). The term MLV describes vesicles consisting of several concentric bilayers ranging in size from 0.2μm to >10μm (Ostro & Cullis, 1989). Sonication of MLV results in a reduction in the size of these vesicles to liposomes consisting of only a single bilayer with diameters ranging from 25-50nm. These liposomes are referred to as small, unilamellar vesicles (SUV). Liposomes with similar structures to SUV but with diameters in the range

100-500nm are termed large unilamellar vesicles (LUV) (Riaz *et al*, 1989). The property of drug encapsulation for these types of liposome are quite different. LUV have a high ratio of encapsulated (aqueous) volume to lipid mass, and thus are the most efficient at trapping water-soluble agents, while SUV exhibit relatively poor solvent trapping, since the ratio of entrapped volume to lipid mass is much lower than for LUV. In fact, a typical SUV preparation has approximately 10% of the capture volume of an equimolar LUV preparation (Karlowsky & Zhanel, 1992). MLV have a high ratio of lipid mass to entrapped volume and are therefore the most efficient carriers of hydrophobic membrane-bound drugs (Talsma & Crommelin, 1992). The liposomal compartment in which a drug may be entrapped is dependent upon the physico-chemical characteristics of the drug itself. Highly polar, water-soluble drugs will tend to become entrapped in the aqueous space of the liposome. Lipophilic drugs can be incorporated into the interior of the bilayer, while amphiphilic drugs can partition across the membrane-aqueous phase interface and may become associated with either or both phases (Figure 5.2). Drugs with intermediate partition coefficients tend to diffuse across the bilayer and show less retention than very hydrophobic or hydrophilic drugs (Karlowsky & Zhanel, 1992). For a given production process, the aqueous space captured by bilayers is directly proportional to the lipid mass. Liposomes containing charged phospholipids entrap an increased aqueous volume due to repulsion between bilayers with like charges, thus causing an increase in inter-lamellar distances (Szoka & Papahadjopoulos, 1980; Alpar *et al.*, 1982).

The encapsulation efficiency of hydrophilic, non-bilayer-interacting drugs depends solely on the entrapped volume and, therefore, on the particle size, number of bilayers and the lipid concentration utilised. The entrapment of charged drug molecules may be increased by the incorporation of an oppositely-charged phospholipid in the bilayer which will result in an electrostatic attraction between the drug and the bilayer surface (Ostro & Cullis, 1989). For hydrophobic, bilayer-bound drugs, the encapsulation efficiency depends on the total lipid used and the fluidity of the bilayer. Fluidity may also determine the leakage rate of entrapped drugs. Liposomes composed of lipids such as PC (lecithin), which is in the relatively disordered liquid crystalline state at room temperature, tend to display increased release rates compared to those composed from saturated lipids such as DSPC, which are in the ordered

gel state at room temperature (Kulkarni *et al*, 1995). The encapsulation of amphiphiles is determined by the mechanism of interaction with the bilayer. If the drug-lipid interaction is based on electrostatic forces, the density of charged phospholipids and the ionic strength and pH of the aqueous medium play a role. Hydrophobic interaction may also occur and the choice of bilayer phase transition properties or phospholipid acyl chain length will be important factors governing the extent of interaction (Talsma & Crommelin, 1992). The incorporation of cholesterol may also increase the encapsulation of hydrophobic drugs, although this is dependent on the specific interaction characteristics of the drug with the bilayer phospholipids (Kulkarni *et al*, 1995).

FIGURE 5.2: *Schematic diagram depicting the compartmental site of drugs following encapsulation (from Alpar, 1989)*

5.1.3 Recent advances in liposomal drug delivery

There are two relatively recent advances in liposome technology which are relevant to the present study. The first is the development of long-circulating liposomes, which came about as a result of research into the enhancement of the *in vivo* stability of liposome preparations. The second is the concept of simultaneous loading and rehydration, which is a form of

passive loading of drugs into liposomes. A brief outline of these concepts are given in the following sections.

5.1.3.1 Developments in the enhancement of *in vivo* stability

After successful encapsulation within liposomes, it is necessary to minimise the leakage of entrapped drugs (Betageri, 1993). The release rate at low temperatures (for example, at 4°C) is seen to be relevant to pharmaceutical stability (Law *et al.*, 1994) and knowledge of the *in-vitro* release profile at 37°C is a prerequisite before *in vivo* investigation may commence (Betageri & Parsons, 1992; Elorza *et al.*, 1993; Taylor *et al.*, 1990). The rate of drug release from liposomes is largely determined by the physico-chemical properties of the drug and the liposomal system. Liposomes are freely permeable to water, but cations are released at a slower rate than anions (Bangham *et al.*, 1965). Aqueous hydrogen bonding may determine the release rate of non-electrolytes (Cohen, 1975). Liposome permeability is a function of the degree of disorder of the lipid bilayer. Phospholipids in the liquid crystalline state show greater permeability to entrapped material than comparative bilayers in the gel state (Betageri & Parsons, 1992; Taylor *et al.*, 1990). Loss of entrapped material is therefore temperature-dependent, with greatest efflux around the phospholipid phase transition temperature (T_m) (reviewed in Crowe *et al*, 1989b). Increased permeability at this temperature is a result of regions of high bilayer disorder where gel and liquid crystalline phases temporarily co-exist (Betageri, 1993). Cholesterol can decrease the efflux rate through interaction with phospholipid hydrocarbon chains by increasing the rigidity of the bilayers; the inclusion of cholesterol at 50 mol% into bilayers has previously been shown to give a gel crystalline system which displays no lipid transition when analysed by DSC (Betageri & Parsons, 1992; Taylor *et al.*, 1990). Electrostatic interactions with charged phospholipids may also affect drug release (Law *et al.*, 1994).

The major factor which has prevented the optimistic expectations of liposomes from being met is that of stability. In the 1970s, many bold predictions were made by numerous workers. At the beginning of the following decade, these claims were bolstered by businessmen and analysts who predicted the rapid development of liposomes as drug carriers. Although initial problems of scale-up, sterilisation and shelf-stability were solved in most

cases, none of the optimistic expectations were realised on the practical level (Lasic, 1996). As a result, confidence in the application of liposomes as a drug delivery system did decline. Many *in-vivo* studies carried out into the pharmacokinetic aspects of liposomal drug delivery only served to highlight the stability problems of many liposomal drug formulations and the role of the immune system in effectively eliminating many liposomes before their therapeutic effect could be mediated. When they are administered as a drug, liposomes are rapidly taken up by the cells of the body's immune system. While this interferes with most applications, it can also be used to target these very cells themselves, which often harbour infection (Lasic, 1996). Indeed, the work of New and colleagues in the late 1970s resulted in improvements in the treatment of parasitic and fungal infections in animal models, when they encapsulated drugs to treat leishmaniasis (a tropical parasitic disease) and Amphotericin B against fungal infections (New, 1981). This work culminated in the production of the first commercially available drug in liposomal form in Europe, that of Amphotericin B embedded into the membrane of small unilamellar vesicles (SUV), which was developed as the product 'AmBisome' by Vestar in California in 1990 (Adler-Moore & Profitt, 1993).

Perhaps the most significant advancement in recent years in the area of liposomal stability is that of the long-circulating liposome, or sterically stabilised liposome (SSL). Lasic and co-workers at Liposome Technology introduced the concept of using polymer-lipids to stabilise liposomes sterically and developed the first pharmaceutical formulation of the anticancer drug epirubicin in sterically stabilised liposomes (Lasic, 1994; Lasic & Needham, 1995). SSL are typically of a diameter less than 100nm and have shown to circulate in humans with half-lives of days, as opposed to tens of minutes or occasionally hours (depending on the mechanical strength of bilayers) in the case of conventional liposomes. Their inherent ability to avoid uptake by immune system cells has earned them the name 'Stealth Liposomes'® (trademark of Liposome Technology Inc., USA). The inclusion of 'Stealth'® lipids into lipid mixtures renders the surface of the resulting liposomes hydrophilic, which decreases the chance of cellular uptake (Gregoriadis, 1993; Bakker-Woudenberg *et al.*, 1993; Allen, 1994a). Such lipids include ganglioside GM_1, hydrogenated phosphatidyl-inositol and the synthetic derivative poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE). All share the common property of a negatively-charged headgroup which is sterically

shielded by the presence of bulky groups. It has been shown that the incorporation of a 5-10% molar quantity of PEG-DSPE (using PEG fraction of mean molecular weight 1900) prolongs the circulation half-life of small (100nm) liposomes and is independent of bilayer fluidity (a result of other lipids present and their relative proportions) and dose effects (Woodle, 1993). Stealth liposomes appear to have overcome the long-standing problem of uptake by the MPS and have opened up new clinical applications, particularly in the treatment of extracellular infections and tumour targetting (Papahadjopoulos *et al.*, 1991; Zalipsky *et al.*, 1994; Allen, 1994; Woodle, 1993; Woodle & Lasic, 1992; Woodle *et al.*, 1992a,b). The abundance of recent review articles in a range of journals by Lasic and co-workers demonstrates the current level of active interest in this area (Lasic 1994, 1996; Lasic & Needham, 1995; Lasic & Papahadjopoulos, 1995). The effect of covalently grafting polymers onto bilayer lipids is depicted in Figure 5.3.

FIGURE 5.3: *Schematic diagram depicting a polymer-grafted bilayer at: (a) low grafting concentration (mushrooms), and (b) high grafting concentrations (brushes). From Hristova & Needham (1996).*

5.1.3.2 The use of passive loading to improve drug loading efficiency into vesicles

The chapter by Crommelin & Schreier (1994) provides an insight into the range of techniques currently used to improve loading efficiencies. These techniques may be divided into categories termed passive loading strategies and active loading strategies. Much of the work reported to date has involved active loading techniques, and the use of pH-gradient-driven loading in particular has appeared to be the most commonly-reported method. However, for the purposes of the present project, it was wished that the use of buffers be avoided, due to the potential of complications resulting from concentration effects during freezing. It was thus decided that for the purposes of increasing drug loading into SSL in the present study, the novel technique known as simultaneous loading-rehydration should be investigated. Simultaneous loading-rehydration is a technique which involves the rehydration of a dried (usually freeze-dried) formulation of preformed, empty vesicles with an aqueous solution containing the chosen drug (or drug marker) compound. This is therefore a passive loading technique, since, according to the categories defined by Crommelin & Shreier (1994) passive loading is where a compound is entrapped in the liposome during formation, or when the liposome structure is severely weakened. In the case of simultaneous loading-rehydration, entrapment occurs upon rehydration of liposomes whose structures have been severely weakened by the freeze-drying process. Dürr and co-workers reported that they had been able to successfully encapsulate the drug doxorubicin (DXR) into preformed vesicles composed of egg lecithin in this manner, achieving an encapsulation of 7.9mg.g^{-1} lipid (expressed as milligrams of drug per gram of lipid used) (Dürr *et al.*, 1994).

The mechanism by which a drug molecule becomes entrapped into (or associated within the bilayer of) dried vesicles on rehydration is dependent of many factors. The nature of the drug (hydrophilicity/lipophilicity, solubility in aqueous medium and in lipids, polarity) choice and compatibility of lipids used (of lipids with each other and of the resulting mixture with the drug concerned), the overall gel- or liquid- crystalline state of the bilayers at the loading temperature (assuming phase separation has not occurred), the type of vesicles formed (which will determine the ratio of aqueous space to lipid volume) are just some of the many factors which will normally determine the success (or otherwise) and extent of drug encapsulation, disregarding any short- or longer- term stability factors.

5.1.4. Stabilisation of Liposomal Formulations by Lyophilisation

Lyophilisation is considered a promising means of extending the shelf-life of liposomes. Indeed, it is acknowledged by many that the best way to stabilise liposomes is to freeze-dry them (Lasic, 1996). A chapter written by Crowe & Crowe (1993) is arguably the most comprehensive work on the lyophilisation of liposomes to date, in which the authors summarise the state of the field, critically review the many issues over which certainty has yet to be established and provide a substantial amount of their own data for further discussion. Initial studies on the preservation of liposomes during drying had their basis in earlier work carried out in the related field of organism preservation, much of which was undertaken by Crowe and co-workers. This work included detailed studies into the protection of natural biological membranes during processes such as freezing and drying. Many organisms which have the ability to survive dehydration (a phenomenon known as anhydrobiosis) accumulate large quantities of disaccharides, the most common of which are sucrose (in the case of higher plants) and trehalose (in the case of animals and lower plants). Survival of these organisms in the absence of water is strongly correlated with the presence of one of these sugars. In the early 1980's, Crowe & Crowe had found that trehalose was effective in the stabilisation of structure and function in intact biological membranes during drying, a finding which was also extended to other membranes. They established that this protective property was apparently particular to disaccharides, and that trehalose was indeed the most effective sugar tested. A similar level of protection was afforded by sucrose, but much more of this sugar was required to give the same level of stabilisation. This series of studies is reviewed in Crowe *et al.* (1992).

Since that time, Crowe & Crowe and other groups of workers have demonstrated the protective effects of trehalose and other saccharides during freezing and dehydration on various types of liposome preparation containing drug marker molecules. The problem of long-term stability of liposomes was initially addressed through the development of methods for preserving unilamellar vesicles, including the materials trapped inside, as freeze-dried powders (Crowe *et al.* (1985, 1986, 1987, 1989a); Crowe & Crowe (1988, 1991); Madden *et al.* (1985); Harrigan *et al.* (1990); Ozer *et al.* (1988); Rudolph (1988); Rudolph & Cliff (1990); Womersley *et al.* (1986)). Water could be added to the lyophilised powders, and

liposomes obtained immediately, which retained as much as 100% of their original contents, compared with the same liposomes before freeze-drying (Crowe *et al.* (1985, 1986)). Although the mechanism of action of protective disaccharides such as trehalose on biological membranes and liposomes has still yet to be fully elucidated, the observations which had been noted for the stabilisation of liposomes in the dried state led Crowe & Crowe to summarise the status of liposome preservation by lyophilisation in their review article (1993).

In an earlier paper, Crowe and co-workers had found that the addition of trehalose at a concentration of 0.3g *per* gram of lipid was sufficient to prevent fusion of unilamellar vesicles composed from POPC and bovine PS (9:1 molar ratio) during freeze-drying, but that 1.8g trehalose *per* gram of lipid was required in order that leakage of the small, water-soluble marker substance (isocitrate) from the same vesicles was prevented (Crowe *et al.*, 1986). However, it was concluded that the effect of increasing carbohydrate concentration was maximal at about 1g/g lipid, and that the addition of further carbohydrate after this point had very little effect. It was also observed that in order to achieve this optimal level of retention, trehalose was required both on the inside and outside of the membrane. Thus, it was necessary to trap some trehalose inside the vesicles during the original preparation. The hypothesis that vesicle fusion always resulted in a release of an entrapped substance was not confirmed in the study described above, but it was found that prevention of aggregation and fusion was essential, but not alone sufficient, for liposome preservation during drying.

Together with co-workers, Crowe & Crowe have more recently demonstrated the effects of trehalose and derivatives on membrane phospholipids in yeast cells in both the intact state and the dried state, with a view to elucidate the specific action of the saccharide in the protective process (Mansure *et al.*, 1994; Leslie *et al.*, 1994; Crowe *et al.*, 1994). The use of disaccharides as virtually the only group of successful protective agents for liposomes during lyophilisation found to date draws an interesting parallel with what Crowe & Crowe and other researchers have reported in the area of protein stabilisation by freeze-drying, a point which is discussed further in the review article by Crowe *et al.* (1987). However, further studies will be necessary before the entire mechanism of lyoprotection for these entities may be fully elucidated.

In individual studies, it has been hypothesised that the amount of protective agent, the optimal residual moisture levels in dried products, liposome size, lipid composition and distribution of solutes about the bilayer all may have a role to play in determination of the stability of a liposomal preparation. In addition to this, process and storage variables may need to be modified with the encapsulation of different active ingredients. Although the elucidation of optimum variables will naturally be very much a long-term process, the review chapter by Crowe & Crowe (1993) highlights the major factors which determine liposome stability during lyophilisation and when stored in the freeze-dried state. A summary of the factors relevant to the present chapter are given in the following sections.

5.1.4.1 Phase transitions of lipids

A lipid may exist in gel- or liquid- crystalline state, its state at any one time being dependent on temperature. In the gel-crystalline state, bilayers of lipid molecules are tightly packed, in a rigid, often (but not exclusively) hexagonal formation, whereas in the liquid-crystalline state, the packing of the bilayer is less rigid and thus more permeable to the flux of compounds both from within the aqueous core to the external medium and *vice versa*. The gel- to liquid-crystalline transition temperature is denoted by T_m, and when heated through this temperature, a lipid will undergo a characteristic increase in heat capacity, accompanying the change from the gel- to the liquid- crystalline state. Thus, the transition temperature for a lipid may be determined using differential scanning calorimetry. This technique is often applied to the analysis of liposome preparations, and may also be used to examine a number of liposome/drug compatability and stability factors (Ford & Timmins, 1989). For aqueous suspensions of liposomes containing drug compounds, the examination of the thermal characteristics of such preparations may be vital in determining storage conditions. As stated in section 5.1.3.1 above, drug leakage from liposome preparations tends to be highest around the transition temperature (T_m) of the preparation. Therefore, heating a liposome-drug preparation through its characteristic transition temperature is not advisable, since this is likely to result in the loss of encapsulated drug. This is a particularly relevant issue in the lyophilisation of liposome preparations, since the characteristic T_m value for a liposome preparation will change throughout the freeze-drying process. The removal of water from the system (and particularly from the polar head groups (PHG) of the lipid molecules) results

in a reduction in lateral spacing of the PHG and increased van der Waals interactions within the hydrocarbon chains. These phenomena subsequently cause the gel- to liquid- crystalline transition temperature to increase, since more energy will be required to cause the closely-packed PHG to overcome the attractive van der Waals interactions and enter into the liquid crystalline state. Crowe & Crowe (1993) use the example of the lipid POPC to illustrate this point. The value of T_m for POPC in the hydrated state is -10°C, whereas in the dried state, it increases to +60°C. Thus, when rehydrated at room temperature, it will undergo a phase transition from the gel- to the liquid- crystalline state. It follows, therefore, that for a drug-loaded liposome preparation consisting of POPC as the sole phospholipid with the transition temperatures quoted above, rehydration of such a preparation at room temperature is likely to be accompanied by leakage of the drug. The incorporation of trehalose into various empty liposome formulations was shown to have a marked effect on the transition temperatures of the lipids investigated (Crowe & Crowe, 1993), and this effect was shown by the authors to be clearly linked to the release of drug marker compounds from drug-loaded single-lipid preparations. The effect of the trehalose was typically to cause an elevation of T_m for the hydrated lipid, but conversely to depress T_m for the same lipid in the dry state. In each case, where the addition of trehalose meant that the preparation did not undergo a phase change on the addition of water at room temperature, retention of the marker compound was dramatically increased.

A further point was put forward by Quinn (1985), who suggested that phase transitions could lead to phase separation of components in a multi-component system. This would also lead to a release of drug or marker compound, as well as a change in the overall liposome structure. This point raises the issue of lipid mixing, an issue which is effectively discussed in the chapter dealing with thermal analysis of liposome preparations in the comprehensive thermal analysis text by Ford & Timmins (1989). Phospholipid mixtures (for example, different phosphatidylcholines (PC), or a derivatised PC with a phosphoglycerol) are often used in liposome preparations. Bilayers containing more than one component have thermal properties which differ from those of the individual ingredients. Phase diagrams may be used to reveal the ideality of mixing. Generally, when two phospholipids containing the same head group differ by two carbon atoms in their fatty acid chains, nearly ideal mixing will take

place. A difference of four carbon atoms shows deviations from ideality and once the chain length differs by six or more carbon atoms, non-ideality occurs, and monotectics may be formed which are indicative of phase separation (Ford & Timmins, 1989). It has been shown that the inclusion of cholesterol into liposome formulations modulates membrane fluidity by decreasing the rotational freedom of phospholipid hydrocarbon chains, thereby reducing bilayer permeability. Previous workers have reported that in phospholipid bilayers containing 50 mol% cholesterol, the phase transition has been lost completely (Betageri & Parsons, 1992; Taylor *et al.*, 1990). In order to minimise the possibility of phase separation occurring in the present studies, cholesterol was utilised in the liposome formulations prepared here.

Crowe & Crowe (1993) reported that studies carried out by themselves and co-workers had demonstrated that the 'phase transition model' was the most effective basis for assessing the success of protective agents for the retention of vesicle contents during freeze-drying. Thermal analysis of a model preparation consisting of POPC and bovine PS (9:1 molar ratio) by DSC had shown three transitions, at temperatures -32°C, +40°C and +57°C. On drying with increasing amounts of trehalose, the thermal energy associated with the -32°C transition steadily increased, while those of the other two transitions steadily decreased and eventually disappeared (Crowe & Crowe, 1988). The authors noted that the increase in thermal energy of the low temperature peak appeared to correlate with the retention of vesicle contents (isocitrate) in a linear fashion. It was concluded from these data, that the lower temperature peak was the transition temperature for the combined POPC/PS/trehalose, and that for the preparation which displayed only this one peak, the dry lipid/trehalose mixture was in the liquid crystalline state at room temperature, and remained in this state on rehydration. Thus, the preparation did not undergo a phase transition during rehydration, and the authors concluded that this explained the retention of vesicle contents.

5.1.4.2 Aggregation and fusion

The phenomena of aggregation and fusion of liposomes, either in the suspension state or during lyophilisation, can lead to leakage of vesicle contents (Crowe & Crowe, 1993). The potential incidence of aggregation or fusion is dependent upon the environmental conditions in which the liposome preparation is stored, as well as the nature of the liposome preparation

(and drug molecule where applicable) itself. Generally, aggregation or fusion tends to occur more readily for liposomes with little or no surface charge, but is far less common in preparations with a high surface charge, since in this situation, liposomes of like charge will have a slight tendency to repel each other. The encapsulation of a charged drug or marker molecule may dramatically affect the overall charge of the liposome, especially if the molecule is amphiphilic and therefore encapsulated partially into the bilayer interior (since hydrophilic portions of the molecule may protrude outwards from the outer bilayer membrane, thus creating a surface charge, see Figure 5.2). Buffer systems may be used in order to counteract attractive effects between liposomes in solution, but the use of buffer salts during lyophilisation is often undesirable, as in the freeze-drying of biological proteins (see chapter 4). Even if such buffer systems do contribute to the stability of liposomal preparations in aqueous suspensions, the concentration of the salts and associated pH changes which occur during freezing may damage liposome structure, leading to burst effects and/or phase separation of lipid components (according to the charged nature of individual components). While this may not be the case for all liposome preparations during the freeze-drying process - indeed, such buffer systems may stabilise certain preparations during the lyophilisation process and even when subsequently stored in the dry state - damage may still result on rehydration of the dried product. Many texts recommend that following drying, liposome preparations are rehydrated carefully according to a specifically-designed procedure (see New, 1990, for examples). It should be remembered that on the addition of the first drop of water to a dried product containing buffer salts and other excipients, the concentration of all water-soluble components suddenly becomes extremely high. This may have similar effects to those described above, leading to damage of liposome structure and leakage of vesicle contents. Therefore, it may be advisable that liposome preparations be freeze-dried in the presence of the least number of excipients possible, at the lowest concentration possible, in order to provide the necessary levels of protection for the process.

5.1.4.3 Proposed mechanisms for the preservation of dry liposomes

Crowe and co-workers originally chose to investigate the use of disaccharides as protective agents for liposomes following their success in examining the freeze-thaw and dehydration

stability of plant cells. The initial belief was that disaccharides would behave as cryoprotectants for liposomes, but would also have the added property of maintaining the hydration of the bilayer. However, practical investigations carried out to test this hypothesis demonstrated that the water content of a sample did not tend to increase or decrease when increasing amounts of trehalose were added (Crowe *et al*, 1987a) and the authors concluded that the retention of water was not required for successful stabilisation of lipid bilayers during dehydration. It was thus suggested that the stabilisation may have been due to a direct interaction between the disaccharide(s) and bilayers, with the saccharide acting as a water-substitute rather than maintaining true hydration of the lipids. This became known as the "water-replacement hypothesis". A similar suggestion had also been made around the same time by Strauss *et al.* (1986). In later studies, Crowe and co-workers demonstrated direct interactions between trehalose and polar headgroups of dry PCs (reviewed in Crowe *et al.*, 1987b), and concluded that these direct interactions involved hydrogen bonding between the -OH groups on trehalose and polar residues in the lipid headgroups. The authors presented several lines of evidence to support this suggestion, one of which was the results from molecular modelling studies, which showed that direct interaction between trehalose and dry DMPC is feasible (Chandrasekhar & Gaber, 1988; Rudolph *et al*, 1990). Later reports by numerous workers (Assadullahi *et al.*, 1992; Park & Huang, 1992; Talsma *et al.*, 1992; Tanaka *et al.*, 1992; Talsma & Crommelin, 1993; Ausborn *et al.*, 1994; Engel *et al.*, 1994; Mobley & Schreier, 1994) have tended to support the water-replacement hypothesis and the theory that direct interaction is the most feasible mechanism by which lyoprotectants afford freeze-dry stabilisation to lipid bilayers.

