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Factors influencing cryoprotective activity and drug leakage from liposomes after freezing

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The effects of freezing and thawing conditions and cryoprotective additives on release of streptomycin from lecithin liposomes following freeze-thaw cycles have been investigated. Drug retention was maximized by slow cooling (approx. 1 °C min^{-1}). At temperatures between 0° and −20 °C, the extent of drug loss was time-dependent indicating incomplete freezing; below −45 °C this effect was abolished and the system was stable. Osmotic gradients across the liposome membrane during freezing were found to have little effect on drug loss. Marked cryoprotective activity was shown by dimethylsulphoxide, glycerol, alanine and glycinebetaine at concentrations of 3% w/v or less. At this concentration sucrose and mannitol had little activity.

Two factors which have limited the development of liposomes as a delivery system for water-soluble drugs are leakage of the entrapped drug across the lipid membrane and instability of the drug in solution within the vesicle. These effects may be retarded by storage at low temperatures, but leakage of drug may result from damage to the membrane by freezing and thawing. This effect may, in turn, be minimized by the use of cryoprotectants which act, at rapid freezing rates, by restricting intraliposomal ice crystal formation, or at slow freezing rates, by restricting osmotic dehydration and the consequent ionic damage which occurs due to ice crystal formation outside, but not inside, the liposome.

There have been several publications in recent years on the use of cryoprotectants to limit freezing and thawing damage to liposomes, with dimethylsulphoxide (DMSO) (Morris 1982), glycerol (Strauss & Ingenito 1980) and sugars or sugar alcohols (Tsyganenko et al 1983; Henry-Michelland et al 1983; Crommelin & van Bommel 1984) as the agents most commonly used. Unfortunately, it is not easy to compare the degree of success achieved by the various groups, not only because of the variety of cryoprotectants, entrapped drugs and liposome types which have been used, but also because full details of the conditions under which the liposomes were frozen have not always been described. In addition, freeze-thaw damage has, in some cases, been assessed solely on the basis of particle size analysis or turbidity changes, despite the possibility that loss of intraliposomal contents may occur as a result of membrane lesions which reseal upon thawing, leaving the appearance of the liposome suspension largely unchanged.

The purposes of this present work, therefore, were to investigate the relative importance of freezing temperature, freezing rate, thawing rate and osmotic pressure differential across the liposomal membrane as possible factors influencing the extent of drug loss, and to examine the cryoprotective potential of several agents used at low concentrations under conditions where the above factors are controlled.

MATERIALS AND METHODS

Liposome preparation

A solution of chromatographically pure egg lecithin (Lipid Products) in (6:1) chloroform–methanol (40 mg in 0·4 ml) was placed in a 250 ml round bottom flask together with a further 0·4 ml of chloroform–methanol and the flask rotated to give a liquid film over the glass; the solvents were evaporated under nitrogen to give a uniform lecithin film. The flask was placed in a vacuum desiccator overnight at 4 °C to remove the last traces of chloroform. A 2% w/v solution of streptomycin sulphate (Fluka) in 0·02 M pH 6·5 phosphate buffer (6·0 ml), containing cryoprotectant where appropriate, was added to the lipid film at 50 °C. The flask was then held at 50 °C for 2 h with intermittent shaking. The resulting liposome suspension was diluted to approximately 4 times its volume with either phosphate buffer or a solution of the relevant cryoprotectant in buffer, then centrifuged at 140000 *g* for 20 min. The liposome pellet was resuspended in the same solution, washed twice

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more and diluted to provide a suspension containing 4 mg ml^{-1} lecithin. This was designated the streptomycin-containing liposome suspension, and was routinely used in subsequent experiments. Drug leakage from the liposomes was insignificant over 24 h at 4 °C.

Liposomes prepared in this way were uncharged (electrophoresis, Rank Brothers Mark I) and multilamellar (electron microscopy). Most vesicles were seen to be between 0·1 μm and 1·0 μm diameter when viewed by electron microscopy, but size distributions determined by Coulter Counter gave larger values because of liposome aggregation.

To measure streptomycin entrapment, liposomes were disrupted using 1% Triton X 100 in 0·075 M NaH_2PO_4 before assay.

