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A comparative investigation of glycinebetaine and dimethylsulphoxide as liposome cryoprotectants

J. HIGGINS, N. A. HODGES*, C. J. OLLIFF AND A. J. PHILLIPS†

Department of Pharmacy, Brighton Polytechnic, Lewes Road, Brighton BN2 4GJ, and †Glaxo Group Research, Ware, Herts SG12 0DJ, UK

The release of streptomycin from lecithin liposomes following a freeze-thaw cycle was used to measure the cryoprotective activities of glycinebetaine and dimethylsulphoxide (DMSO). At concentrations between 4 and 8% w/v in the external solution, glycinebetaine was superior to DMSO at freezing rates faster than 50 °C min^{-1}. At lower rates their activities were similar, and drug loss ranged between 10 and 20% depending upon freezing rate and cryoprotectant concentration. The pattern of streptomycin loss when the concentrations of cryoprotectants inside and outside the liposome were varied indicated that glycinebetaine, in contrast to DMSO, does not diffuse across the liposome membrane. The activity of glycinebetaine was not impaired by the presence in the membrane of cholesterol or charged lipids.

The therapeutic use of drug-containing liposomes would be greatly facilitated by the development of a satisfactory method of storage in which size distribution changes, drug leakage and degradation of both drug and lipid were all restricted to acceptable levels. Both freezing and freeze-drying have been investigated for this purpose with encouraging recent results. Less than 10% drug loss has been reported to occur after freezing of liposomes containing carboxyfluorescein (Fransen et al 1985) and streptomycin (Higgins et al 1986), and, more importantly, after freeze-drying of large unilamellar liposomes containing doxorubicin (Madden et al 1985).

In these and other similar studies, cryoprotectants invariably have been required to minimize membrane damage which arises during the freezing and thawing processes themselves due both to intraliposomal ice crystal formation and to osmotic dehydration and other changes produced by high ionic concentration. DMSO and glycerol are amongst the most widely used cryoprotectants for living cells, with the former probably being more effective (Paul 1975). In liposome systems too, DMSO has usually been found superior to other cryoprotectants. At concentrations of 5–10% it has been shown to afford good or complete protection against freeze-thaw damage under a variety of conditions, whilst glycerol has exhibited lower activity (Strauss & Ingenito 1980; Morris & McGrath 1981; Machy & Leserman 1984).

Glycinebetaine (betaine) is a naturally occurring material which has been implicated in the protection of plant tissues against freezing stress (Coughlan & Heber 1982), and has been shown in preliminary studies, to have potential as a liposome cryoprotectant (Higgins et al 1986).

The purposes of this present work were to investigate some of the factors which may influence the suitability of betaine for the cryoprotection of liposomes and to evaluate its activity in this respect, particularly in comparison with DMSO which was selected as a reference material of wide general application.

MATERIALS AND METHODS

The methods employed for the preparation of streptomycin-containing multilamellar egg lecithin liposomes, their freezing and thawing, and the antibiotic assay have all been described previously (Higgins et al 1986). Cholesterol (25 mole percent) was incorporated into the lipid membrane as required, either alone or in combination with dicetylphosphate (DCP, 10 mole percent) or stearylamine (10 mole percent) to impart a negative or positive membrane charge, respectively. When required, cryoprotectants were added to the buffer entrapped within the liposomes and/or the cryoprotectants were incorporated in the buffer solution in which the liposomes were frozen. Unless specified to the contrary in the text, liposomes were frozen to −45 °C for 10 min and thawed at 600 °C min^{-1}.

After the freeze-thaw cycle the liposomes were diluted five-fold in medium of identical composition to that in which they were frozen and separated from the external solution by centrifugation at 150 000*g* for periods between 15 and 90 min depending upon

* Correspondence.

the external cryoprotectant concentration. The supernatant was carefully removed from the liposome pellet which was dissolved in Triton X 100 to permit measurement of the streptomycin retained. The long centrifugation times required for repeated washing of the liposome pellet in streptomycin-free buffer was the factor which hitherto had limited the external concentration of cryoprotectant used. However, post-thaw washing was found to be unnecessary because control experiments demonstrated that 'carry-over' of extravesicular streptomycin in the pellet was not significant. Elimination of the washing cycle in the present work increased the external concentration of cryoprotectant that could conveniently be used from 3 to 8%.

