J. Pharm. Pharmacol. 1990, 42: 297–301
Received November 13, 1989

The Retention of Entrapped Molecules within Erythrocyte Ghosts During Cryopreservation

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Abstract—In view of the interest in erythrocyte ghosts and carrier erythrocytes as potential drug delivery systems, this work was undertaken to determine conditions facilitating the retention of entrapped molecules during cryopreservation. Upon freeze-thaw treatment intact erythrocytes and erythrocyte ghosts displayed different damage profiles with respect to cryoprotectant concentration. Non-penetrating cryoprotectants showed optimum protection of intact cells at 0·4–0·5 M; this optimum was not observed with ghosts, in which damage decreased with concentration up to 1·0 M. The concentration optimum for intact cells was not abolished by oxidative or reductive treatments suggesting that its absence in ghosts is not due to altered protein-protein or protein-lipid interactions. The extent of freeze-thaw damage to ghosts was influenced by the qualitative ionic composition of a cryoprotectant-free suspending medium, with 10–12% haemolysis observed in the presence of Li^+ and Mg^{2+} but >60% for Na^+, Cs^+, K^+ and NH_4^+ with increasing loss following that order. The release on freezing of entrapped haemoglobin, inulin and sucrose was found to be inversely proportional to their molecular weights.

Erythrocyte ghosts (cells depleted of haemoglobin) and carrier erythrocytes (cells made permeable to exogenous molecules) are currently under investigation as potential drug delivery systems (Green et al 1980; Deloach & Andrews 1986; Ihler & Tsang 1987; Kruse et al 1987; Zocchi et al 1987). Whereas erythrocyte ghosts are prepared by hypotonic lysis, typically at 10–20 m osmol kg^{-1}, carrier erythrocytes are made permeable by the gradual imposition, often by dialysis (Deloach & Ihler 1977; Dale 1987), of hypotonic conditions, typically 100 m osmol kg^{-1}. Both procedures generate metastable pores in the cell membrane, the size of which may be varied according to the pH, temperature and ionic constitution of the medium (Lieber & Steck 1982a, b; Deloach & Andrews 1986, 1987) and thus facilitate the entrapment of pharmacological agents.

We have recently shown that erythrocyte ghosts, like liposomes, may be efficiently cryopreserved at liquid nitrogen temperatures (Brearley et al 1988). The object of this present study was to determine conditions facilitating the retention of entrapped molecules of varying molecular size and to examine the differences between the cryopreservation of erythrocyte ghosts and intact erythrocytes

Materials and Methods

Washing of erythrocytes

Fresh heparinized human blood from a healthy male donor was washed by centrifugation three times in isotonic phosphate buffered saline (PBS, Oxoid), pH 7·2 and adjusted to 50% haematocrit. After each centrifugation the buffy coat was discarded.

Preparation of erythrocyte ghosts

Pink erythrocyte ghosts were prepared with modifications to the hypotonic lysis method of Bjerrum (1979). This involves lysis of erythrocytes in ice-cold media at low osmolalities and ionic strength, restoration of physiological salt concentration and pH conditions (reversal), followed by annealing at 38°C to effect closure of pores generated during lysis.

During the lysis procedure the cells, solutions and centrifuge tubes were maintained strictly at 0°C. Washed cells (2 mL) were added to 20 mL of lysing medium comprising 4 mM $MgSO_4$ and 3·8 mM acetic acid. The lysed cells were kept on ice for 2 min and the ionic conditions were restored by the addition of 2 mL of solution comprising 2·0 M KCl and 25 mM tris(hydroxymethyl)aminomethane. The lysed cells were held on ice for a further 10 min before resealing by incubation at 38°C for 60 min.

After incubation (annealing) ghosts were pelleted at 27 000 *g* for 10 min. The supernatant was discarded and the pellet was washed again by centrifugation in PBS. In some experiments ghosts entrapping radiolabelled markers were prepared by lysis of erythrocytes in lysing medium containing, in addition, either 25 μCi [^{3}H]inulin or 5 μCi [^{14}C]sucrose.

Oxidative and reductive treatment of erythrocytes

Washed erythrocytes were suspended in PBS, at a haematocrit of 2·5%. The cells were incubated at 37°C for 1 h with SH-oxidizing agents (tetrathionate $(S_4O_6)^{2-}$, 20 mM; or diamide, 5 mM) or with reducing agents dithiothreitol (DTT), 10 mM; or glutathione (GSH), 10 mM. After incubation the cells were washed three times in buffer.