The water replacement hypothesis has been called into question by some workers (Green & Angell, 1989; Levine & Slade, 1992). Several workers have emphasised the importance of glass formation in organisms which survive drying, which appears a legitimate emphasis, since it is evident that vitrification is necessary for successful preservation of organisms during deydration (reviewed in Crowe *et al.*, 1992). However, as Crowe *et al.* (1994b) maintain, the two hypotheses are not necessarily mutually exclusive, yet it appears that some workers, either directly or by implication, have suggested that vitrification is the *only*

requirement for preservation of liposomes and proteins during freeze-drying (Koster & Leopold, 1988; Williams & Leopold, 1989; Bruni & Leopold, 1991; Leopold *et al.*, 1992). Data presented by Crowe *et al.* (1994b) showed that for liposomes composed from saturated phospholipids, vitrification was sufficient for retention of vesicle contents during lyophilisation, but that for a preparation composed solely from unsaturated phospholipid, vitrification was necessary but not itself sufficient to afford full freeze-dry protection. The reason for this difference in requirements for the two preparations tested was explained in terms of the difference in phase transition temperatures of the two lipids, and the effect of stabilising additives on T_m. Thus, it was demonstrated that the water-replacement hypothesis and vitrification are not necessarily mutually exclusive.

Rodin & Izmailova (1994) reported that using NMR to study the effect of disaccharides on the stability of PC vesicles at low temperatures, it was possible to examine potential interactions at the molecular level by assessing differences in spectra for individual components (sugars and lipid bilayers) and mixtures. Due to the complexity of the spectra produced by liposomal formulations (particularly mixtures of lipids, drug(s) and additives), it is unlikely that such a method could be utilised other than in the simplest of model cases. However, the NMR peak shifts in the data presented by the authors does seem to indicate that some type of interaction was occurring at the molecular level, even though the evidence does not allow the water-replacement or true hydration to be distinguished, as in the analogous case of protein stabilisation during lyophilisation (see section 4.1).

Therefore, it seems apparent that the phase transition model, where a protective additive influences the phase transition of the preparation *via* direct hydrogen bonding in a vitrified mass, is the most favoured mechanism for protective interaction between saccharides and lipid bilayers.

5.1.5. Objectives of Studies detailed in this Chapter

The primary objective of the work carried out in this chapter was to investigate the effects of freeze-drying and the use of lyoprotectants on the formation, drug encapsulation, remote loading, suspension stability and dry-state stability of sterically stabilised liposomes (SSL),

building on the concepts detailed above in sections 5.1.3.2. and 5.1.3.3, as well as on the results reported by McAllister (1995) from work carried out in these laboratories. For each of the following studies, a brief introduction is given.

5.2 Investigation of simultaneous loading/rehydration of pre-formed, empty sterically stabilised liposomes (SSL), prepared by the DRV method, using polymyxin B (PXB) as a model drug.

5.2.1 Background

As stated above (section 5.1), the passive loading of drugs into pre-formed liposomes may be useful in a number of instances. McAllister (1995) reported the successful loading of the cystic fibrosis drug polymyxin B (PXB) (Figure 5.4) into sterically stabilised liposomes (SSL) for the purpose of pulmonary delivery. These SSL preparations were composed from EPC, cholesterol and DSPE-mPEG$_{1900}$ (EPC:chol in a 2:1 molar ratio; pegylated lipid at 5.0 mol%) and were prepared using the DRV method first reported by Kirby & Gregoriadis (1984), which has more recently been effectively summarised by New (1990). The DRV preparation procedure normally involves the addition of an aqueous drug solution to an aqueous suspension of pre-sonicated SUV prior to a lyophilisation step (see figure 2.9, stage 4). The aim of this experiment was to investigate the possibility of simultaneous loading/rehydration (see section 5.1.3.2) of SSL prepared by the DRV method, using PXB as a model drug, to characterise preparations with respect to size, surface charge and thermal characteristics and to compare these characteristics and drug loading data with a batch of conventional (non-stealth) liposomes prepared under identical conditions but with the omission of the pegylated lipid. Polymyxin B is one of a range of five polymyxins, which are a group of basic polypeptide antibiotics first isolated in 1947 from a spore-bearing soil bacillus (*Bacillus polymyxa*). The structure of the amphiphilic polycation PXB comprises a cyclic heptapeptide moiety attached to a tripeptide side chain which terminates with a fatty acyl residue (Figure 5.4). The potential use of liposomes as carriers for PXB in the treatment of cystic fibrosis lung infections was reported recently by McAllister (1995), although the interaction of PXB and phospholipids has been the subject of many earlier reports, where workers have

investigated the interaction using lipid monolayers or exogenously applied PXB with empty liposomes. McAllister (1995) reported that studies using model liposome systems showed that PXB interacts only weakly with zwitterionic lipids. Imai *et al.* (1975) found that the interaction of PXB with PC liposomes resulted in low levels of release of entrapped marker (glucose) which could be further diminished by the addition of cholesterol. Teuber & Miller (1977) demonstrated that the adsorption of PXB to PC monolayers was much lower than that to other lipids. However, care is needed in the extrapolation of monolayer interactions to liposome systems due to the influence exerted by surface curvature in binding events. It was concluded from these reports, that the compatability of PXB and PC would be sufficiently acceptable for the purposes of the present study.

5.2.2 Materials & Methods

5.2.2.1 Materials

Egg lecithin (egg phosphatidylcholine, EPC), supplied in powder form, was a generous gift from Genzyme Ltd., UK. Polymyxin B (PXB) and cholesterol (chol) were supplied in powder form from Sigma Chemical Co., Poole, UK. DSPE-mPEG$_{1900}$ was a generous gift from Liposome Technology Inc., USA, supplied in dry powder form. Tritiated PXB, which was used as a radioactive tracer for the Sigma PXB, was supplied by NEN products, UK.

5.2.2.2 Preparation of Liposomes

Liposomes were prepared by the DRV method as described in section 2.12, with rehydration of the dried (empty) liposomes (see Figure 2.9, stage 5) carried out using an aqueous solution of PXB (2.00mg/ml) to give a drug:lipid ratio of 7.5μmol:66μmol. For stealth preparations, this ratio was calculated using the amount of lipid excluding the stealth lipid. Half of the batches were subjected to a further freeze-drying cycle (conditions identical to that of the first cycle) in the presence of the PXB rehydrant solution, in order to ascertain whether this extra freeze-drying step would lead to greater loading efficiency. The choice of lipids, drug-to-lipid ratio and method of liposome preparation are outlined in the following sections.

FIGURE 5.4: Structure of Polymyxin B (PXB) (from Lawrence et al., 1993)

5.2.2.2.1 Choice of method of preparation

The DRV method employed in these studies was the procedure described by McAllister (1995), adapted from Kirby and Gregoriadis (1984), and is detailed in Figure 2.9 (section 2.12). The reason for using the DRV method for the purposes of the present study were two-fold. Firstly, the intentions of the studies detailed in this chapter were to examine the role of lyophilisation in the preparation of liposomes and in the stabilisation of pre-formed liposomes loaded with a model drug compound, the application of freeze-drying to remote loading of drugs into SSL and to investigate the effects of saccharide additives on the formation and stability of stealth liposome formulations. With regard to remote loading, the choice of a preparative method which already involved freeze-drying was believed to be a logical step, since it would be relatively simple to examine the effects of adding drug before or after the lyophilisation stage of the preparation protocol on loading efficiency. Secondly, alternative preparative methods such as the REV method proposed by Szoka & Papahadjopoulos (1978) involve the use of organic solvents up to the very final stages of

preparation. In such cases, trace amounts of organic solvent can often prove difficult to remove from the preparations, and consequently could present a number of problems when attempting to study freeze-drying characteristics of such liposomes, leading to possible artifacts in stability data through drug-solvent interaction or loss of lipid (and/or drug) into emulsive regions existing in the preparations. Finally, on the practical level, organic solvents can prove damaging to freeze-drying equipment, in particular to vacuum pumps, whose numerous rubber seals are susceptible to attack by such solvents. Thus, the DRV method was seen to be the method of choice in the present study.

5.2.2.2.2 Choice of lipids

There is a wide range of lipids available to the formulation scientist for the preparation of liposomes, including both natural (isolated) and synthetic lipids. The lipid components employed in the present study were selected according to a number of different criteria. Egg phosphatidylcholine (EPC) was employed, since it is probably the most commonly-used lipid in liposome preparations (see section 5.1.2), much is known about its behaviour in bilayer systems and it could be obtained in a well-characterised form which is assayed for purity and found to be consistently >99% pure. Cholesterol was selected for incorporation into liposome preparations in the present study due to its bilayer rigidifying property, whereby it interacts with acyl carbon moieties on the lipid chain to maintain a gel- crystalline phase. McAllister (1995) found that the inclusion of cholesterol at 33mol% in combination with EPC significantly reduced the energy of the gel- to liquid- crystalline transition. Finally, for the sterically stabilised liposome (SSL) preparation, a derivatised form of DSPE was used; in this case, the DSPE headgroup was covalently coupled to mPEG (MW 1900), giving an overall molecular weight of 2650. The use of this pegylated lipid was believed to endow the liposomes with a surface charge, leading to reduction (or elimination) of aggregation or fusion (Woodle & Lasic, 1992). Lasic (1994) stated that 5mol% of pegylated lipid was required to give stealth liposomes with 'mushroom' formation (Figure 5.3(a)), higher concentrations could be used which give 'brushes' (Figure 5.3(b)) but it was previously found that in the use of DSPE-mPEG$_{1900}$, concentrations of 15mol% or higher began to disperse vesicles with the formation of non-vesicular structures such as tubules (Lasic, 1994). In the same study, it was suggested that the interaction of PEG-PE with bilayers involved

changes in the relative areas of inner and outer monolayers. McAllister (1995) believed that although the concentration of 6.25mol% of DSPE-mPEG$_{1900}$ used in his studies was insufficient to cause gross defects in liposome morphology, an interaction with membrane-bound PXB may have been sufficient to promote membrane defects with a subsequent rise in permeability. In the present study, the ratio of lipid components used in a '33μmol' formulation was 22:11:1.732 (μmol, EPC:chol:DSPE-mPEG$_{1900}$ respectively), which was calculated to give 5.0mol% of pegylated lipid.

5.2.2.2.3 Choice of PXB to lipid ratio

McAllister (1995) has investigated a range of PXB:lipid ratios and has concluded that encapsulation efficiency was highest when a ratio of 8.5μmol PXB to 66μmol lipid was used. The reduced encapsulation efficiencies observed at higher loading ratios (17-34μmol drug to 66μmol lipid) were believed to be due to some interference with vesiculation, or a saturation of membrane binding of PXB. However, given the weak interaction with neutral phospholipids, it was believed that binding to EPC bilayers was not likely to be the major site of encapsulation, since the majority of PXB should be encapsulated within the aqueous volume of the DRV. In addition, the critical micelle concentration of PXB has been previously reported as 6% (w/v) (Lawrence *et al.*, 1993). Since the first rehydration step routinely involved the addition of an aqueous volume of 200μl, a 12mg drug loading would result in the formation of PXB micelles which may not be encapsulated as efficiently as free drug and may interfere with bilayer assembly during the initial rehydration step. In the present study, an 8mg loading was adopted as the maximum drug to lipid ratio when 66μmol of lipid was used. Loadings may be increased with higher lipid quantities (Wichert *et al*, 1992), but the 66μmol was used to maximally encapsulate PXB using minimal phospholipid quantities.

5.2.2.3 Analysis of liposome size

Size distributions of conventional (non-stealth) liposome preparations were determined using photocorrelation spectroscopy, as detailed in section 2.13, whilst those of SSL preparations were determined using laser diffractometry, as described in section 2.14. Since a bias was generally observed in the size distribution towards a small number of larger particles, the

values quoted in the present study represent the number mean size (±s.d.) of the population, which reduces the extent of mean bias resulting from the presence of such particles.

5.2.2.4 Investigation of liposome zeta potentials

Determination of the Zeta potential for all liposome preparations was carried out following dilution of the samples in 0.001M potassium chloride solution, using the Malvern Zetamaster, as detailed in section 2.15.

5.2.2.5 Encapsulation of PXB

Encapsulations were estimated both directly from the resuspended liposome pellet and indirectly from the supernatant washes. Good agreement was seen between direct and indirect values, with variation between the methods typically around ±10%. The results discussed in the present study are those determined by the direct method. McAllister (1995) reported that in early studies, discrepancies had been noted for [^{3}H]-PXB encapsulations determined by indirect and direct means for liposomes which had been prepared by the dehydration-rehydration vesicle (DRV) method. In these cases, the values determined by counting of [^{3}H]-PXB in the supernatant washes of EPC liposomes were approximately 30% greater than those determined directly from the counts of washed liposome samples. The cause of this discrepancy was found to be the freeze-drying step of the DRV process. Free solutions of PXB were found to undergo a loss of approximately one-third of their activity during this lyophilisation step, with a smaller decrease noted after a second cycle. Analysis by HPLC and BCA protocols showed no change in PXB concentration, indicating that a physical loss of drug had not occurred. McAllister concluded that the loss of tritium was possibly due to exchange processes with the buffer used, and that, in any case, the losses of activity were generally reproducible (39.12±4.91%, n=16).

In the present study, measures were taken in order that such discrepancies be avoided. Firstly, all liposomes prepared by the DRV process were lyophilised from solutions of drug (together with a saccharide additive, where appropriate) in water only. Due to the nature of the liposome studies carried out as part of this project, it was believed that the use of buffer salts in liposome suspension medium could potentially lead to significant problems. In

particular, the subsequent rehydration of a dried liposome pellet containing buffer salts would, on addition of the first drop of resuspending medium (often water only), lead to high salt concentrations which could conceivably result in the lysis of liposomes (or a disruption of bilayers) together with unknown charge effects on any PXB present. Secondly, scintillation counting was carried out on both supernatant washes of the liposome preparations as well as on samples of the resuspended pellets themselves. It had been noted that the scintillation fluid used in the present study caused lysis of liposomes and a consequent release of vesicle contents, and that counting discrepancies due to potential 'masking' of [^{3}H]-activity by free lipid was not apparent. The encapsulation values were calculated by taking all sample counts into consideration, and expressing the counts associated with the resuspended washed pellet as a percentage of the total counts (sum total of pellet + first wash supernatant + second wash supernatant). As detailed in previous reports, it was found that two supernatant washes were sufficient to remove any extraneous, unencapsulated drug from the liposome pellet, with typically more than 90% of the unencapsulated drug recovered from the first centrifugation step and more than 90% of the remainder from the second. This suggests that the inclusion of a further centrifugation step would only allow the recovery of such a trace amount of remaining drug (possibly not accurately detectable within experimental limits), that to include such a step may only serve to introduce additional errors into the experiment.

Preliminary investigations were made into the use of extraction procedures whereby liposomes could be lysed and encapsulated (non-radiolabelled) PXB released, which could then be analysed by the BCA/microassay, as detailed in section 2.8. A chapter by Barenholz and Amselem (1993) in a liposome textbook gave reference to a number of methods for such extraction, although these methods had essentially been developed for the extraction of lipids from animal tissue. Two of these methods were investigated to establish their suitability for the lysis and extraction of PXB from liposomes; namely those first reported by Dole (1956) and Bligh & Dyer (1955). Of these two procedures, it was found that the method described by Dole was practically simpler and more effective at causing lysis of liposomes than that reported by Bligh & Dyer. However, on analysis of the extracts by the BCA assay (or microassay) given in section 2.8, the lysates gave consistently high apparent PXB concentrations (25-30% higher than maximum theoretical concentrations) for liposome

preparations containing known amounts of PXB. This increase was believed to have been caused by the presence of extraneous lipid(s) dissolved in the organic solvent mixture which may have participated in the BCA reaction together with PXB. As the scintillation method had proved consistent and in good keeping with analytical data from supernatant washes assayed by the BCA method, it was decided that the extraction method should not be continued in further studies. For the present study, the scintillation method alone was used.

5.2.2.6 Thermal analysis of preparations using DSC

Dried liposome preparations (pre-rehydration) and aliquots of liposome suspensions were subjected to thermal analysis using differential scanning calorimetry (DSC), as detailed in section 2.5 of this report.

5.2.3 Results and discussion

The data from the analyses carried out on liposome preparations in this study are summarised in Table 5.1.

Preparation	Mean Size (μm)	Mean Zeta Potential (mV)	Encapsulation (mg PXB.g^{-1} lipid)
Empty, non-stealth	3.79 (±0.52)	-36.8 (±7.8)	n/a
Empty, non-stealth (FFD†)	3.73 (n=1)	-28.4 (n=1)	n/a
Loaded, non-stealth	3.16 (±0.10)	-18.95 (±5.3)	5.01 (±0.00)
Loaded, non-stealth (FFD†)	2.84 (±0.25)	-14.6 (±6.1)	19.64 (±5.87)
Empty, stealth	0.152 (±0.021)	-35.9 (±2.7)	n/a
Empty, stealth (FFD†)	0.134 (n=1)	-33.8 (n=1)	n/a
Loaded, stealth	0.301 (±0.060)	-18.5 (±8.5)	10.33 (±0.63)
Loaded, stealth (FFD†)	0.353 (±0.074)	-15.7 (±4.2)	19.60 (±0.87)

†Preparations further freeze-dried in presence of rehydrant solution

TABLE 5.1: Size, zeta potential and PXB-loading characteristics of liposome batches

Replicate batches of liposome preparations displayed similar size and zeta potential characteristics, indicating good reproducibility between batches. The (number) mean size of the liposome preparations appeared to be affected both by drug loading and by the incorporation of pegylated lipid. The loading of the positively-charged PXB into the bilayers also caused a marked increase in zeta potential. The projected size for SSL (less than 100nm) is essentially for unilamellar liposomes, since stealth liposomes appear to be most commonly prepared by methods which lead to the production of unilamellar vesicles (Woodle & Lasic, 1992). Since the liposomes in the present study were prepared by the DRV method, the expected vesicle structure would be multilamellar, thus producing liposomes with a larger diameter than SUV. This may explain the large particle diameters observed for preparations in the present study. The presence of the polymer attached to lipid headgroups has been shown to give a liposome a larger spatial volume in aqueous medium, although for PEG with an average molecular weight of 1900, the chain length would increase liposome diameter by less than 10nm (Lasic, 1996). A further suggestion for the relatively large vesicle diameters might be that some of the pegylated lipid had become detached from the liposomes (possibly due to phase separation), thus leading to the presence of non-stealth liposomes in the suspension. While it would not be possible to prove this theory without the use of further techniques, such as the use of radiolabelled stealth lipid, several facts suggest that this is not the case. Firstly, the vesicles are still markedly smaller than those containing no stealth lipid (typically 300nm, compared to 3μm), suggesting that a substantial 'stealth' element is present. Secondly, if phase separation had occurred, then this would most likely have been due to temperature fluctuations within the medium. This would have caused a total loss of stealth lipid from many or all of the liposomes in the suspension, rather than the loss of small amounts of stealth lipid from all liposomes. As such, this phase separation would have led to the existence of discrete groups of liposomes containing either 5mol% pegylated lipid or no pegylated lipid. The size distribution pattern gave no indication of the existence of discrete groups such as these, since the distribution for each preparation was monomodal. Therefore, in the present study, it was concluded that the reason for the liposomes being of more than 100nm diameter could be attributed to the fact that the use of the DRV method had resulted in the production of MLV, as opposed to the more typical

unilamellar vesicles produced by other methods of preparation, and that sterically stabilised liposomes had nevertheless been formed using the DRV method.

For the conventional (non-stealth) liposomes, vesicle size appeared unaffected by the presence of PXB in the preparations. This suggests that either the inclusion of PXB did not have a marked effect on lipid headgroup spacing or interlamellar distance, or that any such effects were compensated by other factors (for example, a decrease in the number of lamallae present in vesicles). It was noted that all zeta potentials for non-loaded liposomes were more negative than typical values given in literature for empty liposomes composed from neutral phospholipids (Gregoriadis, 1993). This was attributed to the EPC used in the present study, which was of unknown purity. In future studies, a form of EPC was used which was >99% pure (see following sections). However, the increase in zeta potential of all preparations when loaded with PXB suggests that some surface interaction may have taken place between PXB and the lipid bilayers. In the case of the stealth liposomes, the marked increase in vesicle size when loaded with PXB was perhaps a result of PXB-bilayer interaction and an increase in interlamella distance, the presence of PEG possibly contributing to a greater degree of interaction than in the conventional liposome preparations, and thus making the size increase due to PXB loading more marked. McAllister (1995) had previously observed that a proportion of PXB in liposome preparations often remained associated with the bilayer, instead of becoming wholly encapsulated within the aqueous compartment. Although it was believed to be only a small fraction of PXB which behaved in this manner, it is likely that, in the present study, PEG coupled to the DSPE headgroups may have interacted with the amphiphilic PXB, resulting in a larger proportion of PXB remaining attached to the outer surface of the liposome than may have been the case for conventional liposome preparations.

The encapsulation values for PXB in stealth and non-stealth preparations are given in Table 5.1. The efficiency of using simultaneous loading/rehydration to load PXB into the stealth liposomes prepared in the present study appeared to be considerably lower than the encapsulation efficiencies reported by McAllister (1995), where the DRV method was followed in the conventional manner. Indeed, even though the loading was seen to increase

following this second lyophilisation step, the levels of encapsulation observed still did not equal those reported by McAllister (1995) for similar stealth preparations which were produced using the conventional DRV method. One possible reason for this difference in efficiency is that the DRV method relies on the breakdown of SUV in the presence of drug during dehydration and their subsequent reformation into MLV upon rehydration in order that high levels of loading are effected. In the simultaneous loading/rehydration procedure followed in the present study, the drug solution was added after the SUV had been broken down by dehydration. In this case, it may be possible that the formation of MLV had occurred before the PXB was able to associate with the bilayers.

The fact that the drug loading was seen to increase following further freeze-drying in the presence of the PXB rehydrant solution suggests that breakdown of the MLV occurred during drying, with the subsequent reformation of bilayers leading to an increased lipid-PXB interaction upon rehydration. The loading efficiency of the stealth liposome preparations was seen to approximately double following this extra lyophilisation step, while that of the conventional liposome preparations apparently increased to four times the original value. Since the only difference in preparations was the inclusion or exclusion of the pegylated lipid, it is likely that the pegylated lipid had played a role in determining the extent of loading efficiency. It is possible that the presence of the pegylated lipid conferred greater stability on the stealth liposomes which rendered these preparations less susceptible to breakdown upon dehydration than conventional preparations. Indeed, SSL are less prone to aggregation or fusion during phase transitions due to the surface charge resulting from the polymer (Woodle & Lasic, 1992). This may explain why the PXB loading doubled for SSL, yet quadrupled for conventional liposomes in the present study.

Thermal analysis using DSC showed that the expected gel- to liquid- crystalline transition temperatures of 41°C and -10°C for EggPC in the dried state and hydrated state, respectively, were apparently non-existent in the current preparations. This suggested that the concentration of cholesterol present in the formulations (~31mol%) was sufficient to rigidify the lipid structure to such an extent that no detectable transition was apparent at these expected temperatures. Even in the case of sterically stabilised liposomes, the

pegylated lipid did not appear to counteract the effect of cholesterol at the concentration used (5mol%), as had previously been reported (Lasic, 1994).