Freeze-thaw induced turbidity changes

Suspensions of multilamellar liposomes (4 mg lecithin mL^{-1}) were prepared using phosphate-buffered saline (PBS—Dulbecco A, Oxoid) and sonicated (Dawe Instruments 1130A) to effect an approx. 95% reduction in turbidity at 420 nm. Aliquots of sonicated suspension (A_{420} 0·8) were diluted fivefold in cryoprotectant/PBS to achieve the required cryoprotectant concentration, and 1·0 mL samples (A_{420} approx 0.16) were frozen at 2·3 °C min^{-1} to −25 °C and maintained at that temperature for 3 h. The ratio of A_{420} after freezing to that before was recorded.

RESULTS

The liposome cryoprotective activity of betaine was compared with that of DMSO by suspending streptomycin-containing liposomes in solutions of both additives at concentrations between 2 and 8% w/v and measuring streptomycin loss following a freeze-thaw cycle. It may be seen from Fig. 1 that when freezing rates below 50 °C min^{-1} were employed the extent of drug loss was similar in the presence of the two cryoprotectants at concentrations between 4 and 8%. At these slow rates 2% betaine appeared to be slightly inferior to 2% DMSO. At faster freezing rates above 50 °C min^{-1} the converse was true and streptomycin loss was lower in the presence of betaine than in DMSO at each of the four concentrations.

When the extent of freeze-thaw damage was assessed using turbidity increase as an index of vesicle aggregation and fusion, betaine and DMSO were again seen to exhibit similar cryoprotection (Table 1). Both agents restricted the turbidity increase to a factor between approximately 2·2 and 2·6 when used at concentrations between 1 and 10% w/v, compared with the value of 7·35 when the liposomes were frozen in buffer alone.

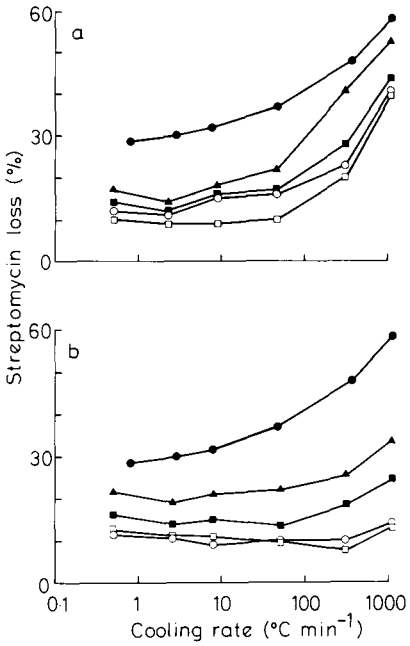

FIG. 1. Streptomycin release from liposomes as a function of a range of concentrations of cryoprotectants in the external solution. 1a DMSO; 1b betaine. Cryoprotectant concentrations (% w/v): 0 ●, 2 ▲, 4 ■, 6 ○, 8 □.

Table 1. The influence of cryoprotectant concentration on turbidity increase of liposome suspensions after freezing.

Cryoprotectant concn & w/v	Turbidity increase* after freezing in	
	DMSO	Betaine
0	7·35	7·35
1	2·59	2·67
2	2·29	2·44
6	2·35	2·27
10	2·24	2·16
15	1·80	2·00

* Ratio of post-freezing to pre-freezing absorbance at 420 nm.