Freezing and thawing

Samples (350 μL) of red cell or ghost suspensions were dispensed into polypropylene tubes (internal dimensions 38 × 4 mm), which were placed in an alloy tube holder and immersed directly in liquid nitrogen. Samples were held at −196°C for 10 min and thawed to room temperature (20°C) by immersion of the tubes directly in a water bath at 55°C.

Temperature profiles were recorded with a copper constantan thermocouple placed in one of the freezing tubes and connected to a chart recorder. The cooling rate which was

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determined from the linear part of the temperature profile between $-15°$ and $-45°C$ was $2600°C\ min^{-1}$.

The extent of freeze-thaw damage (percentage lysis) was determined from the haemoglobin content of the supernatant following centrifugation (11 000 *g* for 5 min) of the sample. Samples of the supernatant were diluted as appropriate and the absorbance at 540 nm determined. Controls were prepared by dispensing samples into tubes which were left at room temperature for the duration of freeze-thawing and treated thereafter as freeze-thawed samples. Total (100%) lysis controls were provided by lysing aliquots of ghost suspension in an appropriate volume of 0·01% Triton-X-100.

Results

Cryoprotection of intact cells and erythrocyte ghosts

Fig. 1 details the loss of entrapped haemoglobin from intact erythrocytes and from erythrocyte ghosts as a function of concentration of three cryoprotective agents. Intact erythrocytes and ghosts show very different damage profiles with respect to the concentration of cryoprotective agent. All of the ten non-penetrating agents tested on erythrocytes (data not shown) showed an optimum for cryoprotection at 0·4–0·5 M. The non-penetrating agents tested included mono- and disaccharides and the *N*-methyl substituted amino acids, sarcosine (monomethylglycine), dimethylglycine and betaine (trimethylglycine). Of the saccharides, galactose, sucrose and trehalose (data not shown) were particularly effective giving less than 10% haemolysis at their respective optima whilst the amino acid derived cryoprotectants were less effective.

In contrast, the penetrating agents glycerol and dimethylsulphoxide (not shown) afforded a progressive increase in protection with increasing concentration.

Erythrocyte ghosts frozen in the absence of any cryoprotective additive appeared to be less stable than their erythrocyte counterparts and exhibited approximately 98% lysis compared to approximately 80% for the intact cells. Ghosts frozen in the presence of cryoprotectants showed a progressive increase in protection irrespective of the penetrating or non-penetrating nature of the additive. The extent of protection afforded by the various agents was comparable to that afforded to intact cells.

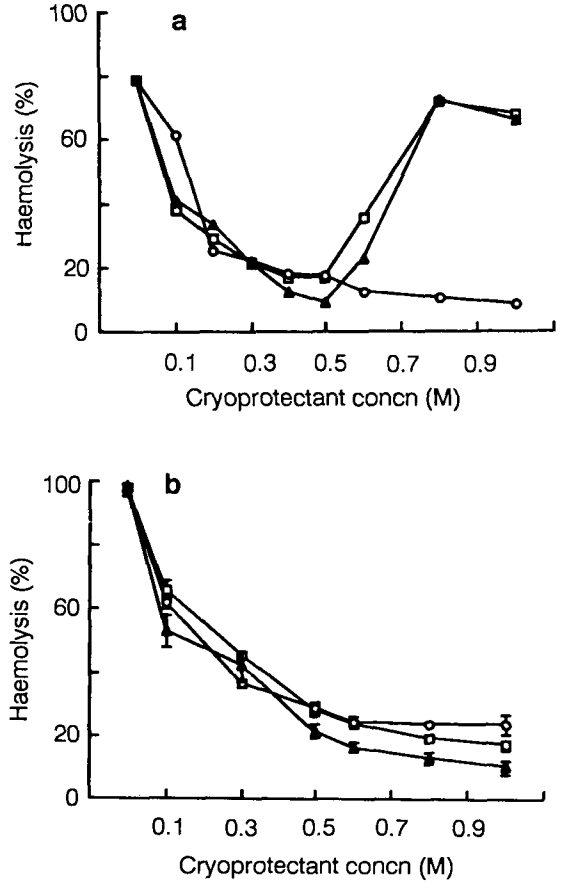

FIG. 1. Effect of cryoprotectant concentration on haemolysis of erythrocytes (a) and erythrocyte ghosts (b) following a freeze-thaw cycle (s.d. less than 5% of the mean, n=4). Betaine □; glycerol ○; galactose △.