5.2.4 Conclusions

Sterically stabilised liposomes were prepared using the DRV method. These liposomes were larger in diameter than typical SSL, which was attributed to method of preparation which is known to produce liposomes with a multilamellar structure. Levels of PXB loading were comparable to those reported by Dürr *et al.* (1994) for the loading of doxorubicin B (DXR) into conventional EPC liposomes, on the basis of mg drug *per* gram of lipid. However, loading efficiency was still far lower than that reported by McAllister (1995) for the loading of PXB into similar liposomes following the original DRV method. Loading was observed to increase in the present study when a second freeze-drying step was included, in which liposomes were dried in the presence of the PXB rehydrating solution. Size and zeta potential of preparations were both observed to increase with drug loading, and both were also affected by the inclusion of pegylated lipid in the preparations. The presence of cholesterol at 33mol% reduced the energy of the phase transition (T_m) of preparations, but did not result in the total elimination of these transitions.

5.3 Investigation of the effects of saccharide additives on the formation, encapsulation efficiency and suspension stability of SSL containing PXB

5.3.1 Background

The aim of this study was to examine the effect of two saccharide additives, trehalose and mannitol, on a number of physical characteristics relating to a model SSL formulation. Firstly, the effect on liposome formation would be studied by examining the size distribution and gross morphology of preparations. Secondly, the effect of additive (and concentration) on the PXB encapsulation efficiency would be examined. Finally, the effect of the additives on the stability of liposomes in aqueous suspension would be assessed by the monitoring of leakage of PXB from the liposomes during storage.

Trehalose is often selected as an excipient in pharmaceutical formulations for its non-reducing nature and relative lack of chemical reactivity which render it stable to degradative reactions with other components (Colaco *et al.*, 1994). As stated in the introduction to the present chapter, trehalose has also been observed to afford stabilisation to liposomes during drying, leading to the total retention of vesicle contents on rehydration. Crowe & Crowe (1993) suggested that the chief reason why trehalose was able to afford protection to liposomes was that its size rendered it able to become inserted between PHG of bilayer lipids and form hydrogen bonds with these groups, thus maintaining PHG spacing even when water was removed on drying. The authors hypothesised that it was this interaction which also caused the changes in dry- and hydrated- state T_m of the bilayers which stabilised preparations by reducing phase transitions.

Mannitol is also a popular choice of excipient in freeze-drying due its crystallising properties which impart rigidity to lyophilised products, which might otherwise display undesirable characteristics such as shrinkage or stickiness. However, mannitol is not as chemically unreactive as trehalose, and its crystallising behaviour can lead to a loss of activity in some sensitive biomaterials with which it is sometimes formulated. It was believed that the use of mannitol in the present study may provide a contrast to the predicted success of trehalose as a lyoprotectant.

5.3.2 Materials & Methods

5.3.2.1 Materials

Egg lecithin (egg phosphatidylcholine, EPC) was supplied at a concentration of 100mg/ml as a 99% pure solution in methanol:chloroform (1:1) by Lipid Products, Surrey, UK. Polymyxin B (PXB) and cholesterol (chol) were supplied in powder form from Sigma Chemical Co., Poole, UK. DSPE-mPEG$^{(1900)}$ was a generous gift from Liposome Technology Inc., USA, supplied in dry powder form. AnalaR grade trehalose and mannitol were supplied by BDH, UK.

5.3.2.2 Preparation of liposomes

Preparation of liposome batches was carried out according to the DRV procedure outlined in section 2.12. PXB was added to the vesicles following sonication and prior to the lyophilisation stage of the preparative procedure (Figure 2.10, stage 3). The PXB:lipid ratio was identical to that given in the previous study: 8mg PXB in a 66μmol preparation. As Crowe & Crowe (1993) had reported that the encapsulation of trehalose into the liposomes was necessary to provide stabilisation to bilayers during later freeze-drying (as well as the addition of trehalose solution to the liposome suspension to provide protection to outer bilayers), it was also believed necessary to encapsulate some additive at the same time as PXB in the present study. The use of a range of additive:lipid weight ratios were investigated in the present study, namely 1:1, 1:2 and 1:4 (wt. additive : wt. lipid) of mannitol and trehalose (each preparation in duplicate) and equal ratios of additive were used on both the interior and exterior of the bilayers. In addition, duplicate batches of liposomes with no additive were prepared.

5.3.2.3 Size distribution analysis

The size distributions of conventional (non-stealth) liposome preparations were determined using photoncorrelation spectroscopy, as detailed in section 2.13, whilst those of SSL preparations were determined using laser diffractometry, as described in section 2.14. As stated in section 5.2.2.3, a skew was generally observed in the size distribution towards a small number of larger particles and thus the values quoted in the present study represent the

number mean size (±s.d.) of the population, which reduces the extent of mean skew resulting from the presence of such particles.

5.3.2.4 Zeta potentials

Determination of the Zeta potential for all liposome preparations was carried out following dilution of the samples in 0.001M potassium chloride solution, using the Malvern Zetamaster, as detailed in section 2.15. Potentials were measured prior to the lyophilisation step of the liposome preparation process and after rehydration of the dried pellet. In this instance, the rehydrated pellet was centrifuged and resuspended in 0.001M KCl solution prior to analysis.

5.3.2.5 Gross morphology using transmission electron microscopy

The gross morphology of a representative sample of the liposome preparations, and of samples taken at various stages during the preparation process, was investigated by transmission electron microscopy using a negative staining technique, as described in section 2.17.

5.3.2.6 Measurement of loading of PXB and leakage into aqueous medium

Encapsulations were estimated indirectly by analysis of the supernatant washes by the BCA assay and microassay protocols, as detailed in section 2.8.

5.3.2.7 Differential Scanning Calorimetry (DSC)

Thermal analysis of preparations was carried out using DSC, in accordance with the method described in section 2.5 of this report.

5.3.3 Results & Discussion

5.3.3.1 Size, zeta potentials and gross morphology of preparations

The size distribution and zeta potential for empty liposome preparations are given in Table 5.2, while those for liposome preparations containing the model drug PXB are shown in Table 5.3, together with encapsulation data. TEM micrographs are given in Figure 5.5.

5.3.3.1.1 Characteristics of empty vesicles prior to lyophilisation

From the data given in Table 5.2, it appears that neither trehalose nor mannitol demonstrated a marked effect on the size distribution of vesicles prior to lyophilisation. This finding would support the theory that any lipid-additive interaction takes place as the solution (suspension) approaches freezing point and the solutes become more concentrated. All preparations exhibited size distributions which are characteristic of SUVs (Gregoriadis, 1991).

ADDITIVE (mass ratio of additive to lipid)	Mean Particle Diameter (nm, ±sd)		Mean Zeta Potential (mV)
	Pre-freeze-drying	Post-freeze-drying	
No additive	32.3±1.4	290±57	-3.2±0.6
Trehalose (1:1)	41.9±4.7	150±13	-1.7±0.2
Trehalose (1:2)	41.5±5.2	134±25	-2.1±0.5
Trehalose (1:4)	39.8±3.0	242±22	-1.5±0.3
Mannitol (1:1)	39.0±2.5	258±45	-2.3±0.3
Mannitol (1:2)	36.8±2.9	316±40	-2.1±0.4
Mannitol (1:4)	37.6±2.7	288±32	-1.4±0.2

TABLE 5.2: Particle size and zeta potential data for empty sterically stabilised liposomes

The use of TEM to examine the gross morphology of a sample of empty vesicles taken prior to the lyophilisation step in the preparation process revealed that many of the vesicles present at this stage were MLV, even though the mean particle size was observed to be between 32-42nm for all preparations using PCS. This discrepancy may have been due to a change in physical change in the vesicles over time whilst in aqueous suspension, since the TEM analysis was carried out later than the particle size analysis. A physical alteration appears plausible for two reasons. Firstly, distilled water was used as the aqueous suspension medium, thereby rendering the liposomes susceptible to subtle changes in micro-environment. Secondly, the stability of the SUV in the aqueous medium may have been outweighed by the stresses of curvature associated with the bilayers (particularly inner bilayers) of such small vesicles. In instances where such stresses are known to exist, the tendency for SUV to break

open and reform as larger, more thermodynamically stable vesicles has been observed (Lasic, 1988). In the case of sterically stabilised liposomes, one might normally expect not to observe such an apparent lack of stability in suspension, especially since such vesicles have been shown to display remarkable *in-vivo* stability, with blood-circulation half-lives of dozens of hours (Lasic, 1996). However, in such studies, the liposomes under investigation will have been administered in isotonic media (or similar buffering systems) where they have already been observed to exhibit acceptable levels of stability; in the present study, the fact that no buffering system was used may account for the relative instability of the liposomes prepared here. It may even be argued that the stresses of curvature for stealth liposomes might be even greater than in conventional liposome preparations, due to the presence of poly(ethylene glycol) (or other similar molecule) protruding into the aqueous medium and (perhaps more crucially) into the internal aqueous space of the liposome itself from the polar headgroups of the stealth lipids present in the bilayer. The Zeta potentials of these preparations also appeared unaffected by the presence of either trehalose or mannitol, the slightly negative potential being attributable to the combination of lipids used in the preparations.

5.3.3.1.2 Characteristics of empty vesicles following lyophilisation and rehydration

The size distributions of the empty vesicle preparations following lyophilisation and rehydration appear to show good batch-to-batch reproducibility (see Table 5.2), but that size was markedly affected by the presence and concentration of additive used. The Zeta potentials (Table 5.2) for the empty liposomes after the freeze-drying and rehydration procedures were in good keeping with those observed prior to these steps (see section 5.3.3.1.1), suggesting that although the liposomes had undergone a distinct alteration in both structure and size, the presence of trehalose or mannitol did not affect the overall surface charges of the liposomes during these processes. The presence of PEG on the outer surfaces of the liposomes may have provided steric hindrance to trehalose and mannitol and thus impeded potential interactions between lipid and additive.

5.3.3.1.3 Characteristics of vesicles in PXB solution prior to lyophilisation

It may be seen from Table 5.3 that the mean particle size of the vesicles in PXB solution prior to lyophilisation was between 28nm and 32nm. Good reproducibility was observed between duplicate batches and the mean sizes were comparable with those of the empty vesicles detailed in section 5.3.3.1.1 above. The mean size did not appear to be related to the type or amount of additive present, but as in the case of the empty liposomes described in section 5.3.3.1.1, this finding would support the theory that any lipid-additive interaction takes place as the solution (suspension) approaches freezing point and the solutes become more concentrated.

ADDITIVE (mass ratio of additive to lipid)	Mean Particle Diameter (nm)		Mean Zeta Potential (mV)	Drug Loading (mg PXB *per* gram lipid)
	Pre-freeze-drying	Post-freeze-drying		
No additive	28.8±2.0	270±21	-2.0±0.8	38.2 (±8.6)
Trehalose (1:1)	31.2±1.4	251±22	0.2±0.3	30.1 (±13.5)
Trehalose (1:2)	31.8±0.5	311±27	1.8±0.7	40.0 (±3.1)
Trehalose (1:4)	29.8±0.5	288±26	0.6±0.3	42.4 (±6.8)
Mannitol (1:1)	30.8±1.1	259±23	1.9±0.2	29.8 (±0.9)
Mannitol (1:2)	31.0±0.9	259±25	0.9±0.3	26.4 (±9.3)
Mannitol (1:4)	29.4±1.7	262±20	0.2±0.5	26.3 (±17.4)

TABLE 5.3: Size, zeta potential and encapsulation data for liposomes containing PXB

The Zeta potentials of the batches also appeared to bear no relation to the type or amount of additive used. The Zeta measurements were between 2.3mV and 3.3mV (data not shown) and the positive values were attributable to the fact that measurements were made in the presence of PXB in the suspension, since PXB exhibits a positive charge at pH neutral conditions. It is possible that some of the free PXB had become associated with the liposome surface following the simple addition of PXB solution to the suspension of empty

SUV following sonication. However, it is not possible to ascertain the extent to which such an association may have occurred, since any shift of the liposome surface potential in a positive direction would be masked by the presence of any free PXB remaining in the suspension, thus rendering the alteration undetectable.

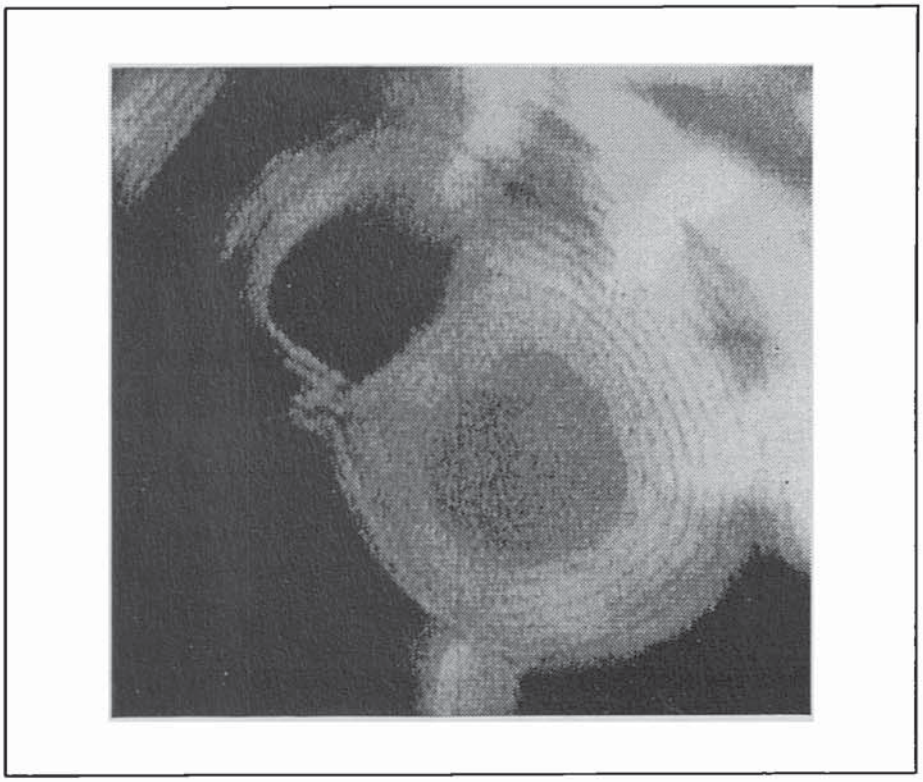


FIGURE 5.5: Transmission electron micrograph of an empty sterically stabilised liposome viewed under negative stain (1cm represents 20nm)

5.3.3.1.4 Characteristics of PXB-loaded vesicles after lyophilisation and rehydration

The data from particle size analysis (see Table 5.3) showed that the mean size of the vesicles following lyophilisation and rehydration ranged from 251nm to 311nm and that the preparations demonstrated good batch-to-batch reproducibility. The Zeta potentials of the preparations following lyophilisation and rehydration were less negative than those of the empty liposomes (see section 5.3.3.1.2), which suggests that some PXB had become associated with the outer bilayer surface during the process, thus causing an increase in overall Zeta potential of the liposomes. The similarity in mean particle size and Zeta

potentials of the preparations in the present study suggested that the type and amount of additive had little or no effect on liposome size or surface charge.

5.3.3.2 Encapsulation of PXB

The encapsulation efficiency for PXB (Table 5.3) in the present study was appreciably higher than that observed for preparations in the simultaneous loading-rehydration study described in section 5.2. The Zeta potentials described above appear to reflect the PXB-encapsulation values determined using the BCA- and micro-BCA- assays in the present study. The gross morphology of the loaded vesicles studied using TEM provided evidence that MLV had been formed by lyophilisation and rehydration of SUV (see section 5.3.3.1), which suggests that the complete preservation of bilayers by trehalose, as described by Crowe & Crowe (1993), did not occur in the present study. Encapsulation data suggested that the extent to which PXB was encapsulated under the conditions of the present study was affected by the type of additive used, but not apparently by the amount used. With regard to the hypothesis highlighted by Crowe & Crowe (1993) that trehalose preserves liposome structure during lyophilisation by maintaining PHG spacing by becoming inserted between PHG, it may have been predicted that in the present study, the process of formation of MLV from SUV by freeze-drying might have been impeded by the presence of trehalose on both the inside and the outside of the bilayers. A logical consequence of this would have been for the resulting levels of PXB encapsulation be very low. In addition, the use of mannitol, which typically crystallises during freeze-drying, was predicted to enhance the breakup of liposome structure during the drying process, thereby leading to greater levels of PXB loading on rehydration than in preparations containing no additive. However, it may be seen from the data shown in Table 5.3 that the levels of PXB encapsulated were lower in the preparations containing mannitol than those containing trehalose or no additive, although it should be noted that the batch-to-batch variability in terms of PXB loading was far higher than for either particle size or Zeta potential.

5.3.3.3 Leakage of PXB into aqueous medium at 4°C

The release profiles for the liposome preparations in this study are shown in Figure 5.6. It is evident from these data that release profiles vary quite substantially between replicate

batches. One factor which may explain this variability is that the present release study was carried out in water only, whereas it would be more typical for such measurements to be carried out in a suitable buffer system. As stated in section 5.2, it was believed necessary to avoid the use of buffer salts in the present study, since the high salt concentrations present on liposome rehydration, and the related problems resulting from a concentration gradient being created by the use of such salts, may lead to possible artifacts which might compromise the reliability of the initial encapsulation data observed prior to the storage stability study.

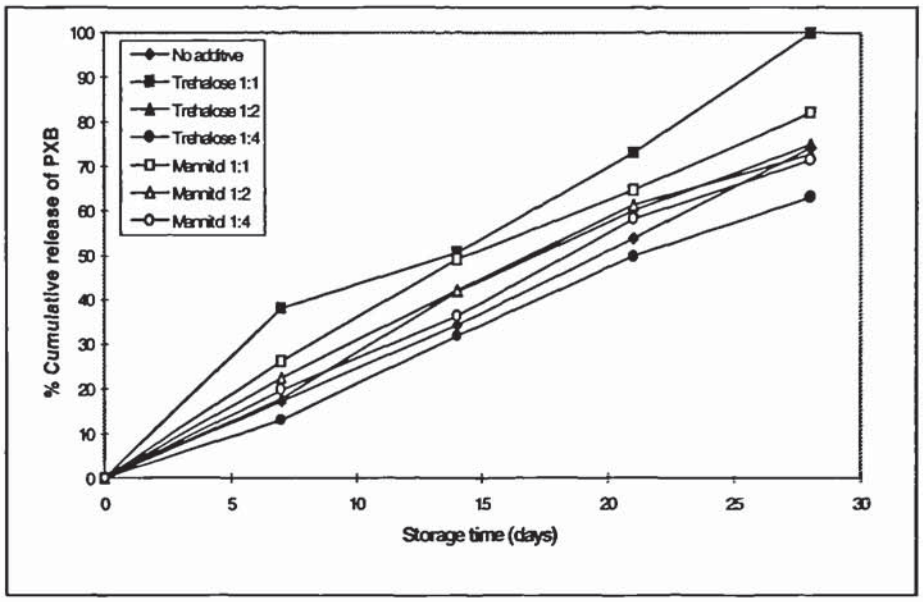


FIGURE 5.6: Leakage of PXB from liposomes into aqueous medium during storage at 4°C

The release profiles infer a zero-order release of PXB, which suggests that the mechanism of release is independent of initial loading concentration. As such, release of PXB might occur by diffusion through voids in the bilayers, as suggested by McAllister (1995). Indeed, the profiles here appeared in good accordance with those observed by McAllister (1995), who reported that both EPC and EPC:chol:DSPE-mPEG vesicles prepared by the DRV method and stored in PBS (pH7.4) released approximately 40% of entrapped PXB in 1 week at 4°C and leakage continued over 4 weeks until almost 55-60% was released. In an extension to this study, it was also found that the incorporation of cholesterol at 33mol% or 50 mol% in EPC vesicles containing no stealth lipid was successful in reducing the leakage of PXB. Initially, release was reduced to 10% at 1 week, followed by a slow phase of release which totalled 12-17% at the end of the 4 week period. Therefore, it appeared that DSPE-mPEG

had the effect of counteracting the rigidifying effect of cholesterol, reversing its stabilising effect in aqueous suspension. It appears also that a similar effect was operative in the present study, although the data from thermal analysis (DSC) suggested that the use of cholesterol at 33mol% had led to a significant decrease in ΔH_{Tm}. This apparent contradiction may be due to a masking of the true T_m by the presence of PEG. The phase transition of hydrated EPC is -15 to -7°C (Szoka & Papahadjopoulos, 1980). Therefore, at 4°C, EPC is in the relatively permeable liquid crystalline state. At this temperature, (that is, above the transition temperature), the inclusion of cholesterol modulates membrane fluidity by decreasing the rotational freedom of phospholipid hydrocarbon chains, thereby reducing bilayer permeability. Previous workers have reported that at 50 mol% cholesterol, the phase transition has been lost completely (Betageri & Parsons, 1992; Taylor *et al.*, 1990; see section 5.1). McAllister (1995) suggested that the mechanism of release of PXB from liposomes was the diffusion through water-filled defects (voids) in the liposome bilayer membrane; however, it is also possible that due to the amphiphilic nature of PXB, some of the drug which was initially present in the outer bilayers of the liposomes may have partitioned into the external aqueous phase. In the present study, it is likely that the such a mechanism of PXB release was operative, for three reasons. Firstly, the liposome preparation in the present study was similar in composition to the SSL preparation studied by McAllister (1995). Secondly, the preparations from the present study displayed no gel- to liquid- crystalline transition when analysed by DSC, thus suggesting that leakage could not be due to vesicles undergoing a phase transitions (or, therefore, phase separation of the individual lipid components). Finally, SSL would not be expected to aggregate or fuse in suspension, due to the charge imparted on the liposome surface by PEG (and also in the present case, by membrane-bound PXB). Therefore, the possibility of PXB leakage due to aggregation or fusion of the vesicles would be slight.

It may be noted from the release profiles in the present study that the use of both trehalose and mannitol generally appeared to lead to a slight increase in both the rate and the extent of release of PXB from the SSL preparations when stored in water at 4°C, compared to the preparation where no additive was used. From the graph of cumulative PXB leakage against storage time (Figure 5.6), it appears that a trend exists between the initial amount of additive

used and the leakage profile. The use of a 1:1 w/w ratio of additive to lipid appeared to increase the rate and extent of leakage more significantly than when lower ratios of additive to lipid were investigated. This may be attributable to osmotic effects resulting from the differences in solute concentration on the inside and outside of the liposomes. This effect would be more pronounced where higher amounts of additive were used, and may possibly have contributed to a 'burst' effect, thus leading to a higher rate (and extent) of leakage of PXB from the liposomes where these higher amounts of additive were used. Further studies will be necessary to reinforce the data produced in this preliminary study.

5.3.4 Conclusions and further work

It may be concluded from the present study that neither trehalose nor mannitol appeared to exert an effect on the formation of SSL or the extent of PXB loading, suggesting a low level of interaction between additive(s) and polar head groups (PHG) of the bilayer lipid components during liposome preparation. This may have been attributable to the presence of PEG on bilayer surfaces and/or interactions between PEG, additive, and PXB. The bilayer association of PXB may have also inhibited PHG-additive interaction due to drug charge or steric hindrance. While apparently little such interaction was observed during vesicle preparation, the addition of both trehalose and mannitol to the prepared PXB-loaded vesicles appeared to slightly increase the rate and extent of PXB leakage from the liposomes into aqueous medium upon storage for one month. However, it should be remembered that the data in the present study were preliminary findings. In addition, it was not possible to select a suitable set of control preparations in order to establish the effects of PEG at the molecular level. It is known that the presence of PEG as part of a 'stealth' lipid affects the size, transition temperature and surface charge characteristics of liposomes. An ideal control study would comprise a set of preparations, each of which differs from the SSL preparations in only one of these characteristics, while remaining similar in the other two. In addition, while PEG has previously been shown to be a successful cryoprotectant in its own right, the behaviour during freezing (and thus freeze-drying) of derivatised PEG which has been covalently coupled to lipid headgroups is not fully understood. Insofar as the remit of this preliminary study was chiefly to examine the effect of trehalose and mannitol on particular

SSL preparations under the chosen conditions, it is believed that this broad objective was met by the present study.

5.4 Investigation of the solid-state stability of PXB-loaded SSL prepared by the DRV method and further lyophilised in the presence or absence of additives.

5.4.1 Background

The previous study in this chapter demonstrated that neither trehalose nor mannitol appeared to exert an effect on the formation of SSL or the extent of PXB loading into liposomes, yet the storage stability data suggested that both additives caused an apparent increase in the rate and extent of subsequent leakage of PXB into aqueous medium during storage of suspensions at 4°C for one month. The aim of the present study was to determine the effects of the excipients trehalose and mannitol on the stabilisation of loaded SSL stored in the lyophilised state. This was carried out by the assessment of the extent of retention of the model tritiated drug ^{3}H-PXB, using a scintillation counting method.