Streptomycin assay

Streptomycin was assayed by HPLC using a Spectra Physics SP8100 chromatograph, SP8440 detector set at 195 nm and a SP4200 computer integrator. A 25 cm column packed with Hypersil ODS was used together with a guard column. The mobile phase was 0·075 M sodium dihydrogen phosphate and 0·02 M 1-pentanesulphonic acid sodium salt with 4% v/v acetonitrile. A flow rate of 1·5 ml min^{-1} and a temperature of 45 °C was used. Dihydrostreptomycin was the internal standard. Replicate determinations of percentage streptomycin entrapment on aliquots from a single liposome suspension gave a coefficient of variation of 0·9%.

Freezing-thawing cycles and temperature measurement

Aliquots of liposome suspension (300 μl) were placed in 400 μl Eppendorf polypropylene centrifuge tubes for freezing experiments. Twelve tubes were placed in a circular test tube holder together with a further tube in which the freezing rate was measured (between −15 and −45 °C) using a copper-constantan thermocouple (Comark) connected to a chart recorder with a reference at 0 °C.

For a freezing rate of 1100 °C min^{-1} the samples were frozen directly in liquid nitrogen, for a rate of 320 °C min^{-1} the samples were placed in ethanol with excess solid CO_2, and for rates between 0·5 and 50 °C min^{-1} the samples were placed in containers with varying degrees of insulation cooled by liquid nitrogen. Samples were thawed in a 40 °C waterbath at a rate of 600 °C min^{-1} unless otherwise stated. After thawing the liposomes were washed in the same liquid as that in which they were frozen. Replicate determinations of percentage streptomycin lost following a freeze-thaw cycle were made on six batches of liposomes and showed a coefficient of variation of 7·0%.

RESULTS

Aliquots of streptomycin-containing liposomes suspended in buffer alone or buffer with 3% w/v glucose were frozen at a rate of 2·5 °C min^{-1} to different temperatures between −5° and −50 °C, and maintained at these temperatures for 5 min before thawing (Fig. 1). The percentage of streptomycin lost from the liposomes increased as the temperature to which the suspension was frozen was reduced (Fig. 1) and the loss was greater in the absence of glucose which, at this concentration, exhibited some cryoprotective activity. When frozen in buffer alone, the freeze-thaw damage to the liposomes as indicated by % streptomycin loss increased rapidly over the temperature range 0 to −20 °C; freezing to lower temperatures produced only a minimal increase in streptomycin loss. In the presence of 3% w/v glucose the temperature range over which the % loss changed markedly was extended down to −40 °C.

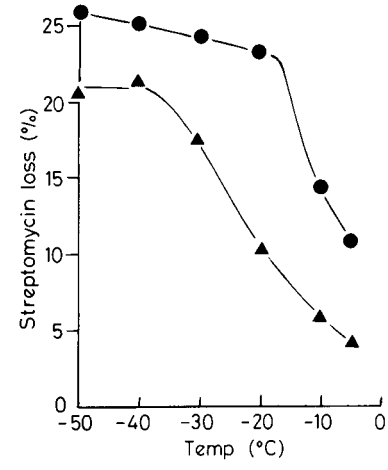

FIG. 1. Effect of freezing temperature on streptomycin loss from liposomes following a freeze-thaw cycle. (●), liposomes suspended in buffer; (▲), liposomes in buffer + 3% glucose.

To determine whether the extent of damage to the liposome membrane in this experiment was dependent upon the freezing temperature itself or on the total period of time during which the liposome suspension was at sub-zero temperatures, the suspension was frozen at 2·5 °C min^{-1} to −20 °C in the presence of various additives and maintained at this temperature for 5 or 30 min; the results are shown in Table 1. In every case the degree of streptomycin loss

Table 1. The influence of holding time at −20 °C on streptomycin leakage from liposomes suspended in iso-osmotic solutions*

Suspending medium additive	Concn in buffer % w/v	% Streptomycin loss after holding at −20° for time 5 min	30 min
Buffer* (control)		†23 ± 2	34
NaCl	0·22	24	31
Alanine	0·60	24	26
Glucose	1·2	28	32
Sucrose	2·3	23	26
Mannitol	1·2	23	30
Glycerol	0·62	23	27
Glycinebetaine	0·79	23	24
DMSO	0·53	19	23

* Additive concentrations determined by freezing point depression (data not shown) to be iso-osmotic with 2% w/v streptomycin; buffer alone is hypo-osmotic with respect to intravesicular liquid.

Results are mean of duplicate determinations except † where n = 6 and 95% confidence limit is given.

increased at the longer holding time which indicated that the system was not stable and complete solidification could not have occurred.

