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# PREPARATION OF ERYTHROCYTE GHOSTS BY A GLYCOL-INDUCED OSMOTIC LYSIS UNDER ISOIONIC CONDITIONS

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SUMMARY

A procedure has been developed for obtaining haemoglobin-free, erythrocyte ghosts under ionic conditions approximating that of the cell cytoplasm. Haemolysis was effected by incorporating glycol into cells suspended in the isoionic medium and then diluting with a large volume of glycol-free medium.

The ghosts were of uniform spherical shape throughout the preparative procedure and were impermeable to macromolecules.

Analysis of polypeptides by sodium dodecyl sulphate-gel electrophoresis at each stage of preparation and comparison with ghosts prepared under hypo-ionic conditions served to distinguish membrane components from those of cytoplasm.

Current knowledge of the composition and structure of erythrocyte membranes has been derived largely from studies of erythrocyte ghosts prepared by haemolysis in hypo-ionic (hypotonic) salt solutions. However, it has long been evident that relatively small variations of haemolysis conditions critically influence the composition and properties of the final ghost preparations [1–4]. The extent of hypo-ionic treatment required to completely free the ghosts of haemoglobin leads to impairment of enzyme activities, changes in permeability characteristics and also to fragmentation of the ghosts. The composition and molecular organisation of the isolated membranes are undoubtedly modified by the non-physiological conditions in which they are isolated (for a review, see ref. 5).

Recently, two different procedures for effecting haemolysis whilst maintaining isoionic conditions have been reported [6, 7]. One has made use of dielectric breakdown of membrane permeability whilst the other has used halothane, an anaesthetic molecule, to induce haemolysis whilst maintaining the preparation in a standard physiological solution with an ionic composition similar to extracellular fluid. The first procedure suffers the disadvantage of requiring special equipment and the second from the uncertainty that the mode of action of the anaesthetic molecule on the membrane is not understood.

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In this paper, we report a procedure for preparing haemoglobin-free ghosts in which an ionic medium approximating to that prevailing within the cell cytoplasm is maintained throughout and haemolysis is induced by using a non-electrolyte to which the membrane is moderately permeable to create an osmotic gradient. A variety of non-electrolytes has been tested, and both glycol and glycerol have proved to be effective for this purpose. With some species of erythrocytes, glycerol might have been expected to be favoured because of the presence of mediated permeation systems, but in fact the variability of this facility constitutes a disadvantage and glycol has provided the greater consistency required for the development of a standard preparative procedure.

## MATERIALS AND METHODS

*Erythrocytes.* Human blood (Group O, $Rh^+$) in citrate-phosphate-dextrose solution, used within three weeks of donation, was centrifuged at $2000 \times g$ for 10 min. The plasma and buffy coat were then discarded. The packed erythrocytes were washed three times with ice-cold, 140 mM NaCl solution buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), pH 7.4.

*Isoionic medium.* The ionic medium in which the cells were loaded and lysed contained 130 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 $\mu$M $CaCl_2$ and 10 mM HEPES (pH 7.4).

*Preparation of ghosts.* An outline of the procedure is given in Fig. 1. 1 vol. of washed packed cells was suspended in 3 vol. of 2 M glycol in isoionic medium and the permeant was allowed to diffuse into the cells for about 10 min at 20 °C. The suspension was chilled in ice-water for 5 min and then diluted with ice-cold, isoionic medium to a final ratio of buffer to cells of 20 : 1. The turbid suspension became clear within seconds, indicating a very rapid lysis of cells. The ghost suspension was then centrifuged at $20\,000 \times g$ for 10 min. The pellet was again suspended in the same volume of fresh, ice-cold, isoionic medium and centrifuged at $20\,000 \times g$ for 10 min. Thereafter, a cycle of loading at room temperature, cooling in ice water and dilution with ice-cold, isoionic medium was repeated until the haemoglobin content of the ghosts was reduced to the desired extent. The ghosts taken for further studies were finally washed once with fresh, ice-cold, isoionic medium to remove glycol. In some preparations, there was a small population of ghosts which were difficult to free of haemoglobin. However, these ghosts were sedimented to the bottom of the final membrane pellet and could, therefore, be easily discarded.

*Chemical assays.* Haemoglobin assays were performed by the pyridine haemochromogen method of Dodge et al. [1].

The total protein was determined by the method of Lowry et al. [8]. Allowance was made for the contribution of HEPES to the developed colour. Bovine serum albumin was used as reference standard. The ghost haemoglobin was subtracted from the total protein to give the nonhaemoglobin protein.

Lipids were extracted by the method of Bligh and Dyer [9] except that 2 M KCl was used instead of water. Organic phosphorus was determined in dried aliquots of the extract by King's method [10].