The feasibility of using freeze-drying to stabilise liposome formulations has been clearly demonstrated in numerous reports, as stated above (section 5.1.4). In parallel with studies in protein stabilisation (see chapter 4), it was found that disaccharides appear to be the best lyoprotectants of all compounds tested. Indeed, Crowe and co-workers reported that trehalose was the best of the disaccharides, which also corresponded closely with work carried out by the group in the field of cell preservation (reviewed in Crowe & Crowe, 1993). There appears to be a lack of reported data describing specifically the preservation of sterically stabilised liposomes (SSL) by lyophilisation, and although many workers have discussed the increased *in vivo* stability of SSL relative to conventional liposomes, the issue of SSL stability *in vitro* has not been widely discussed in major texts or in individual articles. Freeze-drying may offer advantages to SSL in terms of stability, as well as in terms of simultaneous loading/rehydration (discussed in section 5.2). While certain liposomes may display adequate stability when stored in aqueous suspension, many sensitive biomolecules may show limited stability in such environments. In these cases, the stability of a preparation

containing a relatively unstable drug will be limited to the aqueous stability of the drug itself. It is formulations such as this which might best be served by lyophilisation, provided that the drug is stable (or may be stabilised) to the stresses of dehydration. In the present study, it was anticipated that the comparative effects of using trehalose and mannitol would be quite different. It was predicted that trehalose might stabilise the liposomes during the initial freeze-drying step, while mannitol would either contribute to the breakup of vesicles during this stage, or alternatively to have no net effect (since vesicles would typically break up during this stage unless specifically stabilised). This would lead to decreased levels of PXB loading in the preparations where trehalose was added, and conversely an increase (or no net change) in the loading efficiency of PXB into preparations where mannitol was added, compared to those preparations where no additive was used, since the mechanism of drug loading in the DRV method relies on the breakup and subsequent reformation of vesicles during drying and rehydration, respectively. Upon storage of the loaded vesicles, it was anticipated that trehalose might stabilise the vesicles, as demonstrated by Crowe and co-workers for conventional liposomes, thus leading to a decreased rate of PXB release compared to where no additive was used, while mannitol would have the opposite effect.

5.4.2 Materials and methods

5.4.2.1 Materials

Egg lecithin (egg phosphatidylcholine, EPC) was supplied at a concentration of 100mg/ml as a 99% pure solution in methanol:chloroform (1:1) by Lipid Products, Surrey, UK. Polymyxin B (PXB) was supplied in powder form from Sigma Chemical Co,. Poole, UK. Cholesterol (chol) was supplied in powder form from Sigma Chemical Co,. Poole, UK. DSPE-mPEG$_{1900}$ was a generous gift from Liposome Technology Inc., USA, supplied in dry powder form. AnalaR grade trehalose and mannitol were supplied by BDH, UK.

5.4.2.2 Preparation of liposomes

Preparation of liposome batches (in duplicate) was carried out as in the previous study (section 5.3), according to the DRV procedure outlined in section 2.12. PXB was added to the vesicles following sonication and prior to the lyophilisation stage of the preparative

procedure (Figure 2.10, stage 3). The PXB:lipid ratio was identical to that given in the previous study, *i.e.* 7.5µmol PXB in a 66µmol preparation. Since Crowe & Crowe (1993) had reported that the encapsulation of trehalose into the liposomes was necessary to provide stabilisation to bilayers during later freeze-drying (as well as the later addition of trehalose solution to the liposome suspension to provide protection to outer bilayers), additive was encapsulated together with PXB. At the final stage of the DRV process (Figure 2.10, stage 5), lyophilised liposomes were rehydrated with sterile distilled water, centrifuged twice to remove any unencapsulated PXB and additive and the encapsulation efficiency for each batch determined using the scintillation method as described earlier (section 5.2.2.5), in accordance with the procedure given in section 2.16. Following the second centrifugation step, the supernatant was removed for scintillation counting and the pellet resuspended in a solution of the appropriate additive (7.00ml at a concentration of 12.88mg/ml) to give a 1:1 (w/w) ratio of additive to lipid. Preparations were divided into 7 lyophilisation vials (3ml capacity, see section 2.2) to give 1.00ml volume, containing 12.88mg (≡16.5µmole) lipid and 12.88mg additive *per* vial. Preparations were then freeze-dried under identical conditions to stage 4 in the DRV process (section 2.12). Samples were stoppered under full vacuum (~0.2mbar) and stored at 37°C prior to rehydration and analysis as described in section 5.4.2.6 below. Identical batches to the preparations described above, but containing non-radiolabelled PXB, were prepared for characterisation (size distribution and zeta potential analysis).

5.4.2.3 Size-distribution analysis

The size distributions of the SSL preparations were determined using laser diffractometry, as described in section 2.14. As stated in section 5.2.2.3, since a skew was generally observed in the size distribution towards a relatively small number of larger particles, the values quoted are those for the number mean size (±s.d.) of the population, which reduces the extent of mean skew which would otherwise result from the presence of such particles.

5.4.2.4 Zeta potentials

Determination of the Zeta potential for all liposome preparations was carried out following dilution of the samples in 0.001M potassium chloride solution, using the Malvern Zetamaster, as detailed in section 2.15.

5.4.2.5 Measurement of initial loading of PXB

Initial encapsulations were estimated directly by analysis of supernatants and resuspended liposome pellets using the scintillation method as described in section 5.2.2.5, in accordance with the procedure given in section 2.16.

5.4.2.6 Differential Scanning Calorimetry (DSC)

Thermal analysis of lyophilised and hydrated preparations was carried out using DSC, in accordance with the method described in section 2.5 of this report. Hydrated preparations were spun for 30mins at 13,000rpm in a bench-top centrifuge to form a pellet, the supernatant decanted and a sample of the liposome pellet taken for DSC analysis.

5.4.2.7 Assessment of storage stability of loaded SSL at 37°C

Lyophilised products were sequentially rehydrated at room temperature to original volume (1.00ml *per* vial) with sterile distilled water, as described in section 2.12. The amount of PXB released from, and retained by the liposomes was quantified by the scintillation method, as described in section 2.16.

5.4.3 Results and discussion

5.4.3.1 Characterisation immediately following preparation

The particle-size analysis, zeta potential measurements and drug loadings are summarised in Table 5.4 below. It may be seen that the size and surface charge characteristics of the preparations in the present study are similar to those for analogous preparations in previous studies (sections 5.2 and 5.3). The higher drug loading values observed in the present preparations were attributed to the fact that higher concentrations of lipid were used.

Good reproducibility was generally observed from batch to batch in terms of size, zeta potential and drug loading, although the duplicate batches of liposomes containing trehalose displayed notable batch to batch size variation. Neither trehalose nor mannitol appeared to affect any of these characteristics, which compares well with observations made for the effects of these additives in the previous study.

Additive	Mean Particle Diameter (nm)	Zeta potential (mV)	Drug Loading (mg PXB.g^{-1} lipid)
No additive	284.7±12.4	-2.4±0.6	57.51±2.93
Trehalose	252.3±58.3	-2.7±0.1	55.11±2.27
Mannitol	223.8±10.1	-2.4±0.1	54.42±6.64

TABLE 5.4: Size, zeta potential and drug loading characteristics of liposome batches

5.4.3.2 Thermal analysis of dried- and hydrated- liposome preparations

DSC thermograms for samples of dried liposomes showed a gel- to liquid- crystalline transition (T_m) for each preparation, followed by a melting endotherm for preparations containing trehalose or mannitol. The T_m values (mean of duplicate batches, plus or minus the standard error) are summarised in Table 5.5 below. It was noted that the transition temperature for each preparation was in good agreement with that for dehydrated egg PC (41°C; Crowe & Crowe, 1993), indicating that the cholesterol included in each preparation had not completely eliminated the phase transition in the bilayers. It was perhaps more significant, however, that the transition energies for preparations containing trehalose and mannitol were less than one quarter of those for the preparations containing no additive.

Additive	T_m (°C)	Peak energy (ΔH, J.g^{-1})
No additive	41.9 (±1.2)	58.3 (±0.5)
Trehalose	44.2 (±0.3)	13.1 (±1.6)
Mannitol	42.1 (±0.9)	14.4 (±0.6)

TABLE 5.5: Summary of transition temperatures and energies for dried liposome preparations

Crowe & Crowe (1993) had observed that the addition of trehalose to a liposome preparation composed of solely egg PC prior to drying had led to a reduction in the dry-state phase transition temperature of the egg PC to -40°C. Although no event was observed at around this temperature for the current preparations, it is possible that the addition of trehalose had caused a transition in this lower temperature range, but that such a transition

was simply not detected by the apparatus employed here. The authors had also studied the thermal characteristics of a liposome preparation consisting of POPC and bovine PS which typically underwent three phase transitions at three distinct temperatures. It was found that the addition of increasing amounts of trehalose to this preparation led to an increase in the energy of the lowest temperature event and a consequent decrease in the energies of the other two events, until the two higher-temperature events were eventually eliminated (see section 5.1.4.1). In the present study, the temperature limitations of the DSC apparatus employed (minimum temperature attainable -40°C) prohibited analysis below approximately -35°C. With reference to the multi-component liposome study mentioned above, it is feasible that the energy associated with the conjectural lower temperature transition in the present study may have corresponded to the decrease in energy observed for the transition at around 41°C (compared to the preparation containing no additive). This may also have implications in terms of PXB retention, as discussed below. A further point of interest was that a similar decrease in energy associated with the transition at 41°C was also observed for the preparations containing mannitol, suggesting that mannitol may have behaved in a similar manner to trehalose with regard to bilayer transition characteristics. However, further studies would be necessary to confirm these predictions for the preparations containing trehalose and mannitol. DSC thermograms for hydrated liposome pellets displayed no thermal event, except for an ice melt endotherm, for any of the preparations in the present study. Since the samples analysed were concentrated by centrifugation (section 5.4.2.6), it was believed that the lack of a thermal event was not due to the sample having been too dilute. It was predicted that because the dried samples had undergone phase transitions during DSC analysis, hydrated preparations may also display a parallel transition at around the phase transition temperature of hydrated EPC (-5°C, McAllister, 1995). However, this was shown not to be the case.

5.4.3.3 Assessment of solid-state storage stability

Retained PXB levels in liposomes following storage in the dried state at 37°C and subsequent rehydration are given in Table 5.6 below.

Storage Time (days)	Percentage of initial PXB remaining in liposomes (±s.d.)		
	No additive	Trehalose	Mannitol
0	61.1 (±1.0)	50.2 (±6.4)	49.5 (±0.0)
1	55.0 (±4.0)	50.7 (±2.0)	45.9 (±1.5)
4	47.1 (±3.2)	48.0 (±3.0)	38.1 (±3.0)
7	58.0 (±0.7)	47.7 (±1.8)	39.0 (±1.0)
11	52.5 (±16.1)	41.8 (±1.2)	24.1 (±10.7)
14	52.9 (±8.8)	36.5 (±1.5)	25.1 (±1.7)
25	45.4 (±7.7)	21.8 (±4.7)	26.8 (±14.3)

TABLE 5.6: PXB retention by liposome preparations in the presence or absence of excipients following storage in the lyophilised state at 37°C

The data for each preparation on day 0 (zero) represent the amount of PXB associated with the liposomes immediately following the final lyophilisation step. It may be seen from these data that the preparations containing no additive lost approximately 40% of their initial PXB load as a result of this step, while preparations containing trehalose and mannitol lost approximately 50% of their original PXB load. This loss may be attributed to the breakup and subsequent reformation of vesicles during this additional freeze-drying step, causing a release of a significant proportion of the PXB which was initially encapsulated. It was not possible to determine whether the differences in losses observed between batches containing trehalose, mannitol or no additive were due to the effect of the additives on the liposomes or simply due to experimental variability between batches. A plot of the storage stability data (day 1 through to day 25) is given in Figure 5.7, where the level of retained PXB is expressed as a percentage of that remaining at day zero and plotted against storage time. It may be seen from the graph that there was significant vial-to-vial variability within each type of preparation, particularly at the later time points. Due to this variability, it was not possible to determine whether the additives used in the present study had alone accounted for the

differences in stability observed, or whether these differences were simply the result of experimental variation between vials of lyophilised product. While these data should therefore be taken to be preliminary findings, they allow full discussion of all the relevant points to be made.

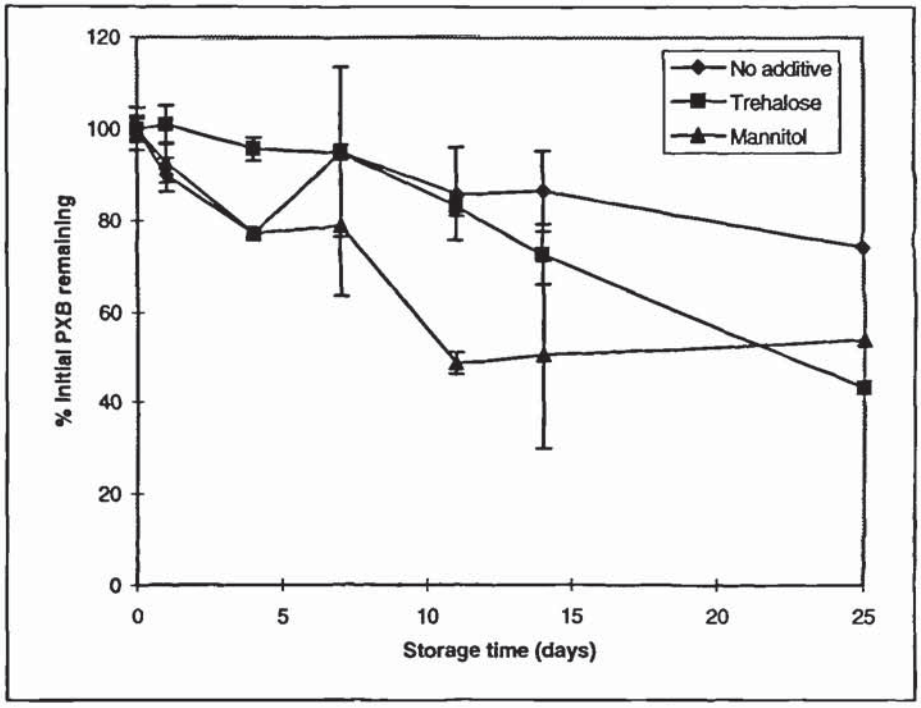


FIGURE 5.7: Retention of encapsulated PXB by SSL following storage at 37°C in the lyophilised state and subsequent rehydration (expressed as percentage of PXB remaining at day zero)

The data presented in Table 5.6 and Figure 5.7 suggest that the storage stability of the liposome preparations lyophilised in the presence of trehalose or mannitol was compromised compared to those preparations which were lyophilised in the absence of additive. It appears from these data that the presence of trehalose or mannitol led to a reduction in both the rate and the extent of liposome breakdown, when assessed by leakage of the PXB marker. While these data are not conclusive, since the variability between liposome batches was high, a number of further observations were made which may be relevant to understanding these data. A discussion of these observations follows.

Thermal analysis of batches of liposomes containing non-radiolabelled PXB using DSC revealed differences in preparations containing mannitol at day 25 compared to similar preparations analysed at day zero. Conversely, thermograms for preparations containing either trehalose or no additive indicated that these preparations had remained unchanged in terms of T_m and transition energy during storage. For the preparations containing mannitol, the energy of the transition around 41°C was seen to increase from 14.4(±0.6) J/g to 33.2 (±1.7) J/g, while for those containing trehalose, the corresponding T_m energy remained similar (14.7J/g on day 25, compared to 13.1J/g on day 0), as did the analogous transition energy for those containing no additive (56.2J/g on day 25 compared to 58.3J/g on day 0). As stated above, the DSC apparatus employed in the present study did not permit analysis below approximately -35°C but it was believed that the putative transition around this temperature may have decreased in reciprocity with the observed increase in energy of the transition around 41°C. If such a change in phase transition temperature occurred during storage, then this would signify thermodynamic changes within the bilayer structure, which would compromise the structural integrity of liposomes and therefore lead to an increase in PXB leakage. Firstly, a phase transition would occur during storage, with more of the product becoming gel- crystalline as the transition at *ca.* -40°C diminished and the transition at +41°C increased in energy. Secondly, a higher proportion of the lipid would consequently undergo a further phase transition on rehydration at 37°C, returning from the gel- to the liquid- crystalline phase. These transitions could account for the decrease in PXB retention during storage for the preparations containing mannitol. A further possibility is that the EPC in the liposome preparation underwent a change in structural arrangement during storage, since PC is known to exist in a number of crystalline forms (McAllister, 1995) and that this rearrangement may have led to the changes in transition temperature and energy. However, further analyses would be necessary to allow the elucidation of these proposals.

Moisture contents were carried out on duplicate preparations following lyophilisation (day zero) using TGA, as described in section 2.6. It was found that moisture contents were 1.95(±0.11)% for preparations containing no additive, 1.75(±0.28)% for those containing mannitol and 5.80(±0.70)% for those containing trehalose (all w/w). This was surprising, since it was believed that such high levels of moisture in the preparations containing trehalose

might lead to the occurrence of degradative reactions during storage. However, the PXB retention data (Table 5.6, Figure 5.7) did not appear to indicate that any such degradation had taken place to a greater extent in the preparations containing trehalose compared to those preparations containing mannitol. In addition, a further observation made in the present study was that following storage in the dried state for 24 hours, the preparations containing trehalose had remained white (all vials), while those containing mannitol or no additive had become yellow in colour (all vials), suggesting that trehalose might have afforded yet further stabilisation to the liposomes. However, this noted observation does not necessarily correlate with the extent of liposome degradation, and it should be remembered that the rate of liposome breakdown observed was markedly different for those preparations containing mannitol, as compared to those containing no additive. One possible explanation for this phenomenon is that the addition of trehalose had prevented 'overdrying', whereas no such prevention had occurred for preparations containing mannitol or no additive. Overdrying is a processing difficulty often encountered in protein systems, as discussed in chapter 4 of the present report. However, since the purported mechanism(s) for the preservation of proteins during freeze-drying and those for the preservation of liposomes appear to share many features (see chapter 1), it may be possible that the overdrying of liposomes had occurred for those preparations containing mannitol or no additive in the present study.

A final point to observe is the possible limitations of the interpretation of the data in the present study. Firstly, the possible chemical degradation of the PXB (either tritiated or non-radiolabelled) which may have occurred during the course of the study was not taken into account in the release data. In addition, it was assumed that the tritiated PXB was fully representative of the total PXB present in the drug loading solution and in subsequent supernatant solutions, which may not have been the case, since it was observed that the levels of radioactivity had reduced significantly during the freeze-drying process, which may be explained by a proton-exchange mechanism occurring between the tritium in the PXB solution and external hydrogen (^{1}H).

5.4.4 Conclusions

It may be concluded that the use of both trehalose and mannitol as excipients appeared to lead to an increase in the rate and extent of liposome breakdown when preparations were stored in the lyophilised state at 37°C for one month, assessed on the basis of leakage of the PXB marker from the liposomes. The trends in the observed stability data were partially attributed to the direct effect of mannitol on the gel- to liquid- crystalline transition temperature of the bilayers during storage and the observed elevated moisture contents in preparations containing trehalose, amongst other factors. However, variation between batches and vials within the study was significant for the storage stability trial and, therefore, the leakage data produced may not alone be a reliable indication of liposome stability under these conditions. Preparations containing mannitol or no additive were seen to become yellow in colour after 24 hours' storage in the dried state, while those containing trehalose did not discolour throughout the entire storage period (25 days). This phenomenon may have been a result of the residual moisture levels in products following lyophilisation, with trehalose preventing 'overdrying' of liposomes (moisture content 5.8%), whereas no such protection was afforded by mannitol (moisture content 1.75%) or where no additive was used (moisture content 1.95%). While all the above issues are believed relevant to the stability of the liposomes in the present study, as well as to that of liposomes in general, the limitations of some of the aspects of the present study were recognised; in particular, the high levels of variability between vials and batches of liposomes and the possibility that the tritiated PXB marker may not have been wholly representative of the non-labelled PXB present in the preparations.

6. THE DEVELOPMENT AND OPTIMISATION OF FREEZE-DRYING CYCLES

SUMMARY

The objective of this final area of study was to analyse the critical temperatures and freezing characteristics of model crystallising and non-crystallising formulations using thermal analysis and freeze-drying microscopy, to use this information to develop more efficient lyophilisation cycles for these products and to assess the practical implications of employing these revised cycle parameters in terms of cycle time and efficiency and product acceptability. It was anticipated that this study might bring together many of the factors which have been discussed in the preceding chapters of work and that a number of analytical methods could be utilised in order to enable cycle development to be carried out. Model crystallising and non-crystallising solutions were initially freeze-dried using a 'conventional' cycle employing low temperatures and high vacuum conditions. Freeze-drying microscopy and freezing resistance analysis were then used to provide evidence of the collapse temperatures and freezing behaviour of these model solutions, which enabled more efficient freeze-drying cycles to be developed, resulting in an overall reduction in drying time for both systems. For the crystallising solution, a reduction in cycle time of 12% was achieved, while for the non-crystallising formulation, the overall cycle time was reduced by 40%. The present chapter demonstrates that a logical approach to the selection of freeze-drying cycle parameters may be effected by the employment of suitable analytical techniques, which can provide information aboutnthe thermal characteristics and freezing (and freeze-drying) behaviour of both simple crytallising and more complex, non-crystallising solutions. Such an approach is necessary when attempting to increase cycle efficiency with a view to eventual cycle optimisation.

6.1 INTRODUCTION

Optimisation of freeze-drying cycles is important for industrial lyophilisation applications, although there appears to be a scarcity of literature relating to optimisation techniques. It appears that while many workers have a basic knowledge of how to operate a freeze-drier, an overall appreciation of the background of the technology and the factors that affect the stability of the product being processed may be limited. Consequently, it is often the case that pharmaceutical companies, where products are freeze-dried at some stage during manufacture, do not realise that the manner in which many of their products are lyophilised could be improved considerably (Franks, 1990). Surprisingly, when applied to sensitive, high-cost bioproducts, the method of lyophilisation for a freeze-dried formulation for a novel compound is often arrived at more by the consideration of freeze-drying conditions for a previous product than by considering the physical and biological requirements of the current product itself. Much effort will have been put into the initial discovery (or design) and development of a potential drug, in terms of both time and financial investment, and a lack of attention to the critical lyophilisation stage may result in an unnecessary, yet substantive product loss. One can only speculate about the number of potential drugs which may have not reached clinical trial stage due to apparent problems in formulation such as the loss of bioactivity on freeze-drying, yet in some of these cases, these apparent problems might have been solved by using a rational approach to the development of suitable formulations and freeze-drying cycles. Conversely, it is likely that many may have been formulated together with co-solutes which, fortuitously, stabilised them during and subsequent to drying or freeze-drying. As discussed in chapter 5 of this report, disaccharides have been shown to act as excellent cryoprotectants and lyoprotectants for the stabilisation of many proteins, both during and subsequent to drying. As such, the extensive use of lactose as a bulking agent may have afforded protection to therapeutic agents from drying stresses.