To determine whether the cryoprotective effects of betaine or DMSO could be further increased, liposomes were prepared with the two agents incorporated at a range of concentrations inside the vesicle together with the streptomycin; these liposomes were frozen in 8% w/v cryoprotectant in the external solution. Again both agents restricted streptomycin loss to approx. 10% (Fig. 2) at freezing rates below 50 °C min^{-1} but exhibited differences in

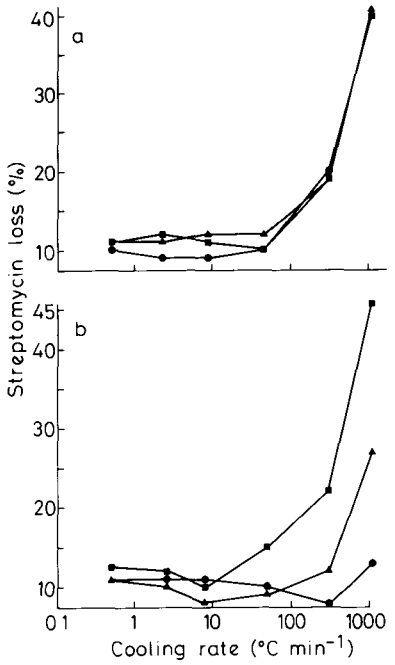

FIG. 2. Streptomycin release from liposomes as a function of rate of freezing with 8% w/v cryoprotectant in the external solution and a range of concentrations within the liposome. 2a DMSO; 2b betaine. Cryoprotectant concentrations (% w/v): 0 ●, 4 ▲, 8 ■.

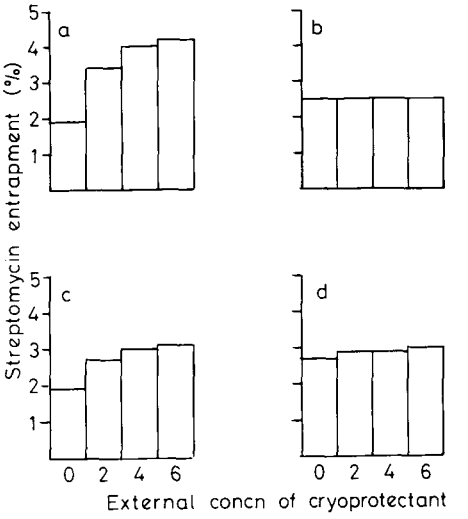

FIG. 3. The effect of external concentration of cryoprotectant (abscissa) on percentage entrapment of streptomycin (ordinate) within liposomes containing 4% w/v cryoprotectant. a, betaine; b, DMSO; c, alanine; d, glycerol.

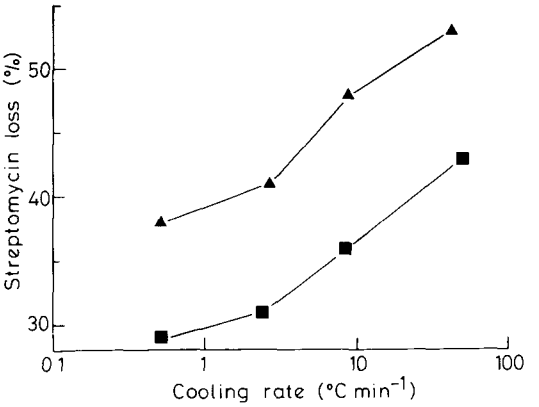

FIG. 4. Streptomycin loss from liposomes initially containing 8% w/v betaine ▲ or 8% w/v DMSO ■ after freezing in buffer.

cryoprotection at higher rates. Whereas drug loss from the liposomes was independent of the nominal concentration of the entrapped DMSO at freezing rates of 310 and 1100 °C min^{-1}, the streptomycin leakage at these rates increased with the concentration of entrapped betaine. DMSO is known to diffuse rapidly across cell membranes and achieve equilibrium within minutes at room temperature (Lovelock & Bishop 1959; Bickis et al 1967). The results observed here would be expected if DMSO were to diffuse across liposome membranes at the same rate. The betaine results show a different trend indicating poor penetrability across the membrane.

This is supported by the data in Figs 3 and 4, which show, respectively, streptomycin entrapment as a function of the external concentration of cryoprotectant during the washing stages of liposome preparation, and streptomycin leakage following freezing in buffer alone of liposomes containing different concentrations of cryoprotectant within the vesicle. From Fig. 3 it may be seen that the concentration of DMSO and glycerol did not influence streptomycin entrapment, but this parameter was dependent upon concentration for betaine. This dependence again suggests that betaine does not readily diffuse across the membrane and establish the same concentration in the internal and external solution.