Oxidative and reductive modification of the erythrocyte membrane

One hypothesis that we have considered to account for the difference in the response of ghosts and intact cells to freeze-thaw stress is that the ghosts show altered cytoskeletal-membrane interactions which render the membrane less sensitive to such stress at high cryoprotectant concentrations. The erythrocyte membrane comprises the lipid bilayer, integral membrane proteins and a filamentous network of proteins, predominantly spectrin, or cytoskeleton. The cytoskeleton through its interactions with other membrane components has an important role in the maintenance of mechanical stability of the membrane (Wolfe 1985). Although the nature of these interactions is only now being unravelled, it is apparent (Haest et al 1978; Smith & Palek 1983; Streichman et al 1988) that the oxidation state of spectrin thiol groups is a critical factor in the thermal stability of the membrane.

The results presented in Fig. 2 and Table 1 show the effect of oxidative and reductive treatment of intact cells on their subsequent response to freeze-thaw stress. Cells treated with oxidizing agents (tetrathionate or diamide) which cause complete crosslinking of spectrin at the concentrations employed here (Haest et al 1978) show greater percentage lysis than their control and reducing agent treated counterparts (Table 1). All the cells showed a qualitatively similar response to cryoprotectant, that is they all demonstrated optimal recovery at a cryoprotectant concentration of 0·4 M (Fig. 2). Thus, it appears that the nature, though not the magnitude, of the stress imposed by freezing and thawing is the same for control and treated membranes. Reducing

Table 1. The effect of oxidative and reductive treatment on freeze-thaw survival of erythrocytes in 0·4 M betaine.

Treatment	Percentage lysis (mean ± s.d.)
Control	28·3 ± 0·8
Diamide	43·9 ± 1·0
Tetrathionate	44·2 ± 1·4
Dithiothreitol	33·2 ± 2·0
Glutathione	31·9 ± 0·9

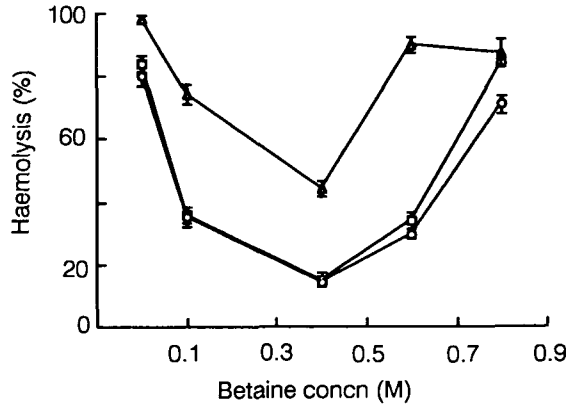

FIG. 2. Effect of oxidative and reductive treatment of erythrocytes on haemolysis after a subsequent freeze-thaw cycle (mean ± s.d., n = 4). Control □; dithiothreitol ○; tetrathionate △.

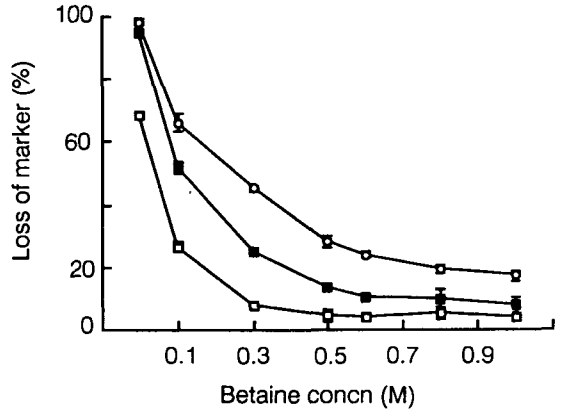

FIG. 3. Percentage release of markers of varying molecular size from erythrocyte ghosts during a freeze-thaw cycle (mean ± s.d., n = 4). Sucrose ○; inulin ■; haemoglobin □.

treatments (DTT and GSH) only slightly increased lysis at the optimum cryoprotectant concentration (Table 1). At superoptimal cryoprotectant concentration lysis was reduced by DTT but not by GSH (data not shown).