*Phase contrast microscopy.* Samples of membranes were examined in cold, isoionic medium with a Zeiss photomicroscope. Exclusion of bovine serum albumin

or dextran was assessed by suspending the ghosts in 2 % bovine serum albumin or 2 % (final concentration) dextran in isoionic medium [11].

*Electron microscopy.* Samples for electron microscopy were fixed in 6.25 % glutaraldehyde, followed by 1 % osmium tetroxide and embedded in Epon. Thin sections were further stained with methanolic uranyl acetate (25 %).

*Sodium dodecyl sulphate-gel electrophoresis.* Sodium dodecyl sulphate-gel electrophoresis was performed according to Weber and Osborn [12]. The delipidated proteins (75 : 25, v/v, ethanol/ether mixture was used for delipidation) were solubilised overnight at 37 °C in 8 M urea, 4 % sodium dodecyl sulphate, 1 % $\beta$-mercaptoethanol, 0.01 M phosphate buffer, pH 7.0, at a membrane phospholipid concentration of 700 nmol/ml. Each 6 % polyacrylamide gel (0.4 × 14 cm) was loaded with solubilised ghost protein equivalent to 35 nmol phospholipid phosphorus. Electrophoresis was carried out at room temperature at a constant current of 7 mA/gel for 4 h. The electrophoresis buffer was 0.1 % sodium dodecyl sulphate in 0.1 M sodium phosphate, pH 7.0. The gels were stained with Coomassie Blue to detect polypeptides or with periodate-Schiff reagent to detect glyco-polypeptides. The destained gels were then scanned at 570 nm in a Unicam SP500 spectrophotometer with a Gilford gel-scanning attachment. Bands were identified and assigned code numbers according to the convention proposed by Steck [13].

## RESULTS

The initial haemolysis and the ease with which haemoglobin was released in subsequent lytic steps were found to be dependent on the level of loading with permeant and on the temperature during loading, lysis and centrifugation. A minimum final concentration of 1.5 M glycol was found to be necessary to produce a preparation of

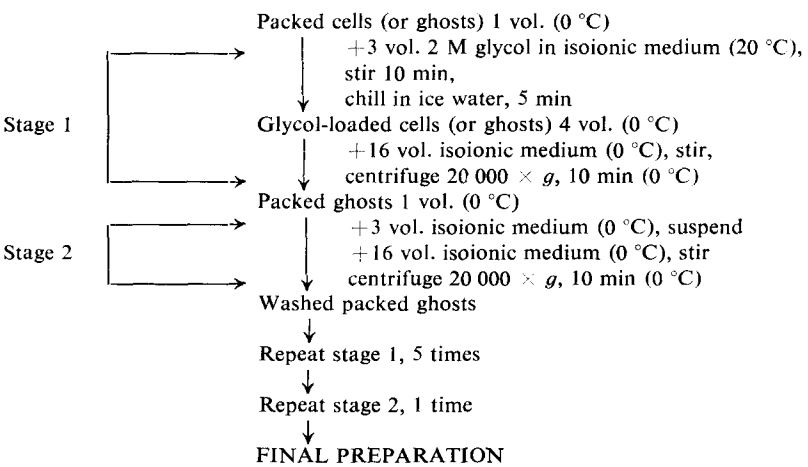


Fig. 1. Schematic outline of the procedure for preparation of haemoglobin-free ghosts by osmotic lysis under isoionic conditions. The isoionic medium contained 130 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 $\mu$M $CaCl_2$ and 10 mM HEPES, pH 7.4. The osmotic gradient necessary for haemolysis was generated by quickly diluting the ice-cold, loaded (1.5 M glycol at equilibrium) erythrocytes or ghosts with ice-cold, glycol-free, isoionic medium.

ghosts of uniform size and shape within a reasonably short time. A temperature of 20 °C or more was required for rapid (equilibrium within 5–10 min) loading of cells or ghosts with glycol but complete haemolysis was achieved only when the subsequent lysis was carried out at reduced temperatures (0 °C).

The number of cycles of loading and lysis necessary for obtaining haemoglobin-free ghosts could be reduced by loading the cells with higher concentrations of permeant. With a loading of 1.5 M glycol, usually six cycles were necessary to get essentially haemoglobin-free ghosts (Fig. 1). Fig. 2 illustrates the typical pattern of haemoglobin release and the nonhaemoglobin protein content of the ghosts at the different stages of preparation. Each sample was washed once with fresh, isoionic medium before assaying for protein and haemoglobin.

When viewed in the phase-contrast microscope, the ghosts appeared as a population of spheres of relatively uniform sizes (Fig. 3a) throughout the preparative procedure. In 2 % albumin or dextran, virtually all of the ghosts appeared pale against the darkened background (Fig. 3b), thus indicating little or no immediate penetration of the ghosts by these macromolecules [11].