The entire area of freeze-drying is little understood, even by those who carry out the process on a regular basis. Franks (1990) speaks of the empiricism associated with the process, and that lyophilisation conditions often need to be established *ab initio* for every new product. Practical experience often demonstrates this statement to be true. However, with a basic understanding of the principles of freeze-drying (such as those outlined in sections 1.1 and

1.2), it is possible to design a freeze-drying process which should give predictable results and stable products on a rational basis by studying critical parameters and behaviour of a formulation. For example, the evaluation of critical processing temperature using thermal analysis (*i.e.* collapse temperature and/or glass transition temperature) will enable a prediction to be made of the maximum allowable (product) temperature during freeze-drying, while the study of freezing behaviour using techniques such as freeze-drying microscopy (see section 1.2) may provide evidence of the microstructure of the frozen matrix. Chang & Fischer (1995) recently reported the successful development of a freeze-drying cycle which dried a solution of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) with glycine and sucrose as excipients, to an apparent level of 0.4% residual moisture in 6 hours. Cycle development was based on characterisation of the frozen formulation by thermal analysis and by the examination of the effects of various lyophilisation process parameters on the sublimation rate of the ice. Thermal analysis showed that the glycine component of the solution gave a metastable glass on freezing, but that annealing to -15°C caused it to devitrify, giving a predominantly crystalline matrix. Shelf temperature (T_s) and chamber pressure (P_c) were then varied until a suitable combination could be arrived at. It was found that although various combinations of shelf temperature and chamber pressure could be used to obtain the same product temperature (T_p) throughout the drying process, the combination of higher shelf temperature and lower chamber pressure was used to maximise the sublimation rate. By controlling the shelf temperature and chamber pressure, the authors were able to maintain the product temperature below T_g' at all times. This example of such a high degree of control over process parameters should be commonplace, but the study of the relationship between T_s and P_c on the practical level for every freeze-drying run for each and every formulation would prove impactical. In addition, some freeze-driers may not allow the operator to have the necessary degree of control in order to achieve this. Perhaps the most significant point to note from the article is that the authors highlight the parameters which could easily be controlled with simple thermo-analytical techniques to achieve a more efficient lyophilisation cycle for their model protein. It is interesting to note that the protein used, rhIL-1ra, did not require the excipients to remain amorphous within the frozen matrix; in the case of many sensitive proteins, the maintenance of a vitrified phase has been shown to be necessary in order to effect lyoprotection (*i.e.* the stabilisation of proteins during freeze-

drying) (Arakawa *et al.*, 1993). Thus, in the work detailed by Chang & Fischer (1995), the final state of the frozen matrix immediately prior to the primary drying process would have been predominantly crystalline. This suggests that the product would have adopted a rigid microstructure, as shown in Figure 1.7(a) (section 1.2), enabling ice crystals to sublime with minimal resistance to vapour flow, drying with retention of structure. However, in the case of many protein formulations, where an amorphous state is essential to maintain biological activity and protein stability, one might not expect that such a high rate of sublimation may be achievable, since the frozen matrix would adopt the type of structure as shown in Figure 1.7(b). Consequently, one would have to conlude that the 'efficient' cycle which Chang & Fischer outline as suitable for rhIL-1ra is unlikely to be achievable for the sensitive proteins requiring the presence of an amorphous matrix.

A comprehensive article by Rowe (1977) outlines the economic considerations of the process which are relevant to the drug development process, providing a basic framework on which production-based decision-making may be based. Some authors have also focussed on the technical, engineering and formulation considerations or have seeked to provide guidelines for the successful freeze-drying of particular categories of products such as proteins or other biologicals (Pikal, 1990a, b; Franks *et al.*, 1991; Adams, 1991). Several methods are available to the formulation scientist to enable a profile of necessary characteristics to be built up for each new product requiring formulation, so that the development of more efficient cycles can be achieved. It is often the case that regulatory constraints placed on pharmaceutical companies can dictate that once a standard manufacturing protocol has been established, alterations to this protocol may require prohibitively extensive revalidation. Such constraints serve to emphasise the importance of ensuring that processes are streamlined before large scale production is begun and the following section seeks to introduce the concepts of cycle development and optimisation.

6.1.1 Increasing Cycle Efficiency: Maximisation and Optimisation

For many compounds, neither freezing nor drying may present a challenge to molecular structure or stability. In such cases, the sole criterion to be satisfied is that the final product is a thermally stable, cohesive cake. To achieve the required results, the freeze-drying cycle

may be essentially 'maximised' (in terms of drying efficiency) by simply considering the factors affecting the physical stability of product microstructure (such as the freezing characteristics of the original solution or suspension), the collapse temperatures both of the frozen matrix and of the dried product following processing, and the ease of sublimation from the frozen mass (which is dependent on the microstructure of the frozen matrix).

However, many novel entities - especially those emerging from molecular drug design and recombinant DNA technology - are bioactive proteins (see Chapter 5) and many of these will encounter freeze-drying during the production process. In such cases, it is not sufficient to consider only the final appearance of the product, since it will also be imperative to maintain biological activity throughout the freeze-drying process and during subsequent storage and reconstitution. It is possible for the appearance of a freeze-dried product to be acceptable in terms of microstructure while the active protein has suffered damage at the molecular level. The topic of protein stabilisation by lyophilisation is discussed further in chapter 5 of this report. Thus, in order to freeze-dry the product to the required level of moisture content (avoiding 'overdrying'), whilst still maintaining biological activity, it is unlikely that the lyophilisation cycle could be maximised. More typically, a compromise will have to be reached in order to reduce cycle time and therefore production costs, while maintaining the level of biological activity of the final product. The term 'optimised' is often used when referring to a cycle which allows all these criteria to be satisfied, without necessarily achieving minimum possible energy requirements and shortest possible cycle time.

There may be a number of reasons why pharmaceutical companies are unable to optimise freeze-drying cycles for their products, while large amounts of both time and money may be invested in the initial formulation development of the therapeutic agent in order to arrive at an efficient production protocol. However, time and money may not be the sole limiting factors in the development of a freeze-drying protocol. A general lack of understanding of freeze-drying as a process also plays its part in explaining why companies may not experience the potential benefits which freeze-drying can offer their formulations. A typical attitude to freeze-drying a solution may be to use very cold and very low pressure (high vacuum) conditions, in the erroneous belief that the conditions are "safe" in terms of avoiding product

collapse. Under such conditions, cycles are often unnecessarily prolonged over several days, whereas with more careful planning and the application of relevant freeze-drying concepts, such as those mentioned in section 1.2, efficiency may be considerably increased and the processing time significantly reduced, whilst maintaining product stability.

A typical ('conventional') freeze-drying cycle may be designed without a great deal of regard for the product in question. The temperature of the shelf will commonly be taken down to almost as low as possible before the highest possible vacuum is applied and the shelf temperature gradually increased (normally stepwise) until the product reaches the shelf temperature. During pre-freezing, the product temperature follows the shelf temperature quite rapidly down to the minimum temperature (typically -40°C), sinc ethe chamber is held at atmospheric pressure. It may then be supposed that the shelf temperature should be kept below 0°C in order to prevent melting of the frozen matrix. However, under high vacuum conditions, heat transfer from the shelf to the vial will be dramatically reduced, since the transfer of heat is reliant upon the conduction of thermal energy from shelf to vial and is significantly influenced by the number of water or gas molecules resident in the drying chamber. Therefore, during primary drying, the product temperature is much slower in responding to the rise in shelf temperature. The use of 'maximum' vacuum conditions (which are dependent on the specifications of the system being used) can often be counterproductive, since the thermal conduction from the shelf to the vial will be significantly reduced and no increase (often a decrease) observed in the rate of drying. Even during the secondary drying phase, it has been demonstrated that it is inefficient to desorb moisture from products at pressures below approximately 0.2 torr and that little is achieved by employing chamber pressures below this value (Pikal, 1990a, b).

Since a temperature increase of only 1°C has been shown to result in at least a 13% reduction in primary drying time (Pikal, 1985), yet product collapse must be avoided, accurate determination of the collapse temperature is critical to process optimisation (Pikal & Shah, 1990). Although the rate of primary drying increases as the product temperature increases, and therefore primary drying should be carried out at the highest temperature possible, the product temperature (T_p) should always be maintained below its characteristic collapse

temperature (T_c). It was recognised by MacKenzie (1965) that products displayed characteristic "maximum allowable temperatures", which were found to be dependent upon product composition and could be altered by adding or removing components or by varying the ratio of the components within the mixture. This was found to be the case for both crystallising and non-crystallising products; for crystallising products, T_{eu} could also be varied in the same manner (since T_c=lowest T_{eu}) and for non-crystallising products, T_g and T_g' also conformed to this pattern of composition-dependent behaviour.

6.1.2 Aim of this Chapter

The objective of this final area of study was to analyse the critical temperatures and freezing characteristics of model crystallising and non-crystallising formulations using thermal analysis and freeze-drying microscopy, to use this information to develop more efficient lyophilisation cycles for these products and to assess the practical implications of employing these revised cycle parameters in terms of cycle time and efficiency and product acceptability. It was anticipated that this study might bring together many of the factors which have been discussed in the preceding chapters of work and that a number of analytical methods could be utilised in order to enable cycle development to be carried out.

6.2 Increasing Cycle Efficiency for a Model Crystallising Product

6.2.1 Background

As described in section 1.2, the formation of ice crystals is a complex phenomenon. In solutions which contain only crystallising solutes, the frozen matrix will adopt a 'rigid' structure, from which ice may sublime through channels created as a result of the sublimation process (Figure 1.4). The aim of this study was to ascertain whether the drying cycle time for a crystallising solution of potassium chloride (10% w/w) could be reduced by altering shelf temperature and chamber pressure conditions in the lyophilisation cycle programme, whilst still maintaining the product temperature below its characteristic collapse temperature throughout processing.

6.2.2 Materials and Methods

6.2.2.1 Materials

AnalaR grade potassium chloride was obtained from BDH, UK.

6.2.2.2 Resistance measurements

A solution of 10% (w/w) potassium chloride was prepared by dissolving 100grams into 1000ml of sterile distilled water for irrigation. Resistance measurements were carried out on the solution, as detailed in section 2.3 of this report, in order to assess the freezing characteristics.

6.2.2.3 Freeze-drying Microscopy (FDM)

Freeze-drying microscopy was carried out on the 10% (w/w) potassium chloride solution, as described in section 2.4 of this report.

6.2.2.4 Freeze-drying

Aliquots (5.0ml) of the 10% (w/w) potassium chloride solution were dispensed into glass freeze-drying vials of 10ml capacity. Sufficient vials were used to fill one tray of the VirTis Genesis EL25 freeze-drier (described in section 2.1). Butyl freeze-drying stoppers (Adelphi

Tubes Ltd, Sussex, UK.) were inserted into the vials in the first stoppering position. Temperature probes were inserted into four of the vials, with each probe in contact with the base of the vial. A conventional cycle was programmed into the microprocessor-controller unit of the freeze-drier, the details of this cycle are given in Figure 6.1. A second batch of solutions was freeze-dried using a modified cycle (see Figure 6.2), based on data obtained from FDM and freezing resistance analyses (see sections 6.2.3.1 and 6.2.3.2 below). In each case, four of the vials were monitored for temperature and these temperatures printed at five minute intervals and whenever the cycle entered a new programmed phase. The drying cycle was judged to be complete once all four monitored temperatures had reached the final programmed shelf temperature.

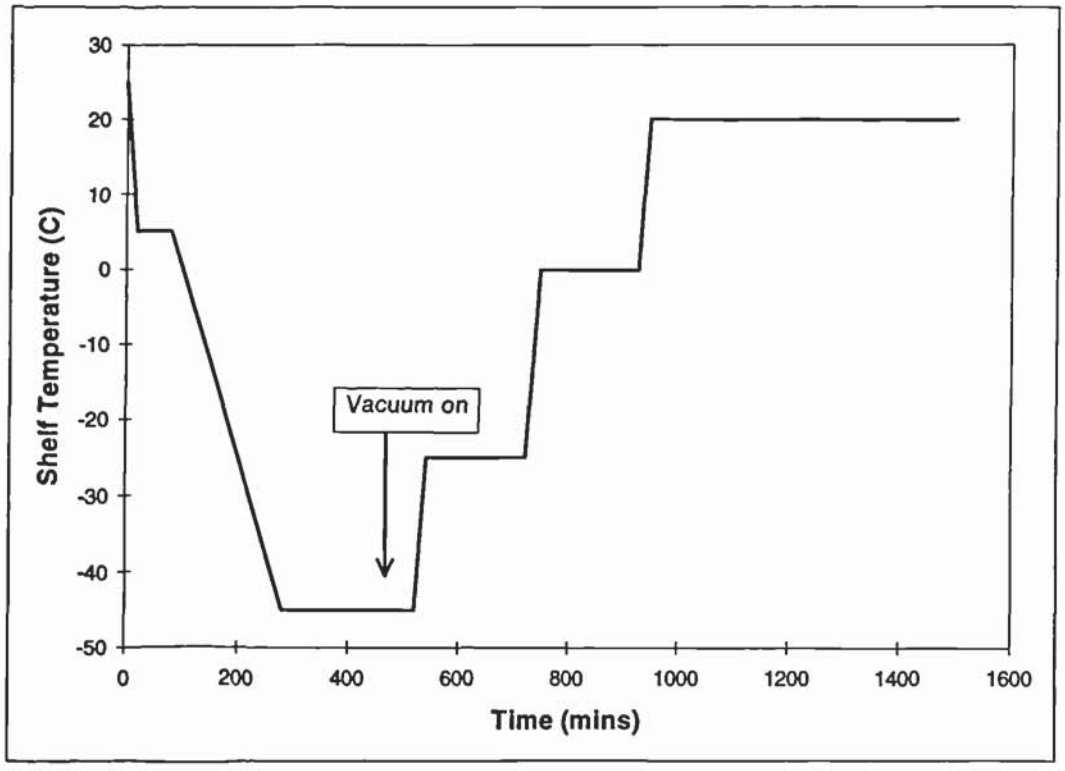


FIGURE 6.1: *Flow diagram representing the 'conventional' freeze-drying cycle*

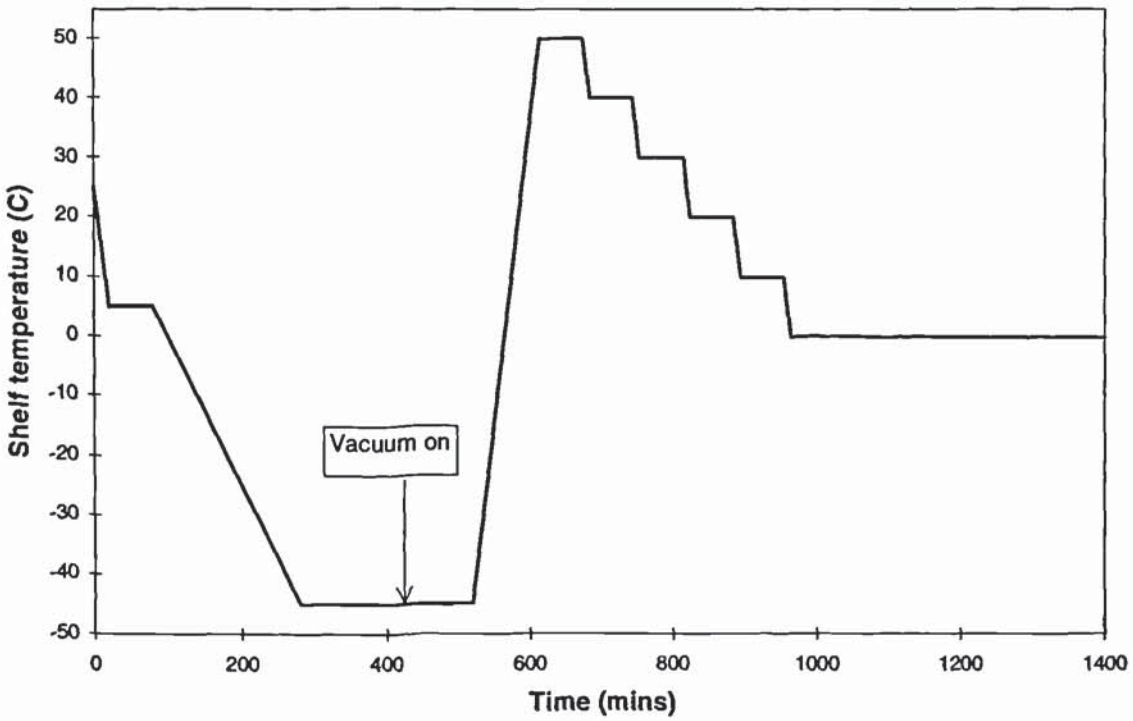


FIGURE 6.2: Flow diagram representing the revised cycle for the lyophilisation of potassium chloride solution

6.2.3 Results & Discussion

6.2.3.1 Resistance measurements

Resistance measurements demonstrated that the eutectic temperature of the frozen mass was -9°C, which was in keeping with the value of -11°C reported by Levine & Slade (1987).

6.2.3.2 Freeze-drying Microscopy (FDM)

Analysis of the KCl solution by FDM showed that the eutectic melting temperature of the frozen mass was -11°C, which was in good agreement with the collapse temperature observed using resistance measurements (see above).

6.2.3.3 Lyophilisation Cycles

The design of the 'conventional' cycle (see Figure 6.1) did not take account the physical properties of the product, but represented conditions which might be regarded as 'typical' in many laboratories. The design of the 'revised' cycle, however, took into account the

information derived from the freezing resistance measurements and freeze-drying microscopy (FDM), and the cycle parameters were designed to maintain the product temperature below its characteristic collapse temperature throughout the process.

The time taken for completion of the conventional freeze-drying cycle for the potassium chloride solution was 1500 minutes. The revised cycle was judged to be complete at 1320 minutes. It may be seen from these results that the time taken for completion of the lyophilisation cycle for potassium chloride was reduced by 12% by the revision of cycle parameters. It should also be noted that the conventional cycle itself was relatively rapid in freeze-drying terms, since the freezing behaviour of the solution resulted in a eutectic mixture of ice and solute crystals (see Figure 1.4).

Eutectic freezing behaviour patterns permit the relatively free flow of water vapour along the channels created in the solid solute microstructure. As the impedance to vapour flow in such a structure is comparatively low, rapid lyophilisation rates may be permitted during drying. Therefore, the use of the revised cycle conditions in this case resulted in a limited increase in cycle efficiency compared to when the conventional cycle was used.

6.2.4 Conclusions

The potassium chloride solution froze to give a structure which contained clearly-defined channels, which allowed subliming ice vapour to freely escape from the frozen matrix. There was therefore little impedance to vapour flow from factors such as skin formation or the persistence of a glassy phase. This may explain why the use of the conventional cycle was sufficient in drying the product in a relatively short time. Indeed, since the product did not lead to the production of a frozen matrix which impeded vapour flow, the use of extra heat input in the initial stages of the developed cycle was perhaps not required.

6.3 Increasing Cycle Efficiency for a Model Non-Crystallising Formulation

6.3.1 Background

The aim of using a more complex, non-crystallising formulation in the present study was to assess the effects of revising cycle parameters in a similar manner to the study described in section 6.2 above. The solution employed here was a formulation containing paracetamol together with a number of excipients. It was anticipated that the resulting frozen matrix of this solution would be of a more complex microstructure than that of potassium chloride alone and would not necessarily contain the same type of sublimation channels. It was predicted that a longer conventional drying stage would be necessary to dry this formulation than that required to dry the crystalline potassium chloride solution in the previous study (see section 6.2). In this study, therefore, a greater potential existed for the reduction of cycle time by the use of modified lyophilisation conditions.

6.3.2 Materials and Methods

6.3.2.1 Materials

All chemicals were supplied by BDH (UK) and were of AnalaR grade, or equivalent.

6.3.2.2 Resistance measurements

A solution was prepared which contained paracetamol (5.0mg/ml), lactose (3.0%, w/w), trimethylamine (Tris, 10mM) and was adjusted to pH8 with HCl. This solution was subjected to resistance measurements, as detailed in section 2.3.

6.3.2.3 Freeze-drying Microscopy (FDM)

FDM of the solution was carried out as described in section 2.4, in order to predict the behaviour of the product during freeze-drying.

6.3.2.4 Freeze-drying

Aliquots (5.0ml) of the formulation solution were dispensed into glass freeze-drying vials of 10ml capacity. Sufficient vials were used to fill one tray of the VirTis Genesis EL25 freeze-drier (described in section 2.1); 125 vials were used for the conventional cycle, while 121 vials were used in the revised cycle. Butyl freeze-drying stoppers (Adelphi Tubes Ltd, Sussex, UK.) were inserted into the vials in the first stoppering position. Temperature probes were inserted into four of the vials, with each probe in contact with the base of the vial. A conventional cycle was programmed into the microprocessor-controller unit of the freeze-drier, identical to that shown in Figure 6.1. A second batch of solutions was freeze-dried using a revised cycle (see Figure 6.3), the conditions of which were based on the data derived from freeze-drying microscopy. In each case, four of the vials were monitored for temperature and these temperatures printed at five minute intervals and whenever the cycle entered a new programmed phase. The drying cycle was judged to be complete once all four monitored temperatures had reached the final programmed shelf temperature.

6.3.3 Results & Discussion

6.3.3.1 Resistance Measurements

Resistance analysis resulted in a trace from which there were no determinable transition temperatures for the paracetamol formulation, which was attributed to the complexity of the solution. Therefore, FDM was employed to enable the assessment of the freezing behaviour and collapse temperature of the product.

6.3.3.2 Freeze-drying Microscopy (FDM)

FDM of the product demonstrated that the collapse temperature of the frozen mass was in the region of -33 to -34°C.

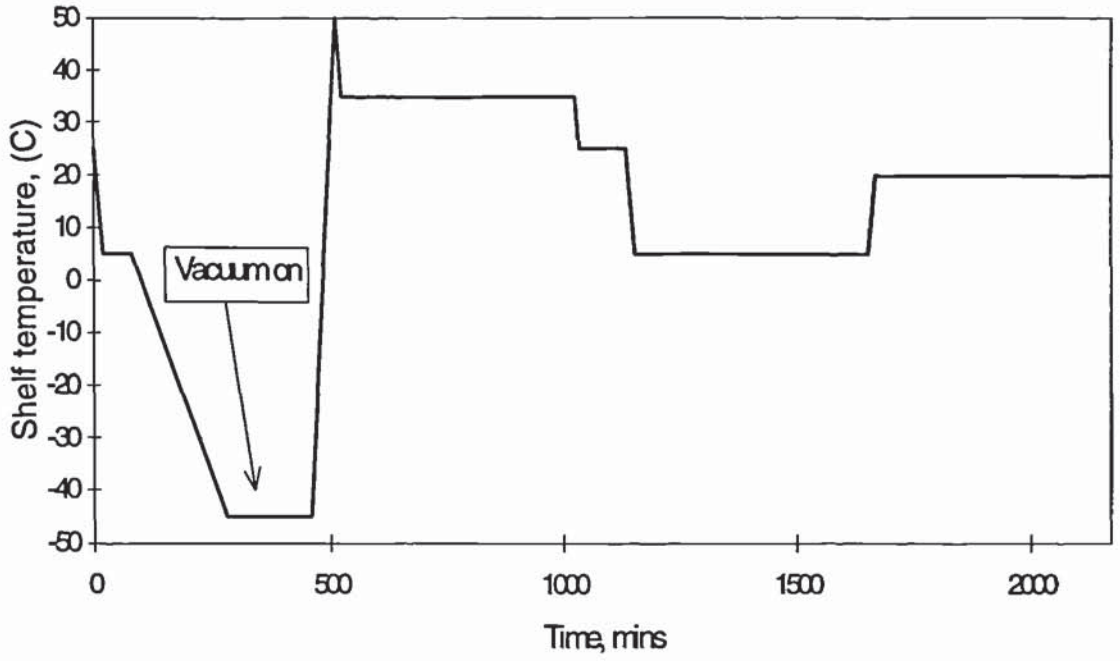


FIGURE 6.3: Flow diagram depicting the revised freeze-drying cycle for the non-crystallising paracetamol formulation

6.3.3.3 Lyophilisation Cycle

The times taken for completion of the freeze-drying cycles for the non-crystallising product for the conventional cycle and the revised cycle were 2401 minutes and 1455 minutes, respectively. It can be seen from these results that the time taken for completion of the lyophilisation cycle for the chosen solution was reduced by almost 40%, by altering the cycle conditions to increase the overall rate of sublimation. The use of the conventional cycle resulted in a longer drying time for the paracetamol formulation than that for the potassium chloride solution, since the frozen mass of the paracetamol formulation was dried by a combination of sublimation of free ice and the sublimation and evaporation of moisture from the glassy phase, as detailed in Table 3.1 (*cf.* simple sublimation for the potassium chloride solution, section 6.2). The microstructure of the frozen matrix was likely to have been dissimilar to that of the frozen potassium chloride solution described in the above section, with the likelihood that the structure of the frozen matrix strongly resembled that of the glass described in Figure 1.4. Such structures present more of a challenge to the sublimation process, since the channels for sublimation are not as open, and thus the subliming vapour must necessarily follow a more convoluted route to escape from the matrix. Sublimation

from an amorphous matrix (or from an amorphous region in an otherwise crystalline matrix) will thus require more thermal energy than that from a corresponding crystalline matrix. Therefore, to dry the paracetamol product in the present study would be more energy-consuming than to dry a simple product containing potassium chloride alone. The use of the more developed cycle in this instance capitalised on the fact that the energy-consuming nature of sublimation from this product led to more scope for reducing cycle time, and that the increased energy input to the products by the use of high shelf temperatures led to an increase in drying efficiency, resulting in a greater time-differential between cycles than in the previous study (section 6.2).