When streptomycin-liposomes which contained 8% betaine or 8% DMSO were suspended in cryoprotectant-free buffer, the extent of drug loss was markedly dependent upon the nature of the vesicle contents. Betaine at 8% was clearly detrimental but the results for DMSO at the same concentration more closely resembled those for buffer alone.

The chemical composition of the liposome membrane and thawing rate were investigated as potential factors influencing the cryoprotective effect of betaine. The leakage of both amaranth and streptomycin from liposomes composed of lecithin alone, and in various combinations with cholesterol, dicetylphosphate and stearylamine was measured after freezing in buffer or buffer plus betaine; the results are shown in Table 2.

Table 2. Influence of liposome composition on percentage loss of entrapped material after a freeze-thaw cycle.

	Loss (%)*			
	Amaranth		Streptomycin	
Liposome composition	Buffer alone	Buffer + 6% w/v betaine	Buffer alone	Buffer + 8% w/v betaine
PC	28† ± 2	11	29	11
PC + 10% DCP	57	23	23† ± 3	5
PC + 10% SA	14	6	45	20† ± 2
PC + 25% chol	39	14	50	15
PC + 25% chol + 10% DCP	58	27	37	22
PC + 25% chol + 10% SA	17	6	55	20

PC = phosphatidyl chloline.
chol = cholesterol.
DCP = dicetylphosphate.
SA = stearylamine.
* Values are the mean of two results except † where n = 6 for which 95% confidence limit is given. Freezing rate 2·5 °C min^{-1}.

To examine the effect of thawing rate on drug loss, streptomycin-containing liposomes were frozen at 1100 °C min^{-1} and aliquots thawed at rates between 0·3 and 600 °C min^{-1} in buffer or 8% betaine. It may be seen from Table 3 that when betaine was present streptomycin loss was reduced and the extent of leakage was thawing rate-dependent. When frozen in buffer alone approximately 60% of the antibiotic was lost regardless of the thawing rate.

Table 3. Influence of thrawing rate on prcentage streptomycin loss from liposomes following rapid freezing.

	Streptomycin loss (%) when frozen* in	
Thawing rate °C min^{-1}	Buffer alone	Buffer + 8% w/v betaine
0·3	57	32
1·5	62	30
3·0	62	25
30	61	20
113	58	14
300	57	15
600	58	14

* Freezing rate 1100 °C min^{-1}.
Results are the mean of two determinations.

DISCUSSION

Cryoprotectants have been classified as 'penetrating' or 'non-penetrating' depending upon their rate of diffusion across biological membranes (Taylor et al 1974; McGann 1978). The former are used at high molar concentrations, and are thought to act colligatively and primarily protect against 'solution effects' occurring at low cooling rates, whereas the latter are active at lower concentrations and afford protection at fast cooling rates (Meryman et al 1977).

Suspension of liposomes in hyperosmolar solutions of a non-penetrating cryoprotectant may result in significant water loss by osmotic dehydration both before and during the early stages of freezing. Such effects may explain the good cryoprotection observed with betaine (Fig. 1) which displays the characteristics of a non-penetrating agent. McGann (1978) attributed the protective effect of hydroxyethyl starch during freezing of Chinese hamster fibroblasts to a similar mechanism.

The good activity exhibited by DMSO at slow freezing rates between 0·1 and 10 °C min^{-1} (Fig. 1) is in accord with the earlier findings of Morris & McGrath (1981) and is the expected result for a diffusible cryoprotectant.

The therapeutic efficacy of a drug administered as a liposomal preparation is markedly influenced by the particle size distribution of the vesicles (Juliano & Stamp 1975). Thus the maintenance of the optimum size distribution following a period of storage by freezing or freeze-drying is an important aspect of stability, particularly if liposomes were included in aerosols for the treatment of respiratory disease (McCullough & Juliano 1980). The light scattering properties of a liposome suspension alter as the size distribution changes, and increased turbidity following freezing is normally observed as a result of the aggregation or fusion of small liposomes. The ability of cryoprotectants to restrict this increase has been used as a measure of their ability to maintain the initial size distribution after freezing (Strauss 1984). From Table 1 it may be seen that DMSO and betaine show similar activity in this respect.