Electron micrographs (Fig. 4) of glycol-lysed ghosts showed a clear trilamellar feature at high magnification. There was also a substantial filamentous component at the cytoplasmic face of the membrane.

The final haemoglobin-free membrane preparation contained 1.4 mg of protein per μmol lipid phosphorus (Fig. 2). The total of estimated lipid (phospholipid+cholesterol) was 0.8 mg per mg of protein.

The polypeptide patterns revealed by sodium dodecyl sulphate-gel electrophoresis were recorded after each loading and lysis under isoionic conditions (Fig. 5) and compared (Fig. 6) with three different ghost preparations obtained under hypoionic conditions (15 imosM $NaHCO_3$ and 40 imosM $NaHCO_3$ [3]; 40 imosM $NaHCO_3$+1 mM EDTA [14]). Substantial variations in the intensities of bands

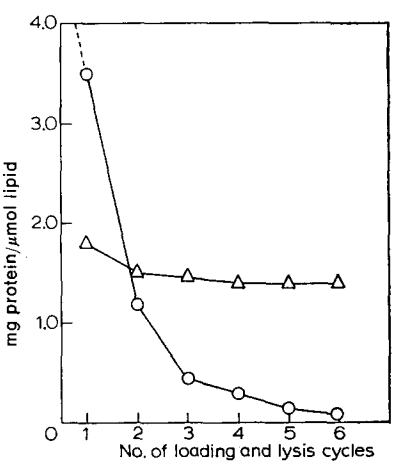


Fig. 2. Retention of haemoglobin (○–○) and non-haemoglobin protein (△–△) by glycol-lysed, isoionic erythrocyte ghosts after each cycle of loading and lysis (stage 1 in Fig. 1). Prior to assays, ghost samples were washed once with ice-cold, isoionic medium (stage 2 in Fig. 1) to remove glycol.