*Rating**	*Conventional cycle (2401 mins)*	*Revised cycle (1455 mins)*
Good	17 vials (14%)	63 vials (52%)
Acceptable	48 vials (38%)	21 vials (17%)
Poor	60 vials (48%)	37 vials (31%)
Total:	125 vials (100%)	121 vials (100%)

*Ratings: Good - No ablation or shrinkage; Acceptable - slight ablation or shrinkage; Poor - Marked ablation or shrinkage

TABLE 6.1: Acceptability ratings of vials of lyophilised paracetamol formulation

6.3.4 Conclusions

The present study demonstrated how the modification of freeze-drying conditions for a non-crystallising product could lead to a decreased drying time and thus increased lyophilisation cycle efficiency such a product. As predicted from the assessment of the microstructure of the frozen matrix, both sublimation and desorption were necessary to effect the dehydration of the frozen mass, whereas in the case for the crystallising model solution of potassium chloride described in the previous section (6.2), sublimation alone was sufficient to effect drying, since the freezing of this solution resulted in the creation of sublimation channels. Thus, the use of modified cycle conditions in the present study capitalised on the fact that the drying process for a non-crystallising product requires more energy than that for simple crystallising products, therefore presenting a greater opportunity for increasing drying

efficiency by the adoption of higher shelf temperatures than for the potassium chloride solution detailed in the previous study (section 6.2).

The present chapter demonstrates that a logical approach to the selection of freeze-drying cycle parameters may be effected by the employment of suitable analytical techniques, which can provide information aboutnthe thermal characteristics and freezing (and freeze-drying) behaviour of both simple crytallising and more complex, non-crystallising solutions. Such an approach is necessary when attempting to increase cycle efficiency with a view to eventual cycle optimisation.

7. CONCLUDING REMARKS

In overview, the objectives of this project were: to examine the thermal characteristics of a range of single excipients, combinations of excipients and of model formulations in the light of their observed freeze-drying behaviour and stability; to assess the lyophilisation characteristics of model polymeric proteins, with particular emphasis on the stabilisation of these proteins during the freeze-drying process by protective additives and the elucidation of the mechanism of stabilising interactions at the molecular level; to evaluate the use of freeze-drying in the preparation and stabilisation of stealth liposomes, and to analyse the critical temperatures and freezing characteristics of model crystallising and non-crystallising formulations using thermal analysis and freeze-drying microscopy, to use this information to develop more efficient lyophilisation cycles for these products and to assess the practical implications of employing these revised cycle parameters in terms of cycle time and efficiency and product acceptability. The present chapter attempts to present the major conclusions from this work in the context of these original objectives.

The thermal characteristics of excipients, particularly saccharides, and their critical temperatures (T_g', T_g) were studied using DSC. In particular, the examination of the thermal characteristics of binary blends of fructose-sucrose and of trehalose-mannitol revealed a dependency of glass transition temperature on the proportion of each component in the mixture. The study of the thermal characteristics of lyophilised binary saccharide mixtures, each containing mannitol together with an amorphous excipient, revealed that the solid-state glass transition temperatures of such mixtures following a heating and quench-cooling cycle were all below 30°C, suggesting that the solid-state stability of lyophilised products containing such mixtures when stored under ambient conditions may be compromised. The use of more sensitive thermal techniques such as microcalorimetry may provide further information on the thermal behaviour of products containing such mixtures, which may enable suitable processing and storage conditions of such materials to be selected on a rational basis.

The applicability of FPLC to the analysis of the quaternary structure (and thus stability) of L-asparaginase in the presence of trehalose or lactose, following lyophilisation and

reconstitution, was demonstrated. However, the use of FPLC as the sole method for this purpose was found to lack quantification. The development of an assay to measure the biological activity of L-asparaginase enabled a further study to be carried out which examined the effects of a range of excipients, at a series of concentrations, on the biological activity and quaternary structure of the protein. This study demonstrated a marked similarity in the protective abilities of the excipients tested when compared on a weight basis. Glucose, mannitol and PEG (10kDa) were also observed to stabilise the protein, which contrasts with the reported findings of a similar study carried out using PFK as a model protein (Carpenter & Crowe, 1989), where only disaccharides were observed to provide stabilisation to biological actuvuty during drying. Molecular modelling of the spatial arrangement of the L-asparaginase monomer and tetramer structures, and specifically the prediction of the positions of highly polar residues (HPR) on the molecule, allowed the calculation of theoretical amounts of additive required to give full protective interaction (based on the assumption that one molecule of additive would be required for each exposed HPR) and thus stabilisation of the protein during lyophilisation. These calculated amounts of additive corresponded closely to those amounts found to be required experimentally, although it was not possible to confirm whether the amounts required in practice were due to the assumptions made in the calculation, or due to other factors, or a combination of both. Although hydrogen bonding appears to be the most favoured explanation for protein-co-solute interaction in published reports, it seems likely that other factors also have a part to play in determining the success or failure of a particular excipient to provide stabilisation to proteins during lyophilisation. Indeed, it appears from the data produced in the present report, that it is perhaps unrealistic to generalise about such interactions, and that individual proteins require their own specific requirements to be met in this regard. Further studies would be necessary in order that the interaction at the molecular level might be more fully elucidated.

The intrinsic stability of L-asparaginase during freeze-drying was determined using the assay for biological activity mentioned above. It was found that the higher the original protein concentration, the higher the proportion of protein remaining biologically active following lyophilisation and subsequent reconstitution (initial concentration of 23.6mg/ml led to a

retention of 78% of the original activity following lyophilisation, while only 15% activity was retained when the protein was lyophilised from a solution of initial concentration 2.95mg/ml). It was believed that the mechanism of 'intrinsic stabilisation' of L-asparaginase may be similar to the mechanism of protein-co-solute interaction occurring in solutions containing L-asparaginase together with excipients. The use of sensitive analytical techniques such as microcalorimetry applied to the study of the behaviour protein solutions during cooling (to freezing) may allow the further elucidation of the mechanism of 'self-interaction' for L-asparaginase.

The freeze-dry stability of LDH in the presence of a range of additives was determined using a biological assay which measured the retained activity of LDH following lyophilisation and reconstitution. In addition, the stability of the protein stored in the lyophilised state (together with additives) was assessed on the same basis. It was found that different additives provided differing levels of protection to LDH both during the lyophilisation process and in subsequent storage and these differences in protective abilities were attributed to the crystallising (or non-crystallising) characteristics and the chemical reactivity or reducing capability of the additives. The fact that the requirements for PFK reported by Carpenter & Crowe (1989) and the requirements for L-asparaginase and LDH observed in the present study have been shown to apparently differ further underlines the hypothesis that differences in protein structure and conformation lead to differences in specific stabilising requirements for proteins at the molecular level.

The investigation of alternative techniques such as SDS- and Native-PAGE, gel filtration/separation and equilibrium dialysis was carried out in order to determine whether such techniques were able to provide further information about the affinity of proteins for specific additives, to elucidate the observations made in the studies described above. However, limited success was achieved using these techniques, and thus the data are not presented in this report.

The successful use of the freeze-drying process in the preparation of conventional liposomes has been reported in literature. It was demonstrated in the present study that SSL could also

be prepared using the DRV method of Kirby & Gregoriadis (1984), which inherently involves lyophilisation. In this case, multilamellar stealth liposomes were produced, and the encapsulation/incorporation of a model marker compound polymyxin B (PXB) was successfully achieved. The encapsulation (incorporation) of PXB into SSL using a simultaneous loading-rehydration technique based on the DRV method was also investigated, and the effects of the potentially stabilising excipient trehalose and the potentially destabilising excipient mannitol on the drug loading and subsequent leakage into the external aqueous medium was assessed. It was shown that PXB could be loaded into ready-prepared SSL using the simultaneous loading-rehydration technique which had previously been shown by Dürr *et al.* (1994) to be successful for the encapsulation of doxorubicin B into conventional (non-stealth) liposomes, although the levels of PXB loading using simultaneous loading-rehydration were lower than those achieved when the original DRV method was used. It was demonstrated that the use of either trehalose or mannitol appeared to have no appreciable effect on either PXB loading efficiency or on the subsequent leakage of PXB from the vesicles into surrounding aqueous medium upon storage at 4°C over one month. However, these additives were observed to affect the stability of SSL when stored in the lyophilised state for one month at 37°C, assessed on the basis of leakage of PXB from the vesicles upon rehydration. The data produced in the present work suggested that the liposomes which were prepared and stored in the absence of additive were the most stable, since a lower proportion of PXB was released than from those preparations containing trehalose or mannitol. Limitations of the studies were discussed, which included the appreciation of the materials used, such as the tritiated PXB, which may have undergone some degree of proton exchange with aqueous media, and the variability of the system as a whole, which appeared to reflect the levels of variability typically present in biological systems. Further studies involving SSL might include the investigation of lipid mixture composition and the inclusion of different stealth lipids on liposome preparation and stability, as well as the potential for incorporating a wider range of therapeutic agents into such lipid systems and the use of additives for the purposes of stabilisation during, and subsequent to, processing.

A logical stepwise approach was used to develop freeze-drying cycles for model solutions. The practical implications of revising freeze-drying cycle parameters for model crystallising and non-crystallising formulations, in terms of cycle time and efficiency and product acceptability, were investigated. 'Conventional' cycles followed a classic shelf temperature and pressure pattern, while the 'revised' cycles were designed to minimise drying time and thus maximise efficiency by the use of higher shelf temperatures and chamber pressures compared to those employed in the conventional cycle. In this case, cycle parameters were selected to increase the rate of drying, whilst maintaining product temperature below the collapse temperature as determined by FDM. It was demonstrated that the type of microstructure of the product in the frozen state had a significant impact on the end result of the optimisation process. The drying time for a model crystallising solution (potassium chloride) using a 'conventional' freeze-drying cycle was not significantly reduced by the adoption of a more radical programme, since the crystalline microstructure of the frozen solution had encouraged rapid primary drying (sublimation) even under conventional cycle conditions. However, a much greater time differential was observed between conventional and revised cycles for the model non-crystallising formulation. In this case, the more complex microstructure of the frozen solution was not conducive to rapid primary drying, since primary involved desorption as well as sublimation from the frozen matrix. The adoption of more radical cycle parameters capitalised on the fact that the drying process for a non-crystallising product requires more energy than that for a simple crystallising product due to the more complex mechanism of primary drying in the non-crystallising product. This therefore presented a greater opportunity for increasing drying efficiency for the non-crystallising product than for the potassium chloride solution studied earlier. These studies using model solutions demonstrate that the application of a logical approach to the selection of lyophilisation parameters may be effective in reducing drying time for a wide range of products, since the models selected in the present project could be seen to adopt the two extreme patterns of freezing behaviour, leading to the formation of two distinctly different types of microstructure on freezing. In practice, products will generally adopt one of these behavioural patterns, or a mixture of both. Although the optimisation work of the present project was limited practically to the cycle development for products exhibiting the two extreme types of freezing behaviour, using one vial type, and a constant fill-volume, in

reality, efficient freeze-drying involves the achievement of a balance between several parameters including vial type, fill volume, overall product concentration, initial shelf temperature, shelf-cooling rate, ultimate shelf temperature, the inclusion of an annealing stage, regulation of heat input during drying and chamber pressure, many of which are often overlooked by the regular operator of a freeze-dryer.

The studies in this report demonstrate practically how analytical techniques such as freeze-drying microscopy and differential scanning calorimetry may be employed to determine important product characteristics and thus provide a logical basis for cycle development. This project serves to raise the current awareness of lyophilisation technology, providing an insight into the physical basis underlying the process itself, as well as describing how the use of the process in particular pharmaceutical applications may lead to potential benefits. Further to this, it is hoped that many of the studies undertaken in this project demonstrate that the freeze-drying process is a predictable and controllable process and that with a basic understanding of a small number of fundamental product and process parameters, a stepwise approach to cycle development may be taken which should consistently yield an end-product which conforms to prescribed pharmaceutical characteristics, irrespective of the complexity of the formulation.

8. REFERENCES

Adams, G.D.J. (1990) Residual Moisture and the Freeze-Dried Product, CAMR Report Document, CAMR, Porton Down, Wiltshire, UK.

Adams, G.D.J. (1991) Freeze-drying of Biological Materials, *Drying Technology*, **9**(4), 891-923

Adams, G.D.J. (1995) Practical Aspects of Formulation, Lyophilization Course Notes, Lyophilization Technology, 12-15 June 1995, The Center for Professional Advancement, Amsterdam, p.B163

Adams, G.D.J. (1996) Lyophilization of Vaccines, In: A. Robinson, G.H. Farrar & C.N.Wiblin (Eds.), *Vaccine Protocols*, Humana Press, New Jersey, ISBN 0-89603-334-1, pp.167-185

Adams, G.D.J. and Irons, L.I. (1993) Some implications of Structural Collapse during Freeze-Drying Using *Erwinia carotovora* L-Asparaginase as a Model, *J. Chem. Tech. Biotechnol.*, **58**, 71-76

Adler-Moore, J.P. and Proffitt, R.T. (1993) Development, characterization, efficacy and mode of action of AmBisome, a unilamellar liposomal formulation of amphotericin B, *J. Liposome Res.(USA)*, **3**(3), 429-450

Allen, T.M. (1994a) Long-circulating (sterically stabilised) liposomes for targeted drug delivery, *Trends in Pharmacological Sciences*, **15**, 215-220

Allen, T.M. (1994b) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system, *Adv. Drug. Del. Rev.*, **13**, 285-309

Allison, A.C. and Gregoriadis, G. (1974) Liposomes as immunological adjuvants, *Nature*, **252**, 252

Alpar, H.O. (1989) Liposomes as Drug Carriers, *Pharmaceutical Jnl.*, **243**(6548), 254-255

Arakawa, T. and Timasheff, S.N. (1985) Mechanism of Poly(ethylene glycol) Interaction with Proteins, *Biochem.*, **24**(24), 6756-6762

Arakawa, T., Bhat, R. and Timasheff, S.N. (1990a) Preferential Interactions Determine Protein Solubility in Three-Component Solutions: The $MgCl_2$ System, *Biochemistry*, **29**(7), 1914-1923

Arakawa, T., Bhat, R. and Timasheff, S.N. (1990b) Why Preferential Hydration Does Not Always Stabilize the Native Structure of Globular Proteins, *Biochemistry*, **29**(7), 1924-1931

Arakawa, T., Carpenter, J.F., Kita, Y.A. and Crowe, J.H. (1990c) The Basis for Toxicity of Certain Cryoprotectants: A Hypothesis, *Cryobiology*, **27**, 401-415

Arakawa, T., Kita, Y. and Carpenter, J.F. (1991) Protein-Solvent Interactions in Pharmaceutical Formulations, *Pharm. Res.*, **8**(3), 285-291

Arakawa, T., Prestrelski, S.J., Kenney, W.C, and Carpenter, J.F (1993) Factors affecting short-term and long-term stabilities of proteins, *Adv. Drug. Del. Rev.*, **10**, 1-28

Assadullahi, T.P., Hamid, K. and Hider, R.C. (1992) *In-vitro* stability of liposomes in the presence of polymers, trehalose and sodium taurocholate, *J. Microencapsulation*, **9**(3), 317-327

Atkins, P.W. (1989) *Physical Chemistry*, 3rd edition, Oxford University Press, Oxford, UK., ISBN 0-19-855186-X, p.144

Ausborn, M., Schreier, H., Brezesinski, G., Fabian, H., Meyer, H.W. and Nuhn, P. (1994) The protective effect of free and membrane-bound cryoprotectants during freezing and freeze-drying of liposomes, *J. Cont. Rel.*, **30**, 105-116

Bakker-Woudenberg, I.A.J.M., Lokerse, A.F., ten Kate, M.T., Mouton, J.W., Woodle, M.C. and Storm, G. (1993) Liposomes with prolonged circulation and selective localisation in *Klebsiella pneumoniae*- infected lung tissue, *J. Infectious Diseases*, **168**, 164-171

Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids, *J. Molec. Biol.*, **13**, 238-252

Barenholz, Y. and Amselem, S. (1993) Quality control assays in the development and clinical use of liposome-based formulations, In: Liposome Technology, Volume I (2nd edition), CRC Press, Boca Raton, USA, pp. 527-616

Bellows, R.J. and King, C.J. (1972) Freeze-Drying of Aqueous Solutions: Maximum Allowable Operating Temperature, *Cryobiology*, **9**, 559-561

Betageri, G.V. and Parsons, D.L. (1992) Drug encapsulation and release from multilamellar and unilamellar vesicles, *Int. J. Pharm.*, **81**, 235-241

Betageri, G.V. (1993) Liposomal encapsulation and stability of dideoxyinosine triphosphate, *Drug. Dev. Ind. Pharm.*, **19**(5), 531-539

Bligh, E.J. and Dyer, W.J. (1955) A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, **37**, 911-917

Bruni, F. and Leopold, A.C. (1991) Glass transitions in soybean seed: Relevance to anhydrous biology, *Plant Physiol.*, **96**, 660-663

Buckton, G. (1995) Applications of isothermal microcalorimetry in the pharmaceutical sciences, *Thermochimica Acta*, **248**, 117-129

Carpenter, J.F and Crowe, J.H (1988) The mechanism of protection of proteins by solutes, *Cryobiology*, **25**, 244-255

Carpenter, J.F. and Crowe, J.H (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried protein, *Biochemistry*, **28**, 3916-3922

Carpenter, J.F., Crowe, J.H. and Arakawa, T. (1990) Comparison of Solute-Induced Protein Stabilization in Aqueous Solution and in the Frozen and Dried States, *J. Dairy. Sci.*, **73**(12), 3627-3636

Carpenter, J.F., Arakawa, T. and Crowe, J.H. (1991) Interactions of Stabilizing Additives with Proteins During Freeze-Thawing and Freeze-Drying, *Dev. Biol. Std.*, **74**, 225-239

Carpenter, J.F., Prestrelski, S.J. and Arakawa, T. (1993) Separation of Freezing- and Drying- Induced Denaturation of Lyophilized Proteins Using Stress-Specific Stabilization. I. Enzyme Activity and Calorimetric Studies, *Arch. Biochem. Biophys.*, **303**(2), 456-464

Carpenter, J.F., Prestrelski, S.J., Anchordoguy, T.J. and Arakawa, T. (1994) Interactions of Stabilizers with Proteins During Freezing and Drying, *In:* J.L. Cleland & R. Langer (eds.), *Formulation and delivery of proteins and peptides*, American Chemical Society Symposium Series, Washington DC, pp.134-147

Ceh, B. and Lasic, D.D. (1995) A Rigorous Theory of Remote Loading into Liposomes, *Langmuir*, **11**, 3356-3368

Cevc, G. (1993) Lipid properties as a basis for membrane modeling and rational liposome design, In: G. Gregoriadis (ed.), *Liposome Technology*, Vol. I, CRC Press, Boca Raton, Florida, pp.1-35

Chandrasekhar, I. and Gaber, B.P. (1988) Stabilization of the bio-membrane by small molecules: interaction of trehalose with the phospholipid bilayer, *J. Biomol. Struct. Dyn.*, **5**, 1163-1168

Chang, B.S. and Randall, C.S. (1992) Use of Subambient Thermal-Analysis to Optimize Protein Lyophilization, *Cryobiology*, **29**(5), 632-656

Chang, B.S., Randall, C.S. and Lee, Y.S. (1993) Stabilization of Lyophilized Porcine Pancreatic Elastase, *Pharm. Res.*, **10**(10), 1478-1483

Chang, B.S. and Fischer, N.L. (1995) Development of an Efficient Single-Step Freeze-Drying Cycle for Protein Formulations, *Pharm. Res.*, **12**(6), 831-837

Cleland, J.L., Powell, M.F. and Shire, S.J. (1993) The development of stable protein formulations: a close look at protein aggregation, deamidation and oxidation, *Crit. Rev. Ther. Drug Carrier Systems*, **10**, 307-377

Cohen, B.E. (1975) The permeability of liposomes to non-electrolytes, *J. Membrane Biology*, **20**, 205-234

Colaco, C.A.L.S., Smith, C.J.S., Sen, S., Roser, D.H., Newman, Y., Ring, S. and Roser, B.J. (1994) Chemistry of Protein Stabilization by Trehalose, *Am. Chem. Soc. Symposium Series*, **567**, 222-240

Constantino, H.R., Langer, R. and Klibanov, A.M. (1995) Aggregation of Lyophilized Pharmaceutical Protein, Recombinant Human Albumin: Effect of Moisture and Stabilization by Excipients, *Biotechnology*, **13**(5), 493-496

Crommelin, D.J.A. and Schreier, H. (1994) Liposomes, In: J. Kreuter (ed.), *Colloidal Drug Delivery Systems*, Marcel Dekker, New York, 73-190

Crowe, J.H. and Crowe, L.M. (1988) Factors affecting the stability of dry liposomes, *Biochim. et Biophys. Acta*, **939**, 327-334

Crowe, J.H. and Crowe, L.M. (1991) Stabilization of dry liposomes by carbohydrates, *Dev. Biol. Std.*, **74**, 285-294

Crowe, J.H. and Crowe, L.M. (1993) Preservation of Liposomes by Freeze-Drying, In: G. Gregoriadis (ed.), *Liposome Technology*, Vol. I, CRC Press, Boca Raton, Florida, pp.229-252

Crowe, J.H., Spargo, B.J. and Crowe, L.M. (1987a) Preservation of dry liposomes does not require retention of residual water, *Proc. Natl. Acad. Sci. USA.*, **84**, 1537-1540

Crowe, J.H., Crowe, L.M., Carpenter, J.F. and Aurell Wistrom, C. (1987b) Stabilization of dry phospholipid bilayers and proteins by sugars, *Biochem. J.*, **242**, 1-10

Crowe, J.H., McKersie, B.D. and Crowe, L.M. (1989a) Effects of free fatty acids and transition temperature on the stability of liposomes, *Biochim. et Biophys. Acta*, **979**, 7-12

Crowe, J.H., Crowe, L.M. and Hoekstra, F.A. (1989b) Phase transitions and permeability changes in dry membranes during rehydration, *J. Bioenerg. Biomemb.*, **21**, 77-95

Crowe, J.H., Carpenter, J.F., Crowe, L.M. and Anchordoguy, T.J. (1990) Are Freezing and Dehydration Similar Stress Vectors? A Comparison of Modes of Interaction of Stabilizing Solutes with Biomolecules, *Cryobiology*, **27**, 219-231

Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1992) Anhydrobiosis, *Annu. Rev. Physiol.*, **54**, 579-599

Crowe, L.M., Crowe J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Preservation of freeze-dried liposomes by trehalose, *Arch. Biochem. Biophys.*, **242**(1), 240-247

Crowe, L.M., Womersley, C., Crowe J.H., Reid, D., Appel, L. and Rudolph, A. (1986) Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates, *Biochim. et Biophys. Acta*, **861**, 131-140

Crowe, L.M., Spargo, B.J., Ioneda, T., Beaman, B.L. and Crowe, J.H. (1994a) Interaction of cord factor (α,α'-trehalose-6,6'-dimycolate) with phospholipids, *Biochimica et Biophysica Acta*, **1194**, 53-60

Crowe, J.H., Leslie, S.B. and Crowe, L.M. (1994b) Is Vitrification Sufficient to Preserve Liposomes during Freeze-Drying?, *Cryobiology*, **31**(4), 355-366

D'Andrea, G., Salucci, M.L. and Avigliano, L. (1996) Effect of lyoprotectants on ascorbate oxidase activity after freeze-drying and storage, *Process Biochemistry*, **31**(2), 173-178

de Kok, P.M.T. and Rosing, E.A.E. (1994) Reactivity of Peptides in the Maillard Reaction, *Am. Chem. Soc. Symposium Series*, **543**, 158-179

Dole, V.P. (1956) A relation between nonesterified fatty acids in plasma and the metabolism of glucose, *J. Clin. Invest.*, **35**, 150-154

Dürr, M., Hager, J. and Löhr, J.P. (1994) Investigations on mixed micelle and liposome preparations for parenteral use based on soya phosphatidylcholine, *Eur. J. Pharm. Biopharm.*, **40**(3), 147-156

Elorza, B., Elorza, M.A., Sainz, M.C. and Chantres, J.R. (1993) Comparison of Particle Size and encapsulation parameters of three liposomal preparations, *J. Microencapsulation*, **10**(2), 237-248