To investigate the freezing temperature required to provide a stable system and eliminate the time-dependent effect of streptomycin-loss, aliquots of the liposome suspension were frozen in buffer alone at varying rates to temperatures between −45° and −196 °C, and then rapidly frozen further from the nominated temperature down to −196 °C. The results are shown in Table 2. This experiment was repeated

Table 2. Effects of freezing rate on streptomycin leakage from liposomes suspended in buffer.

Initial freezing temperature	Rate applied in freezing to initial temp.	% Streptomycin loss Freezing to initial temp. only	Freezing to initial temp. and then to −196 °C*
−45	0·78	30	27
−45	2·9	31	29
−107	7·7	31	33
−165	47	37	37
−77	336	47	49
−196	1150	†59 ± 2	—

* Freezing rate applied from initial temperature to −196 °C was −1150 °C min^{-1}.

Results are the mean of duplicate determinations except † where n = 6 for which 95% confidence limit is given.

with the liposomes suspended in an iso-osmotic (1·2% w/v) concentration of glucose in buffer. The results were qualitatively and quantitatively similar.

The effect of thawing rate on freeze/thaw damage was examined using liposomes frozen to −50 °C at 2·5 °C min^{-1} and thawed at rates between 0·15 and 750 °C min^{-1}. Streptomycin loss fell from approximately 32% to 26% as the thawing rate increased within this range, thus the effect was less than that due to freezing rate (Table 2). When % streptomycin loss was plotted (ordinate) against $\log_{10}$ of thawing rate in °C min^{-1}, a linear relationship resulted conforming to the equation $Y = 31{\cdot}5 - 2{\cdot}173\,X$ (correlation coefficient −0·940).

The effects of relative osmolarities of intra- and extraliposomal liquids on freeze/thaw damage were investigated by suspending streptomycin-containing liposomes in phosphate buffer at eight concentrations between zero (water) and 0·075 M. A concentration of 0·045 M was determined by freezing point depression to be iso-osmolar with the liposomally entrapped 2% w/v streptomycin sulphate in 0·02 M phosphate buffer. Freezing at 2·4 °C min^{-1} to −50 °C followed by thawing at 600 °C min^{-1} resulted in 23% streptomycin loss from liposomes suspended in the iso-osmotic concentration. The corresponding value for suspension in water was 22% loss, and no consistent trend was apparent for the eight suspensions, which, as a whole, gave a mean % loss of 24·1 with a standard deviation of 2·6.

The cryoprotective activities of the materials listed in Table 1 were compared by measuring percentage streptomycin loss from liposomes suspended in 3% w/v solutions in 0·02 M phosphate buffer following freezing at 2·6 °C min^{-1} to −20 and −45 °C (Table 3); concentrations higher than 3% w/v gave solutions of such density that it was not possible to separate the liposomes by centrifugation.

Table 3. Percentage loss of streptomycin from liposomes after a freeze-thaw cycle in the presence of various additives 3% w/v.

Additive	% Streptomycin loss −20 °C*	−45 °C*
Buffer control	†23 ± 2	†26 ± 2
Alanine	14	16
Glucose	19	23
Sucrose	24	29
Mannitol	24	26
Glycerol	15	19
Glycinebetaine	16	15
DMSO	10	12
Polyvinylpyrrolidone (PVP) Pharmaceutical grade Av. Mol wt 40 000	21	23

* Holding time 5 min.

Results are the mean of duplicate determinations except † where n = 6 for which 95% confidence limits are given.

DISCUSSION

The extent to which entrapped material may leak from liposomes at sub-zero temperatures depends upon the rate of temperature drop and the period of

time during which there is liquid water present so that the system remains osmotically active. If the temperature is sufficiently low the system may effectively be stabilized because movement of water by osmosis and of entrapped drug by diffusion through intact membranes and leakage through ruptured membranes, is halted.

Strauss & Ingenito (1980) reported that the extent of liposome membrane damage following a freeze/thaw cycle depended upon the temperature to which the suspension was frozen. A similar effect is seen here in Fig. 1. If however both cooling rate and thawing rates are kept constant in experiments such as this, it is not possible to achieve different final temperatures without variable periods of exposure below 0 °C. If, therefore, the system remains osmotically active throughout the exposure period at all temperatures investigated, variations in percentage loss may largely be a reflection of exposure time rather than temperature.