The effect of ionic species

The ionic constitution of the medium is an important factor in determining pore size in erythrocyte ghosts (Lieber & Steck 1982a, b). We have tested (Table 2) the effect of various chloride salts added to ghost suspensions on the recovery of entrapped haemoglobin after freeze-thawing. For this experiment erythrocyte ghosts were suspended in media comprising 200 mM salt and 10 mM HEPES, pH 7·2. The extent of leakage of haemoglobin from the ghosts is indicated in the Table. Of the Group I metal chlorides tested, K was the most damaging giving 88% loss of haemoglobin, and Li the least (12%). The extent of damage decreased in the order K > Cs > Na > Li. Magnesium chloride (11%) was the least damaging of all the salts tested and NH_4Cl (90%) the most. In all cases the loss of haemoglobin from control (non-frozen) samples was less than 5% of the total haemoglobin.

When erythrocyte ghosts were freeze-thaw treated in the additional presence of 300 mM sucrose as cryoprotectant (Table 2) the effect of the salts was abolished, the loss of haemoglobin being reduced to less than 3% of the total haemoglobin irrespective of the nature of the ionic species.

Table 2. The effect of ionic species (200 mM) on freeze-thaw damage to erythrocyte ghosts.

	Percentage lysis (mean ± s.d.)	
Ionic species	Buffer alone	Buffer plus +300 mM sucrose
NH_4Cl	90·1 ± 5·4	5·5 ± 1·4
LiCl	12·1 ± 4·3	6·2 ± 2·1
NaCl	62·3 ± 4·7	5·9 ± 1·8
KCl	88·4 ± 6·7	5·9 ± 2·0
CsCl	73·0 ± 6·0	6·1 ± 2·0
$MgCl_2$	10·8 ± 3·9	4·9 ± 1·8

Nature of entrapped marker

Considering the nature of the haemolytic hole and the potential of erythrocyte ghosts as agents of drug delivery it is pertinent to inquire of the permeability of freeze-thawed ghosts to solutes of differing molecular size. To this end glycine betaine, as a typical non-penetrating agent, was tested as a cryopreservative of erythrocyte ghosts entrapping sucrose; mol.wt 342, Stokes radius 5·2 Å (Schultz & Solomon 1961), inulin; mol. wt 5200, Stokes radius 20 Å and haemoglobin; mol wt 66 000, Stokes radius 40 Å (Deloach & Andrews 1987).

The release of markers from control (unfrozen) ghosts was dependent upon the nature of the marker but independent of cryoprotectant concentration. Sucrose release from control ghosts was between 9 and 11%, inulin release was between 3 and 6% whilst haemoglobin showed the least leakage, less than 2% irrespective of cryoprotectant concentration.

Upon imposition of freeze-thaw stress sucrose loss was progressively reduced from 98% in the absence of cryoprotectant to 17% at 1 M betaine (Fig. 3). Inulin release was similarly reduced from 95 to 8% whilst haemoglobin release was the lowest of the three markers, 68% in the absence of betaine and less than 7% at 0·3 M and above.

Discussion

In the absence of major functional differences between erythrocyte ghosts and intact cells (Bjerrum 1979) we have considered two possible explanations of the different response (Fig. 1) of ghosts and intact cells to freezing stresses at high cryoprotectant concentration. One hypothesis is that the presence of stable pores within the ghost membrane attenuates the extent of osmotic stress imposed by the hypertonic cryoprotectant solution during freezing and thawing. This hypothesis is not easily tested for ghosts because of the incomplete closure of the pores generated during ghost preparation. The conditions required to effect complete closure of the pores have not been identified; rather, the evidence (Lieber & Steck 1982a, b) suggests, to the contrary, that the pores, though variable in size, are stable and permanent features of erythrocyte ghosts.

An alternative hypothesis is that the generation of ghosts

by osmotic lysis of intact cells causes some aberration of membrane component interactions. We have also considered that the disruption of these interactions may be mimicked by oxidative or reductive treatment of the membrane. It has been shown (Haest et al 1978) that treatment of intact cells with thiol oxidizing agents releases constraints imposed by spectrin upon phosphatidylserine and phosphatidylethanolamine such that the phospholipids may move from the inner half of the bilayer to the outer half. These effects can be blocked by thiol alkylating agents such as *N*-ethylmaleimide. Furthermore, the thermal stability of the membrane can similarly be manipulated by oxidative or alkylating treatment of spectrin thiol groups (Smith & Palek 1983; Streichman et al 1988).