Engel, A., Bendas, G., Wilhelm, F., Mannova, M., Ausborn, M. and Nuhn, P. (1994) Freeze-drying of liposomes with free and membrane-bound cryoprotectants - the background of protection and damaging processes, *Int. J. Pharm.*, **107**, 99-110

Fielding, R.M. (1991) Liposomal drug delivery: advantages and limitations from a clinical pharmacokinetic and therapeutic perspective, *Clinical Pharmacokinetics*, **21**, 155-164

Finegold, L., Franks, F. and Hatley, R.H.M. (1989) Glass/Rubber Transitions and Heat Capacities of Binary Sugar Blends, *J. Chem. Soc. Faraday Trans. 1*, **85**(9), 2945-2951

Flosdorf, E.W. and Mudd, S. (1935) Procedure and apparatus for preservation in the lyophile form of serum and other biological substances, *J. Immunol.*, **29**, 389-425

Ford, A.W. and Allahiary, Z. (1993) The adverse effect of glycation of Human Serum Albumin on its Preservative Activity in the Freeze-Drying and Accelerated Degradation of Alkaline Phosphatase, *J. Pharm. Pharmacol.*, **45**, 900-906

Ford, A.W. and Dawson, P.J (1994) Effect of Type of Container, Storage Temperature and Humidity on the Biological Activity of Freeze-dried Alkaline Phosphatase, *Biologicals*, **22**, 191-197

Ford, J.L. and Timmins, P (1989) Pharmaceutical Thermal Analysis: techniques and applications, Ellis Horwood, Chichester, UK, pp.154-156

Franks, F. (1982) The properties of aqueous solutions at subzero temperatures, In: F. Franks (ed.), *Water: A Comprehensive Treatise*, Vol.7, Plenum Press, New York, pp.215-338

Franks, F. (1987) Physical-chemistry of small carbohydrates - Equilibrium solution properties, *Pure Appl. Chem.*, **59**(9), 1189-1202

Franks, F. (1990) Freeze-drying: from empiricism to predictability, *Cryo-Letters*, **11**, 93-110

Franks, F. (1994) And then it was freeze-dried (editorial), *Cryo-Letters*, **15**, 75-76

Franks, F., Hatley, R.H.M., Mathias, S.F. (1991) Materials Science and the Production of Shelf-Stable Biologicals, *Pharm. Tech. Int.*, **3**(8), 24-29

Frohwein, Y.Z., Friedman, M., Reizer, J. and Grossowicz, N. (1971) Sensitive and Rapid Assay for L-Asparaginase, *Nature New Biology*, **230**, 158-159

Fujita, Y. and Noda, Y. (1979) Effect of hydration on the Thermal Stability of Protein, as measured by Differential Scanning Calorimetry. Lysozyme-D_2O system, *Bull. Chem. Soc. Japan*, **52**(8), 2349-2352

Gatlin, L.A. and DeLuca, P.P. (1980) A Study of the Phase Transitions in Frozen Antibiotic Solutions by Differential Scanning Calorimetry, *J. Parent. Drug Assoc.*, **34**, 398-408

Gerlsma, S.Y. (1968) Reversible denaturation of Ribonuclease in Aqueous Solutions As Influenced by Polyhydric Alcohols and Some Other Additives, *J.Biol. Chem.*, **243** (5), 957-961

Goldberg, R.N. and Tewari, Y.B. (1989) Thermodynamic- and transport-properties of carbohydrates and their monophosphates - the pentoses and hexoses, *Jnl. Physical and Chemical Reference Data*, **18**(2), 809-880

Greaves, R.I.N. (1946) The Preservation of Proteins by Drying, *Medical Research Council Special Report Series No.258*, HMSO, London

Green, J.L. and Angell, C.A. (1989) Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly, *J. Phys. Chem.*, **93**, 2880-2882

Green, R.W. (1947) The Adsorption of Water Vapour on Casein, *Trans.Royal Soc. N.Z.*, **77**(2), 313-317

Gregoriadis, G. (1976a) The Carrier Potential of Liposomes in Biology and Medicine (First of Two Parts), *New. Engl. J. Med.*, **295**, 704-710

Gregoriadis, G. (1976b) The Carrier Potential of Liposomes in Biology and Medicine (Second of Two Parts), *New. Engl. J. Med.*, **295**, 765-770

Gregoriadis, G. (1988) Fate of injected liposomes: observations on entrapped solute retention, vesicle clearance and tissue distribution *in-vivo*, In: G. Gregoriadis (ed.), *Liposomes as drug carriers - recent trends and progress*, John Wiley & Sons, Chichester, pp.3-18

Gregoriadis, G. (1990) Immunological adjuvants: a role for liposomes, *Imunology Today*, **11**(3), 89-97

Gregoriadis, G. (1993) Liposomes: a tale of drug targeting, *J. Drug Targeting*, **1**, 3-6

Gregoriadis, G. (1995) Fate of liposomes *in-vivo* and its control: a historical perspective, In: D. Lasic & F. Martin (eds.), *Stealth Liposomes*, CRC Press, Boca Raton, Florida, pp.7-12

Gregoriadis, G. and Ryman, B. (1972) Lysosomal localisation of β-fructofuranosidase-containing liposomes injected into rats. Some implications in the treatment of genetic disorders, *Biochem. Biophys. Res. Comm.*, **129**, 123-133

Hageman, M. (1988) The Role of Moisture in Protein Stability, *Drug Dev. Ind. Pharm.*, **14**(14), 2047-2070

Hancock, B.C. and Zografi, G. (1994) The Relationship Between the Glass Transition Temperature and the Water Content of Amorphous Pharmaceutical Solids, *Pharm. Res.*, **11**(4), 471-477

Harrigan, P.R., Madden, T.R. and Cullis, P.R. (1990) Protection of liposomes during dehydration or freezing, *Chem. Phys. Lipids*, **52**(2), 139-149

Hatley, R.H.M., Franks, F., Brown, S., Sandhu, G., and Gray, M. (1996) Stabilization of a pharmaceutical drug substance by freeze-drying: a case study, *Drug Stability*, **1**(2), 73-85

Hellman, K., Miller, D.S. and Cammack, K.A. (1983) The effect of freeze-drying on the quaternary structure of L-Asparaginase from *Erwinia carotovora, Biochim. Biophys. Acta*, **749**, 133-142

Hobbs, P.V. (1974) *Ice Physics*, The Clarendon Press, Oxford, UK.

Hristova, K. and Needham, D. (1995) Physical properties of polymer-grafted bilayers, In: D. Lasic and F. Martin (eds.), *Stealth Liposomes*, CRC Press, Boca Raton, USA, ISBN 0-8493-8383-8, pp.35-49

Hsu, C.C., Ward, C.A., Pearlman, R., Nguyen, H.M, Yeung, D.A. and Curley, J.G. (1991) Determining the Optimum Residual Moisture in Lyophilised Protein Pharmaceuticals, *Dev. Biol. Stand.*, **74**, 255-271

Imai, M., Inoue, K. and Nojima, S. (1975) Effect of polymyxin B on liposomal membranes derived from Escherichia coli lipids, *Biochim. et Biophys. Acta*, **375**, 130-137

Irwin, W.J. and Iqbal, M. (1991) Solid-state stability: the effect of grinding solvated excipients, *Int. J. Pharm.*, **75**, 211-218

Izutsu, K., Yoshioka, S. and Takeda, Y. (1991) The effects of additives on the Stability of freeze-dried β-galactosidase stored at elevated temperature, *Int. J. Pharm.*, **71**, 137-146

Izutsu, K., Yoshioka, S. and Terao, T. (1993a) Decreased Protein-Stabilizing Effects of Cryoprotectants Due to Crystallization, *Pharm. Res.*, **10**(8), 1232-1237

Izutsu, K., Yoshioka, S. and Terao, T. (1993b) Stabilization of β-galactosidase by amphiphilic additives during freeze-drying, *Int. J. Pharm.*, **90**, 187-194

Izutsu, K., Yoshioka, S. and Kojima, S. (1994a) Physical stability and protein-stability of freeze-dried cakes during storage at elevated temperatures, *Pharm. Res.*, **11**(7), 995-999

Izutsu, K., Yoshioka, S. and Terao, T. (1994b) Stabilizing effect of amphiphilic excipients on the freeze-thawing and freeze-drying of Lactate Dehydrogenase, *Biotech. Bioeng.*, **43**, 1102-1107

Izutsu, K., Yoshioka, S. and Terao, T. (1994c) Effect of Mannitol Crystallinity on the Stabilization of Enzymes during Freeze-drying, *Chem. Pharm. Bull.*, **42**(1), 5-8

Izutsu, K. and Yoshioka, S. (1995) Stabilization of protein pharmaceuticals in freeze-dried formulations, *Drug Stability*, **1**(1), 11-21

Izutsu, K., Yoshioka, S. and Kojima, S. (1995) Increased stabilising effects of amphiphilic excipients on the freeze-drying of lactate dehydrogenase (LDH) by dispersion into sugar matrices, *Pharm. Res.*, **12**(6), 838-843

James, C. (1995) A Spoonful of Sugar Helps the Medicine Go Down, *The Independent*, IND 10/10/95, p.15

Karlowsky, J.A. and Zhanel, G.G. (1992) Concepts on the use of liposomal antimicrobial agents: applications for aminoglycosides, *Clinical Infectious Diseases*, **15**, 654-667

Kayes, J.B. (1988) Disperse systems, In: M.E. Aulton (ed.), *Pharmaceutics - The Science of dosage form design*, Churchill Livingstone Publishing Ltd, London, pp.81-118

Kirby, C. and Gregoriadis, G. (1984) Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes, *Biotechnology*, **2**, 979-983

Koenigbauer, M.J. (1994) Pharmaceutical Applications of Microcalorimetry, *Pharm. Res.*, **11**(6), 777-783

Koster, K.L. and Leopold, A.C. (1988) Sugars and desiccation tolerance in seeds, *Plant. Physiology*, **88**, 829-832

Kramhöller, B., Pischetsrieder, M. and Severin, T. (1993) Maillard Reactions of Lactose and Maltose, *J. Agric. Food. Chem.*, **41**, 347-351

Kulkarni, S.B., Betageri, G.V. and Singh, M. (1995) Factors affecting microencapsulation of drugs in liposomes, *J. Microencapsulation*, **12**(3), 229-246

Lasic, D.D. (1988) The Mechanism of Vesicle Formation, *Biochem. J.*, **256**, 1-11

Lasic, D.D. (1994) Sterically Stabilized Vesicles, *Angewandte Chem. Int. Ed. Engl.*, **33**, 1685-1698

Lasic, D.D. (1996) Liposomes - an industrial view, *Chemistry & Industry*, **6**, 210-214

Lasic, D.D. and Needham, D. (1995) The "Stealth" Liposome: A Prototypical Biomaterial, Chemical Reviews, **95**(8), 2601-2628

Lasic, D.D. and Papahadjopoulos, D. (1995) Liposomes revisited, *Science*, **267**(No.5202), 1315-1316

Law, S.L., Tang, T.F., Chang, P. and Lin, C.H. (1994) Release characteristics of mitoxantrone-containing liposomes, *Int. J. Pharm.*, **103**, 81-85

Lawrence, S.M., Alpar, H.O., McAllister, S.M. and Brown, M.R.W. (1993) Liposomal (MLV) Polymyxin B: Physicochemical Characterization and Effect of Surface Charge on Drug Association, *J. Drug Targeting*, **1**, 303-310

Lee, J.C. and Lee, L.L.Y. (1981) Preferential Solvent Interactions between Proteins and Polyethylene Glycols, *J. Biol. Chem.*, **256**(2), 625-631

Lee, L.L.Y. and Lee, J.C. (1987) Thermal Stability of Proteins in the Presence of Polyethylene Glycols, *Biochem.*, **26**, 7813-7819

Lehninger, A.L. (1982) *Principles of Biochemistry*, Worth Publishers, New York, p.96

Leopold, A.C., Bruni, F. and Williams, R.J. (1992) Water in dry organisms, In: G.N. Somero, C.B. Osmond and C.L. Bolis (eds.), *Water and Life*, Springer-Verlag, Berlin, pp.161-169

Leslie, S.B., Teter, S.A., Crowe, L.M. and Crowe, J.H. (1994) Trehalose lowers membrane phase transitions in dry yeast cells, *Biochimica et Biophysica Acta*, **1192**, 7-13

Levine, H. and Slade, L. (1987) Water as a plasticizer: physico-chemical aspects of low-moisture polymeric systems, In: F. Franks (ed.), *Water Science Reviews*, Vol. 5, 79-185

Levine, H. and Slade, L. (1988) Another view of trehalose for drying and stabilizing biological materials, *BioPharm*, **5**, 36-40

Liapis, A.I. and Bruttini, R. (1994) A theory for the primary and secondary drying stages of the freeze-drying of pharmaceutical crystalline and amorphous solutes: comparison between experimental data and theory, *Separations Technology*, **4**, 144-155

Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265-275

MacKenzie, A.P. (1964) Apparatus for microscopic observations during freeze-drying, *Biodynamica*, **9**, 223-231

MacKenzie, A.P. (1966) Basic Principles of Freeze-Drying for Pharmaceuticals, *Bulletin of the Parenteral Drug Association*, **20**(4), 101-127

MacKenzie, A.P. (1975) Collapse during freeze-drying. Qualitative and quantitative aspects, In: S.A. Goldblith, L. Rey & W.W. Rothmayr (eds.), *Freeze-drying and advanced food technology*, Academic Press, London, pp.277-307

MacKenzie, A.P. (1976) Principles of Freeze-Drying, *Transplantation Proceedings*, **8**(2, Supplement 1), 181-188

MacKenzie, A.P. (1977) The Physico-chemical basis for the freeze-drying process, *Dev. Biol. Std.*, **36**, 51-67

MacKenzie, A.P. (1985) A Current Understanding of the Freeze-drying of representative aqueous solutions, *Proceedings of the Meeting of Commision C1, Tokyo*, Issued by the International Institute of Refrigeration, Paris, pp.155-163

McAllister, S.M. (1995) Liposomes as carriers for polymyxins in the treatment of cystic fibrosis lung infections, Ph.D. Thesis, Aston University, UK.

McFadyen, P. (1986) Electrophoretic mobility and zeta potential of colloidal particles, *International Laboratory*, **9**, 32-42

Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Protection of large unilamellar vesicles by trehalose during dehydration: retenrion of vesicle contents, *Biochim. Biophys. Acta*, **817**, 67-74

Manning, M.C., Patella, K. and Borchardt, R.T. (1989) Stability of Protein Pharmaceuticals, *Pharm. Res.*, **6**(11), 903-915

Mansure, J.J.C., Panek, A.D., Crowe, L.M. and Crowe, J.H. (1994) Trehalose inhibits ethanol effects on intact yeast cells and liposomes, *Biochimica et Biophysica Acta*, **1191**, 309-316

Marlborough, D.I., Miller, D.S. and Cammack, K.A. (1975) Comparative study on conformational stability and sub-unit interactions of two bacterial asparaginases, *Biochim. Biophys. Acta*, **386**, 576-589

Martin, F.J. (1990) Pharmaceutical manufacturing of liposomes, In: P.Tyle (ed.), *Specialized drug delivery systems: Manufacturing and production technology*, Marcel Dekker, New York, pp.267-316

May, J.C., Grim, E., Wheeler, R.M. and West, J. (1982) Determination of Residual Moisture in Freeze-Dried Viral Vaccines - Karl Fischer, Gravimetric and Thermogravimetric Methodologies, *J.Biol.Std.*, **10**, 249-259

Mellor, J.D. (1978) *Fundamentals of Freeze-Drying*, Academic Press, London

Meyer (Jr.), A.S. and Boyd, C.M. (1959) Determination of Water by Titration with Coulometrically Generated Karl Fischer Reagent, *Analyt. Chem.*, **31**, 215-219

Miller, M., Rao, J.K.M., Wlodawer, A., Gribskov, M.R. (1993) A left-handed crossover involved in amidohydrolase catalysis. Crystal structure of *Erwinia chrysanthemi* L-asparaginase with bound aspartate, FEBS Letters, **328**(3), 275-279

Mobley, W.C. and Schreier, H. (1994) Phase transition temperature reduction and glass formation in dehydrated lyophilized liposomes, *J. Cont. Rel.*, **31**, 73-87

Müller, R.H. (1991) *Colloidal carriers for controlled drug delivery and targeting. Modification, characterization and in-vivo distribution*, CRC Press Inc., Boca Raton.

Nail, S.L. and Johnson, J.W. (1992) Methodology for in-process determination of residual moisture in freeze-dried products, *Dev. Biol. Std.*, **74**, 137-151

Nail, S.L. and Gatlin, L.A. (1993) Freeze-Drying: Principles and Practice, In: K. Avis, L. Lachman and H. Lieberman (eds.) *Pharmaceutical Dosage Forms: Parenteral Medications*, Vol II., Marcel Dekker, New York, pp.163-233

Nelson, E.W. and Newton, R.F. (1941) The Heat Capacity of Glucose Glass, *J. Am. Chem. Soc.*, **63**, 2178-2182

New, R.R.C. (1981) Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes, *J. Antimicrob. Chemoth.*, **8**(5), 371-377

New, R.R.C. (1990) Preparation of Liposomes, In: R.R.C. New (ed.) *Liposomes - a practical approach*, Oxford University Press, New York, pp.1-32

Ostro, M.J. and Cullis, P.R. (1989) Use of liposomes as injectable drug delivery systems, *American Journal of Hospital Pharmacy*, **46**, 1576-1587

Özer, Y., Talsma, H., Crommelin, D.J.A. and Hincal, A.A. (1988) Influence of freezing and freeze-drying on the stability of liposomes dispersed in aqueous media, *Acta Pharm. Technol.*, **34**, 129-133

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy, *Proc. Natl. Acad. Sci. USA.*, **88**, 11460-11464

Park, Y.S. and Huang, L. (1992) Cryoprotective activity of synthetic glycophospholipids and their interactions with trehalose, *Biochim. et Biophys. Acta*, **1124**, 241-248

Pauling, L. (1945) The Adsorption of Water by Proteins, *J.Amer.Chem.Soc.*, **67**, 555-557

Pikal, M.J., Lukes, A.L. and Lang, J.E. (1977) Thermal Decomposition of Amorphous β-Lactam Antibiotics, *J. Pharm. Sci.*, **66**, 1312-1316

Pikal, M.J., Lukes, A.L., Lang, J.E. and Gaines, K. (1978) Quantitative Crystallinity Determinations for β-Lactam Antibiotics by Solution Calorimetry: Correlations with Stability, *J. Pharm. Sci.*, **67**, 767-773

Pikal, M.J. (1990a) Freeze-Drying of Proteins, Part I: Process Design, *BioPharm*, **3**(8), 18-27

Pikal, M.J. (1990b) Freeze-Drying of Proteins, Part II: Formulation Selection, *BioPharm*, **3**(9), 26-30

Pikal, M.J. and Shah, S. (1990) The collapse temperature in freeze-drying: dependence on measurement methodology and rate of water removal from the glassy phase, *Int. J. Pharm.*, **62**, 165-186

Pikal, M.J., Dellerman, K.M., Roy, M.L. and Riggin, R.M. (1991) The effects of formulation variables on the stability of freeze-dried human growth hormone, *Pharm. Res.*, **8**, 427-436

Pikal, M.J. (1994) Freeze-Drying of Proteins - Process, Formulation and Stability, In: J.L. Cleland & R. Langer (eds.), *Formulation and delivery of Proteins and Peptides*, ACS Symposium Series, Washington DC, pp.120-133

Prestrelski, S J., Arakawa, T. and Carpenter, J.F. (1993a) Separation of Freezing- and Drying- Induced Denaturation of Lyophilized Proteins Using Stress-Specific Stabilization. II. Structural Studies Using Infrared Spectroscopy, *Arch. Biochem. Biophys.*, **303**(2), 465-473

Prestrelski, S.J, Tedeschi, N., Arakawa, T. and Carpenter, J.F. (1993b) Dehydration-induced Conformational Transitions in Proteins and Their Inhibition by Stabilisers, *Biophys. J.*, **65**, 661-671

Quinn, P.J. (1985) A lipid phase-separation model of low temperature damage to biological membranes, *Cryobiology*, **22**, 28-38

Reading, M. (1993) Modulated differential scanning calorimetry - a new way forward in materials characterisation, *Trends in Polymer Science*, **1**(8), 248-253

Riaz, M., Weiner, N. and Martin, F. (1989) Liposomal disperse systems, In: H.A.Lieberman, M.M. Reiger and G.S. Baker (eds.), *Pharmaceutical Dosage Forms: disperse systems*, Marcel Dekker Inc., New York, pp.567-602

Rodin, V.V. and Izmailova, V.N. (1994) NMR Study of the Effect of Disaccharides on the Stability of Vesicles from Phosphatidylcholine with Respect to Low Temperatures, *Colloid Journal.*, **56**(6), 745-751

Rowe, T.W.G. (1977) Optimization in Freeze-Drying, *Dev. Biol. Std.*, **36**, 79-97

Rudolph, A.S. (1988) The freeze-dried preservation of liposome-encapsulated hemoglobin: a potential blood substitute, *Cryobiology*, **25**, 277-280

Rudolph, A.S. and Cliff, R.O. (1990) Dry storage of liposome-encapsulated hemoglobin: a blood substitute, *Cryobiology*, **27**, 585-589

Rudolph, B.R., Chandrasekhar, I., Gaber, B.P. and Naguomo, M. (1990) Molecular modelling of saccharide-lipid interactions, *Chem. Phys. Lipids*, **53**, 243-249

Shah, N.K. and Ludescher, R.D. (1993) Influence of hydration on the internal dynamics of hen egg white lysozyme in the dry state, *Photochemistry and Photobiology*, **58**(2), 169-174

Shalaev, E.Y., Varaksin, N.A., Rjabicheva, T.G. and Aborneva, I.V. (1996) Solid-liquid state diagrams of the pseudo-ternary systems hydrolysed protein-sucrose-water: Implication for Freeze-Drying, *Cryoletters*, **17**, 183-194

Shaw, D.J. (1991) *Introduction to colloid and surface chemistry* (3rd edition), Butterworth-Heinemann, Oxford, UK, p.156, ISBN 0 7506 0338 0

Skrabanja, A.T.P., de Meere, A.L.J., de Ruiter, R.A. and van den Oetelaar, P.J.M. (1994) Lyophilization of Biotechnology Products, *PDA J. Pharm. Sci. Technol.*, **48**(6), 311-317

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.T. and Goeke, N.M. (1985) Measurement of proteins using bicinchoninic acid, *Analytical Biochemistry*, **150**, 76-85

Strauss, G., Schurtenburger, P. and Hauser, H. (1986) The interaction of saccharides with lipid bilayer vesicles: stabilization during freeze-thawing and freeze-drying, *Biochim. et Biophys. Acta*, **858**, 169-180

Sutherland, D.T. (1995) Lyophilization Course Notes, Lyophilization Technology, 12-15 June 1995, The Center for Professional Advancement, Amsterdam, p.E18

Szoka, F. and Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, *Proc. Natl. Acad. Sci. USA.*, **75**(9), 4194-4198

Szoka, F. and Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes), *Ann. Rev. Biophys. Bioeng.*, **9**, 467-508

Talsma, H. and Crommelin, D.J.A. (1992) Liposomes as drug delivery systems, Part I: Preparation, *Pharmaceutical Technology*, **4** (October), 96-106

Talsma, H., van Steenburgen, M.J. and Crommelin, D.J.A. (1992) The Cryopreservation of Liposomes: 2. Effect of Particle Size on Crystallization Behavior and Marker Retention, *Cryobiology*, **29**, 80-86

Talsma, H. and Crommelin, D.J.A. (1993) Liposomes as drug delivery systems, Part III: Stabilization, *Pharmaceutical Technology*, **5** (January), 48-59

Tanaka, K., Takeda, T. and Miyajima, K. (1991) Cryoprotective Effect of Saccharides on Denaturation of Catalase by Freeze-Drying, *Chem. Pharm. Bull.*, **39**(5), 1091-1094

Tanaka, K., Takeda, T., Fuji, K. and Miyajima, K. (1992) Cryoprotective Mechanism of Saccharides on Freeze-Drying of Liposome, *Chem. Pharm. Bull.*, **40**(1), 1-5

Taylor, K.M.G., Taylor, G., Kellaway, I.W. and Stevens, J. (1990) Drug entrapment and release from multilamellar and reverse-phase evaporation vesicles, *Int. J. Pharm.*, **58**, 49-55

te Booy, M.P.W.M, de Ruiter, R.A. and de Meere, A.L.J. (1992) Evaluation of the Physical Stability of Freeze-Dried Sucrose-Containing Formulations by Differential Scanning Calorimetry, *Pharm. Res.*, **9**(1), 109-114

Teuber, M. and Miller, I.R. (1977) Selective binding of polymyxin B to negatively-charged lipid monolayers, *Biochim. et Biophys. Acta*, **467**, 280-289

Timasheff, S.N. and Inoue, H. (1968) Preferential Binding of Solvent Components to Proteins in Mixed Water-Organic Solvent Systems, *Biochemistry*, **7**(7), 2501-2513

Torchillin, V.P. (1985) Liposomes as targetable drug carriers, *CRC Critical Reviews in Therapeutic Drug Carrier Systems*, **2**(1), 65-115

Towns, J.K. (1995) Moisture content in proteins: its effects and measurement, *Journal of Chromatography. A*, **705**, 115-127

Uedaira, Hatsuho and Uedaira, Hisashi (1980) The Effect of Sugars on the Thermal Denaturation of Lysozyme, *Bull. Chem. Soc. Jpn*, **53**

Vaara, M. and Viljanen, P. (1985) Binding of Polymyxin B nonapeptide to Gram-negative bacteria, *Antimicrobial Agents and Chemotherapy*, **27**(4), 548-554

Vemuri, S., Yu, C.D. and Roosdorp, N. (1994) Effect of Cryoprotectants on Freezing, Lyophilization and Storage of Lyophilized Recombinant $Alpha_1$-Antitrypsin Formulations, *P.D.A. Jnl. Pharm. Sci. Technol.*, **48**(5), 241-246

Volkin, D.B. and Klibanov, A.M. (1989) , In: T.E. Creighton (ed.), *'Protein Function: A Practical Approach'*, Oxford University Press, Oxford, UK, pp.1-24

Wang, Y.C.J. and Hanson, M.A. (1988) Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers, *J. Parenter. Sci. Technol.*, **42**(Supplement), S3-S26

Watwe, R.M. and Bellare, J.R. (1995) Manufacture of Liposomes: A review, *Current Science*, **68**(7), 715-724

Wendlandt, W.W. (1986) *Thermal Analysis*, 3rd edition, Chemical Analysis Series, Wiley, London.