It is widely recognized that freeze/thaw damage may result from two main sources, viz ice crystal formation and osmotic effects (Siminovitch & Chapman 1971). The former is a particular problem at high cooling rates because nucleation of intracellular ice occurs during freezing, with an increase in crystal size during thawing, particularly if the reheating is slow. Thus, both freezing and thawing are likely to contribute to liposomal membrane damage. Osmotic damage arises at slow cooling rates where ice crystal formation occurs outside the liposome but not within. This results in a slowly increasing concentration of solutes outside the liposome and osmotic dehydration of the vesicle which results in its shrinkage, and stress in the membrane. It has also been stated that drug loss is facilitated during the phase change of the lipid from liquid crystal to gel (Weinstein et al 1976 and references, therein; Juliano & Leyton 1980). The phase transition temperature for lecithin extends over several °C and is dependent upon the purity, chemical composition and lipid: water ratio; it has been reported to be between 0 and −15° (Strauss & Ingenito 1980; Crommelin & van Bommel 1984). Using liposomes of dipalmitoyl phosphatidylcholine, cholesterol and dicetylphosphate in a 7:2:1 molar ratio, Morris & McGrath (1981) showed by cryomicroscopy that shrinkage, distortion and thus osmotic activity were retained at temperatures down to −25 °C. Thus it is likely that the effect which is seen in Fig. 1 is due to increased length of exposure in an osmotically active system in which leakage was promoted by phase transition of the lecithin. This is supported by the data in Table 1 in which streptomycin loss was shown to increase in each of 9 suspensions as the holding time was increased from 5 to 30 min.

It can be seen from Table 2 that after the liposome suspension was frozen to −45 °C at each of two rates, subsequent cooling in liquid nitrogen had no effect on streptomycin loss. Thus all the drug loss and hence all the membrane damage occurred between 0 and −45 °C with the exposure during rapid cooling from −45° to −196 °C not contributing to the overall damage. At temperatures below −45 °C the system was stable, i.e. solidification was complete. The results indicate that it was the initial freezing rate which determined the extent of drug loss rather than the initial temperature to which the suspension was cooled. Had it been the latter, the pattern of results in Table 2 would have given 30–32% drug loss on cooling to −77 °C, whereas a figure of 47% resulted.

The data in Table 2 show substantially reduced membrane damage occurring at slow freezing rates. In this respect the results are similar to those observed when living cells are frozen (Miller & Mazur 1976) but in marked contrast to the findings of Strauss & Ingenito (1980) who reported little difference between rates 0·3, 4·0 and 750 °C min^{-1}.

The beneficial effect of rapid thawing may be attributed to a restriction of the time available during which intraliposomal ice crystal nuclei can increase in size. In this respect, too, the results reported here parallel the situation in living cells.

Freezing drug-containing liposomes in water rather than an iso-osmotic solution may be expected to result in greater membrane damage as a consequence of the increased stress on the lipid membrane following water influx. Similarly, suspension in hyperosmolar solutions may result in increased stress as a result of shrinking of the liposome. The fact that no detectable effect was observed either in water or hyperosmolar solutions may have been due to the use of liposomes prepared from lecithin alone, the membranes of which are more flexible than those of cholesterol-containing liposomes.

In selecting the concentration at which agents should be used to minimize freeze/thaw damage to liposomes, the beneficial cryoprotective effect which may result from an increase in concentration must be balanced against the risk of increased drug leakage at room temperature. Both glycerol (Morris & McGrath 1981) and DMSO (Strauss & Ingenito 1980) have been reported to promote membrane instability at concentrations normally used for the cryoprotection of cells. It is clear, both from Table 1 and Table 3 that betaine, DMSO and alanine show

cryoprotection at concentrations of 3% w/v or lower. DMSO shows some cryoprotective activity even at 0·53% (Table 1), whilst at 3% the amount of streptomycin lost is less than half that in the buffer control. At such concentrations no increase in drug leakage was observed for any of these agents over 24 h at 4 °C. It is noteworthy that glycerol when used at the same concentration by weight was inferior to DMSO as a cryoprotectant; Paul (1975) states that this is probably the common experience when freezing cells.

The sugars, glucose and sucrose had little or no effect, although on a molar basis they were present at a very low concentration. Tsyganenko et al (1983) reported that these sugars when used at 10–15% w/v were superior to glycerol, polyvinylpyrrolidone and polyethylene glycol 400 as cryoprotectants.

Neither betaine nor alanine are commonly used as cryoprotectants although each has previously been reported to show activity in the protection of chloroplast membranes (Coughlan & Heber 1982; Heber et al 1971). This present work indicates that these two compounds are as effective as glycerol in protecting egg lecithin liposomes from freeze-thaw damage under the conditions used.

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