The evidence in Fig. 1 which suggested that betaine is a non-penetrating cryoprotectant is further supported by the data in Figs 2–4. The differences in cryoprotection by DMSO and betaine in Fig. 2 are again seen at fast freezing rates when streptomycin leakage was independent of the initial concentration of entrapped DMSO. Because the liposomes were suspended in 8% w/v DMSO it is probable that this concentration was rapidly achieved inside the vesicle

regardless of the initial internal value. Betaine, however, showed better activity when the ratio of external to internal concentration was high; this again is explicable on the basis of osmotic dehydration.

A high value for the external to internal betaine concentration ratio during preparation promoted streptomycin entrapment in the liposomes (Fig. 3). A qualitatively similar effect was observed for alanine, but not for DMSO and glycerol. In every case, 4% of the cryoprotectant being studied was initially entrapped within the liposomes which were then washed in solutions containing 0–6% of the agent. Assuming DMSO to be freely and rapidly diffusible the small quantity contained within the liposomes which were suspended in buffer alone would rapidly equilibrate with the bulk of the external solution to give an extremely low internal final concentration. Suspension in buffer of liposomes containing 4% betaine or alanine appears to reduce streptomycin entrapment due, probably, to liposome rupture as the internal contents would be hyperosmotic with respect to the outside, and influx of water would place the membrane under stress. The detrimental effects of a high intraliposomal betaine concentration relative to the outside are also seen in Fig. 4 which shows the effect on streptomycin loss of freezing liposomes which contained 8% betaine or 8% DMSO in buffer alone. The high internal osmolarity of betaine-containing liposomes relative to the suspending medium would be expected to result in swelling due to water uptake and the imposition of stress on the membrane. This may account for the higher susceptibility to freeze-thaw damage that they display compared with those (nominally) containing 8% DMSO.

When the effect of liposome membrane composition on betaine activity was investigated (Table 2) the results showed that the cryoprotective activity was not influenced by cholesterol, dicetylphosphate or stearylamine. In every case liposomes frozen in betaine retained a higher proportion of entrapped material than those frozen in buffer. When cholesterol alone was added to the phospholipid membrane, increased drug leakage was observed in every case, whereas DCP and stearylamine had beneficial effects on drug retention where the entrapped material was of opposite charge to that of the lipid. Thus negatively charged DCP promoted the retention of the base streptomycin but facilitated leakage of the negatively charged amaranth. The increased leakage when cholesterol was present in the membrane is in accord with the reports of Morris & McGrath (1981) and Strauss (1984); this effect is probably due to diminished elasticity in the membrane rendering it more susceptible to rupture under stress.

Following rapid freezing the extent of betaine cryoprotection was seen to increase with increasing thawing rate (Table 3). When frozen in 8% betaine, liposomes exhibited only 14% streptomycin loss at a thawing rate of 600 °C min^{-1} compared with more than twice that value of 0·3 °C min^{-1}. During slow thawing the liposomes were exposed for longer times to conditions favouring the recrystallization of intracellular ice crystals hence greater damage is expected under these circumstances. A high external betaine concentration is likely to cause dehydration of the liposome and thus reduce the amount of water available for this crystallization. In the absence of a cryoprotectant approximately 60% streptomycin loss occurred at all thawing rates which suggests that all the membrane damage occurred during the freezing part of the cycle and no further damage arose during thawing.

The results of this work clearly show betaine to be an effective liposome cryoprotectant which has activity similar to that of DMSO at slow rates of freezing and is superior at higher rates. Not only does it retard freeze-thaw induced drug leakage from liposomes, but it also minimizes size distribution changes to an extent similar to DMSO. Its activity is not influenced by the composition of the liposome. Betaine has been administered to humans at a dose of 6 g day^{-1} in the treatment of homocysteinuria (Wilcken et al 1983), demonstrating that it has low toxicity. These factors combine to make it a material worthy of further investigation for the cryoprotection of liposomes and, possibly, mammalian cells.

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