Wichert, B.V., Gonzalez-Rothi, R.J., Straub, L.E., Wichert, B.M. and Schreier, H. (1992) Amikacin liposomes: characterization, aerosolization, and *in-vitro* activity against *Mycobacterium avium intracellulare* in Alveolar Macrophages, *Int. J. Pharm.*, **78**(2-3), 227-235

Williams, N.A. and Dean, T. (1991) Vial breakage by frozen mannitol solutions: correlation with thermal characteristics and effect of stereo-isomerism, additives and vial configuration, *J. Parenter. Sci. Technol.*, **45**, 94-100

Williams, R.J. and Leopold, A.C. (1989) The glassy state in corn embryos, *Plant Physiol.*, **89**, 977-981

Womersley, C., Uster, P.S., Rudolph, A.S. and Crowe, J.H. (1986) Inhibition of dehydration-induced fusion between liposomal membranes by carbohydrates as measured by fluorescence energy transfer, *Cryobiology*, **23**, 245-249

Woodle, M.C. (1993) Surface-modified liposomes: assessment and characterization for increased stability and prolonged blood circulation, *Chem. Phys. Lipids*, **64**, 249-262

Woodle, M.C. and Lasic, D.D. (1992) Sterically stabilized liposomes, *Biochim. Biophys. Acta*, **1113**, 171-199

Woodle, M.C. and Papahadjopoulos, D. (1989) Liposome preparation and size characterization, *Methods in Enzymology*, **171**, 193-217

Woodle, M.C., Storm, G., Newman, M.S., Jekot, J.J., Collins, L.R., Martin, F. and Szoka (Jr.), F.C. (1992a) Prolonged Systemic Delivery of Peptids Drugs by Long-Circulating Liposomes: Illustration with Vasopressin in the Brattleboro' Rat, *Pharm. Res.*, **9**(2), 261-265

Woodle, M.C., Mathay, K.K., Newman, M.S., Hidayat, J.E., Collins, L.R., Redemann, C., Martin, F. and Papahadjopoulos, D. (1992b) Versatility in lipid compositions showing prolonged circulation with sterically stabilised liposomes, *Biochim. et Biophys. Acta*, **1105**, 193-200

Yoshioka, S., Aso, Y., Izutsu, K.I. and Terao, T. (1993) Stability of β-Galactosidase, a Model Protein Drug, Is Related to Water Mobility as Measured by ^{17}O Nuclear Magnetic Resonance (NMR), *Pharm. Res.*, **10**(1), 103-108

Zalipsky, S., Brandeis, E., Newman, M.S. and Woodle, M.C. (1994) Long-circulating, cationic liposomes containing amino-PEG-phosphatidylethanolamine, *FEBS Letters*, **353**, 71-74

APPENDIX I: Typical printout from the Malvern Mastersizer E

Version 1.2a — Tue, Feb 27, 1996 12:52PM

SSL+PXB, 7 days @ 4C :Run Number 1

Sample T1:1, vortexed prior to sizing

Sample File Name: KRW , Record: 3 — Source: Analysed
Measured on: Tue, Feb 27, 1996 12:52PM Last saved on: Tue, Feb 27, 1996 12:52PM

Presentation: 2OFD
Polydisperse model

Number Result
Kill Result Low = 0 High = 4

Focus = 45 mm.

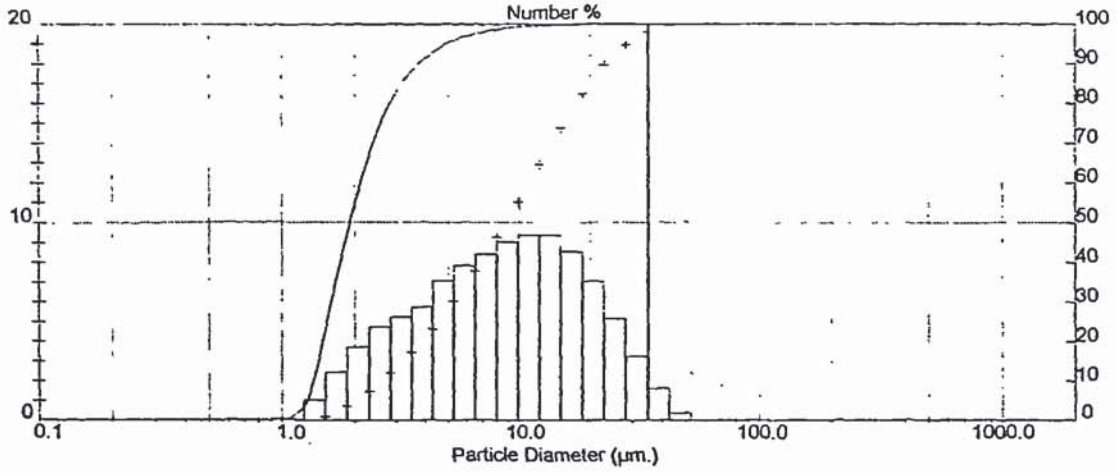
Residual = 0.408 %
d (0.5) = 1.81 µm
D [4, 3] = 10.57 µm
Sauter Mean (D[3,2]) = 5.84 µm
Specific Surface Area = 1.0277 sq. m. / gm

Concentration = 0.002 %
d (0.1) = 1.35 µm
Span = 1.25

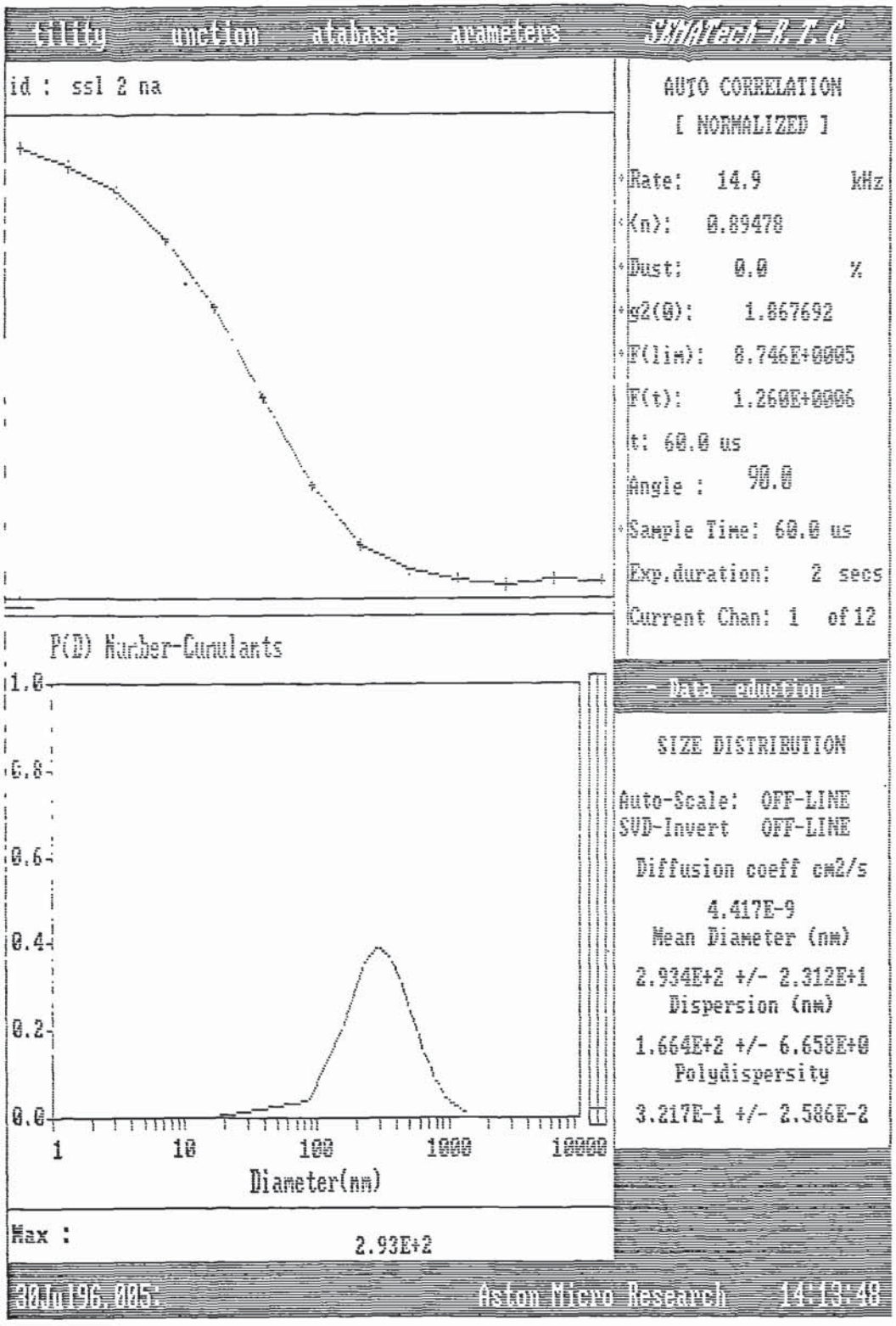
Obscuration = 3.24 %
d (0.9) = 3.74 µm

Mode = 1.51 µm
Density = 1.00 gm. / c.c.

Size (Lo) µm	Result In %	Size (Hi) µm	Result Below %
0.05	0.00	0.12	0.00
0.12	0.00	0.15	0.00
0.15	0.00	0.19	0.00
0.19	0.00	0.23	0.00
0.23	0.00	0.28	0.00
0.28	0.00	0.35	0.00
0.35	0.00	0.43	0.00
0.43	0.00	0.53	0.00
0.53	0.00	0.65	0.00
0.65	0.00	0.81	0.00
0.81	0.01	1.00	0.01
1.00	3.47	1.23	3.48
1.23	19.32	1.51	22.80
1.51	24.43	1.86	47.23
1.86	19.74	2.30	66.98
2.30	13.38	2.83	80.36
2.83	7.87	3.49	88.23
3.49	4.62	4.30	92.85
4.30	3.04	5.29	95.89
5.29	1.82	6.52	97.71
6.52	1.04	8.04	98.75
8.04	0.60	9.91	99.35
9.91	0.33	12.21	99.68
12.21	0.18	15.04	99.85
15.04	0.09	18.54	99.94
18.54	0.04	22.84	99.98
22.84	0.01	28.15	99.99
28.15	0.01	34.69	100.00
34.69	0.00	42.75	100.00
42.75	0.00	52.68	100.00
52.68	0.00	64.92	100.00
64.92	0.00	80.00	100.00



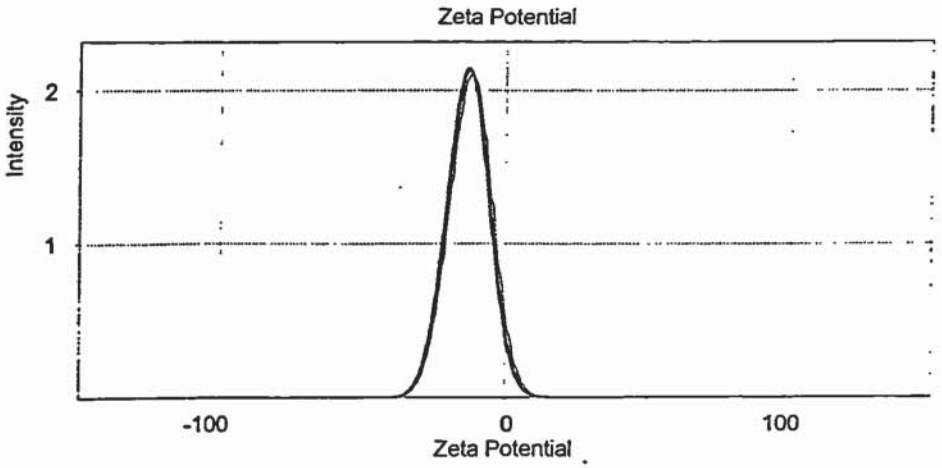
APPENDIX II: Typical printout from the SEMATech Photon Correlation Spectrometer



APPENDIX III: Typical printout from the Malvern Zetamaster

SSLs, reconst. with PXB (i.e. 1xFD).
Prepared 2/6/95.
Vortexed, to reduce aggregation.
File data from Live zeta Record 15
Cell type ZEM010 Cross Beam Mode F(ka) = 1.50 (Smoluchowsky)
Data taken on 6/05/95 at 11:58:49
Cell voltage 140.0 Current 0.3 mA Conductivity 0.11 mS
Temperature 20.5 Viscosity 0.990 Dielectric Constant 79.0
Count rate 2944051.0 ph 0.00 fka 1.50 Cell Position 83.33

Zeta Potential -12.6 StDev 7.4



Zeta	Intensity	Zeta	Intensity	Zeta	Intensity
50.0	0.0	16.7	0.0	-16.7	18.6
45.8	0.0	12.5	0.1	-20.8	12.2
41.7	0.0	8.3	0.6	-25.0	6.0
37.5	0.0	4.2	2.1	-29.2	2.2
33.3	0.0	-0.0	5.8	-33.3	0.6
29.2	0.0	-4.2	11.9	-37.5	0.1
25.0	0.0	-8.3	18.4	-41.7	0.0
20.8	0.0	-12.5	21.3	-45.8	0.0

Peak 1 mean -12.6 width 7.3

Run	Pos.	KCps	Mob.	Zeta	Width	Time
1	16.7	3178.6	-0.828	-11.8	7.4	11:54:49
2	33.3	2988.9	-0.905	-12.8	7.3	11:55:49
3	50.0	2783.4	-0.877	-12.4	7.3	11:56:49
4	66.7	2920.5	-0.923	-13.1	7.3	11:57:49
5	83.3	2944.1	-0.888	-12.6	7.4	11:58:49
Average		2963.1	-0.884	-12.5	7.3	
+/-		142.8	0.036	0.5	0.1	

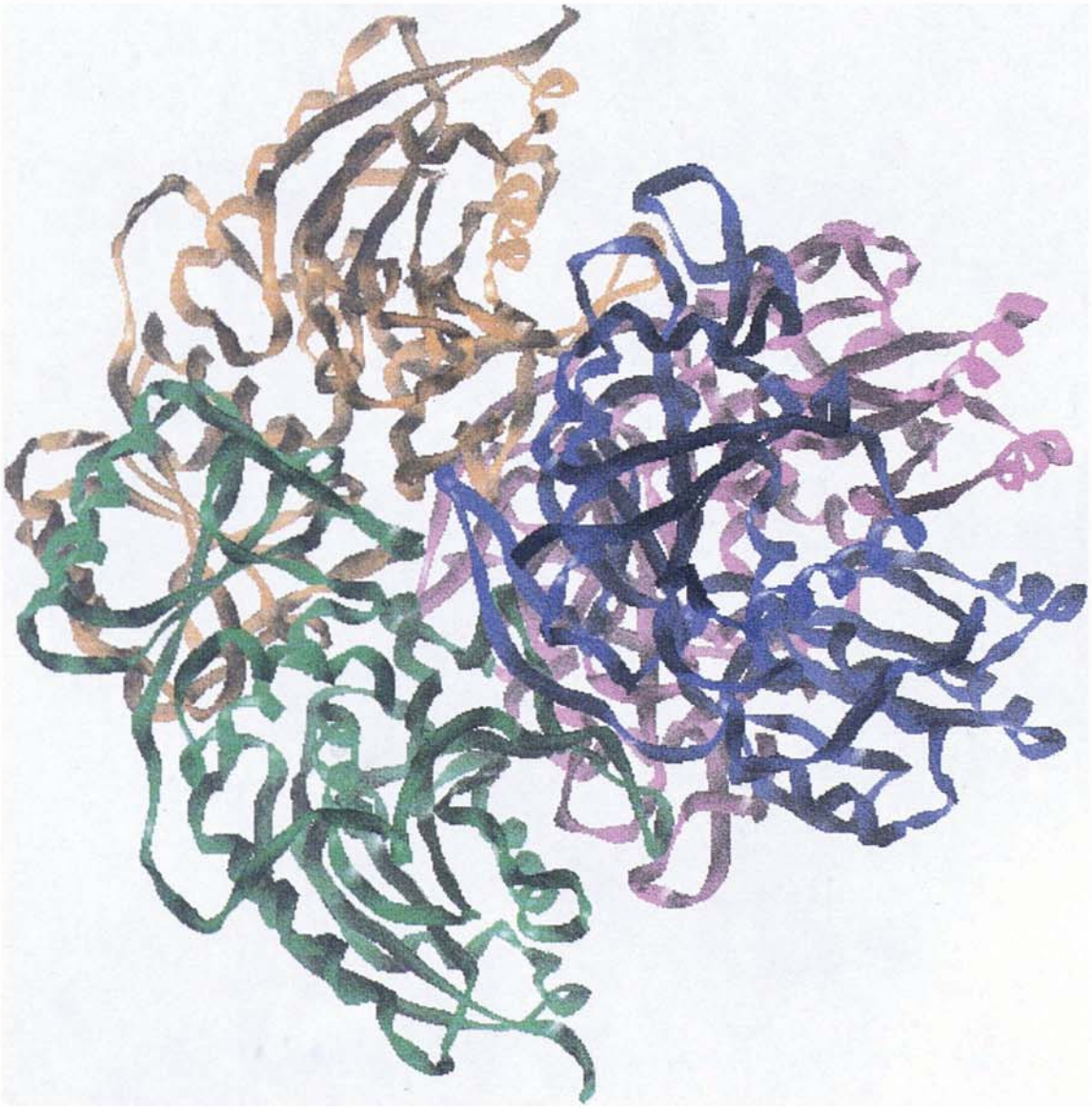
Malvern Instruments England
ZetaMaster Version PCS: v1.2

Monday 5 June 1995

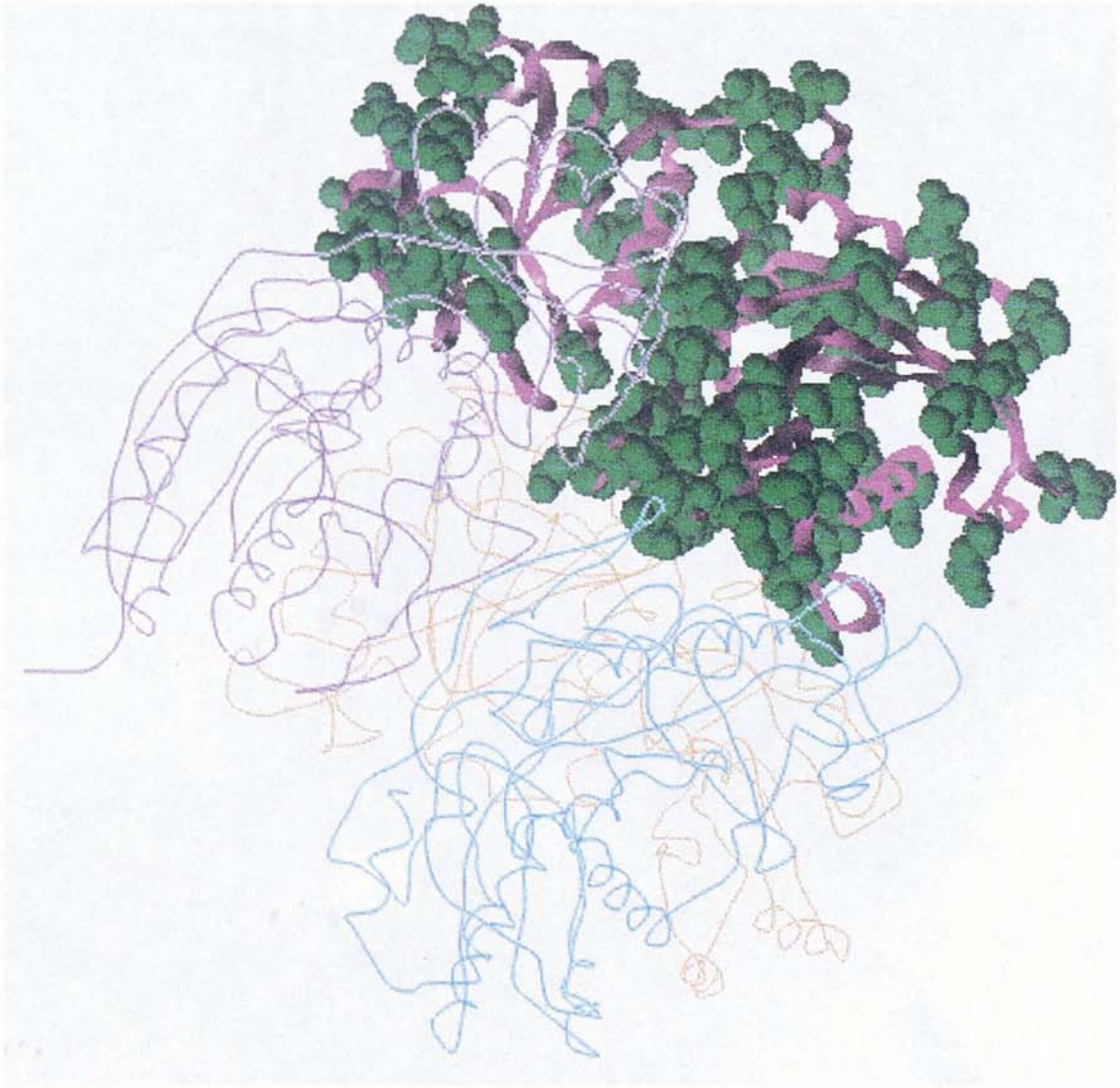
APPENDIX IV: Key to abbreviations for amino-acid residues (from Lehninger, 1982)

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Glu	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX V: Structure of L-asparaginase tetramer depicted in ribbon form, with each monomer sub-unit highlighted in a different colour.



APPENDIX VI: L-Asparaginase tetramer, with one monomer sub-unit highlighted in pink, the HPR exposed in unassociated form are depicted in green. The remaining three sub-units are depicted as ribbons.



APPENDIX VII: L-Asparaginase tetramer, with one monomer sub-unit highlighted in pink, the HPR exposed in the associated form are depicted in green. The remaining three sub-units are depicted as ribbons.