Our inability to abolish the concentration dependent optimum of cryoprotection of intact erythrocytes by oxidative and reductive treatments of the cells (Fig. 2) suggests that the absence of such an optimum in erythrocyte ghosts is not a consequence of altered protein-protein or protein-lipid interactions. In the absence of other likely functional differences between intact cells and erythrocyte ghosts, we suggest that the optimum of cryoprotection observed in intact cells is a consequence of critical osmotic stresses imposed during the freeze-thaw cycle upon intact cells, but which are not imposed upon ghosts presumably because of the presence of stable pores in their membrane.

The nature of pores generated in erythrocyte ghosts has been studied by Lieber & Steck (1982a, b) who determined the size of pores in ghosts prepared by the method of Bjerrum (1979) to be 9 Å in size. In their exhaustive study the authors concluded that the size of the pores was controlled by the balance between opposing forces within the membrane. Hole closure was enhanced by the binding of cations to anionic groups in the membrane.

It is well known that the surface areas and hence pressure of monolayers of phospholipids is controlled to some extent by the charge repulsion of like-charged groups in neighbouring phospholipid headgroups.The strength of such interactions is dependent on the charge shielding properties of the medium between neighbouring phospholipid headgroups. The ability of different electrolytes to structure or destructure water (Verral 1973; Franks 1983) in the immediate vicinity of phospholipid headgroups or other charged groups on the membrane surface may thus influence the nature of the haemolytic hole and the subsequent freeze-thaw stability of the membrane. The extent of these effects might be expected to follow a lyotropic series (Record et al 1978) related to the hydrated ion radii of the Group I cations (Franks 1983). It is of some note, therefore, that the efficacies of the monovalent ions (Table 2) do not follow the hydrated ionic radii $Li > Na > K > Cs$ of these ions.

Alternatively, there is growing evidence that cations interact directly with membrane components (Cunningham et al 1988). Divalent and trivalent cations are known to bind to both zwitterionic and charged phospholipids (Hauser et al 1975, 1977a; Lis et al 1981). Monovalent ions also appear to bind to negatively charged surfaces (Hauser et al 1977b; Jacobson & Pahapadjopoulos 1977) and furthermore, there is evidence of interactions with phosphatidylcholine bilayers (Lis et al 1975; Chapman et al 1977).

There are also theoretical treatments of the selectivity of equilibrium binding of cations by ion exchangers reviewed by Eisenman (1969). These treatments predict a whole range of selectivity isotherms for binding of monovalent cations, the specific sequence depending upon the sizes of the interacting species and their resultant charge densities.

Interestingly, the specific sequence $Li > Na > Cs > K$ is also afforded by an analysis of the free energy of transfer of monovalent cations from water into a ligand structure giving cubic co-ordination (Conway 1981, and references therein). The binding of cations to a membrane surface could readily be considered in such terms. The relevance of the application of such theories to ion-phospholipid membrane interactions is further suggested by the results of a recent study by Eklund et al (1989). In this study the elevation of the thermotropic subtransition of dipalmitoylphosphatidyglycerol bilayers by monovalent ions followed the precise sequence $Li > Na > Cs > K$ which we have described. In a similar manner the ability of monovalent cations to cause aggregation of digalactosyldiacylglycerol vesicles followed the same sequence at low turbidity values (Webb et al 1988).

The presence of stable pores in ghost and carrier erythrocyte is a matter of great importance considering the applications of such systems as agents of delivery of a wide range of pharmaceutical agents including drugs (Kruse et al 1987) and enzymes (Deloach & Ihler 1977; Morelli et al 1979; Dale 1987). We have demonstrated that the pores generated during the preparation of erythrocyte ghosts can be maintained at liquid nitrogen temperatures and be subsequently restored on thawing in a cryoprotectant concentration dependent manner. Liquid nitrogen storage may similarly be possible for carrier erythrocytes. We have ascertained that the extent of damage incurred by erythrocyte ghosts in the absence of cryoprotectants during a freeze-thaw cycle is strongly dependent upon the cationic constitution of the suspending medium, and in a manner which is not explained simply by consideration of the hydrated ionic radii.

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