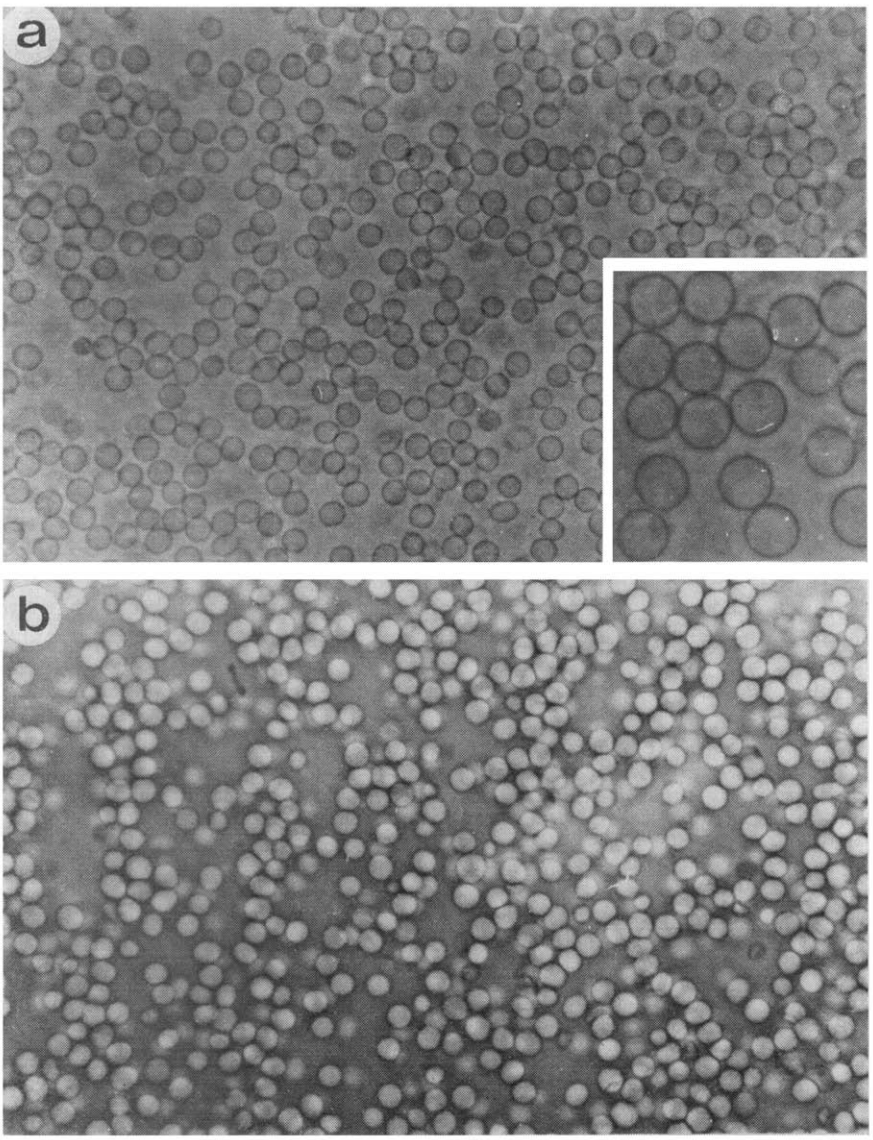


Fig. 3. Phase-contrast micrographs of glycol-lysed, isoionic ghosts suspended in (a) ice-cold, isoionic medium and (b) 2 % dextran (final concentration) in ice-cold, isoionic medium. The ghosts maintained these morphological features throughout the preparative procedure. Final magnification: × 650; inset, × 1500.

2.2, 4.3*, 6, 7 and 8 were observed. Band 6 was prominent in all of the hypo-ionic ghost preparations but was rapidly eliminated during the first stages of glycol-induced lysis. Bands 4.3, 7 and 8 also decreased substantially during the series of glycol-

* First of a triplet of bands referred to by Steck [13] as the "4.5 zone".

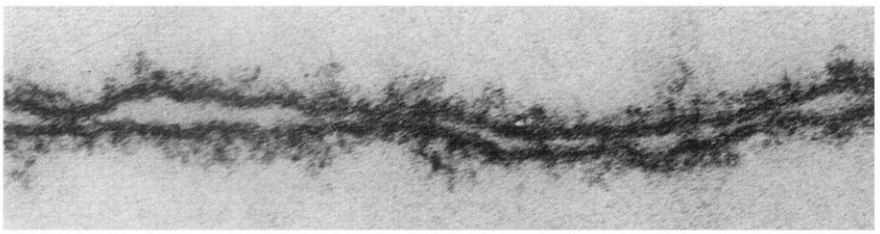

Fig. 4. Electron micrograph of glycol-lysed, haemoglobin-free erythrocyte ghost membrane prepared under isoionic conditions. Final magnification: × 180 000.

induced lyses. However, bands 4.3 and 7 appeared to reach a constant level whereas band 8 continued to diminish in parallel with the haemoglobin band. Component 2.2 was relatively weak in the gels from hypo-ionic ghosts but appeared considerably more intense in the isoionic preparations (Fig. 6.)

Variations of the concentration of glycol used in the preparation of haemoglobin-free ghosts had no appreciable effect on the polypeptide composition and overnight treatment with hypo-ionic salt solutions (140 mM-10 mM, including 5 mM HEPES buffer, pH 7.4) of a haemoglobin-free preparation (lysed with 1.5 M glycol) did not effect any significant extraction of polypeptide components.

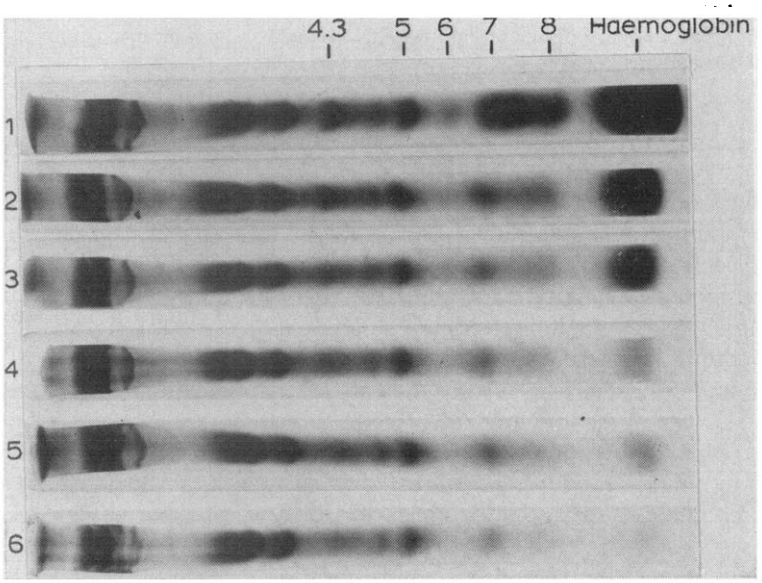


Fig. 5. Sodium dodecyl sulphate-gel electrophoretic patterns of the glycol-lysed, isoionic, erythrocyte ghosts obtained after each cycle (1–6) of loading with glycol and lysis (stage 1 in Fig. 1). Each ghost sample was washed once with ice-cold, isoionic medium (stage 2 in Fig. 1). Solubilised ghost protein equivalent to 35 nmol membrane phospholipid was applied to each gel. The polypeptide bands stained with Coomassie Blue were numbered according to Steck [13]. The first of the triplet bands referred to as the '4.5' zone was numbered 4.3.

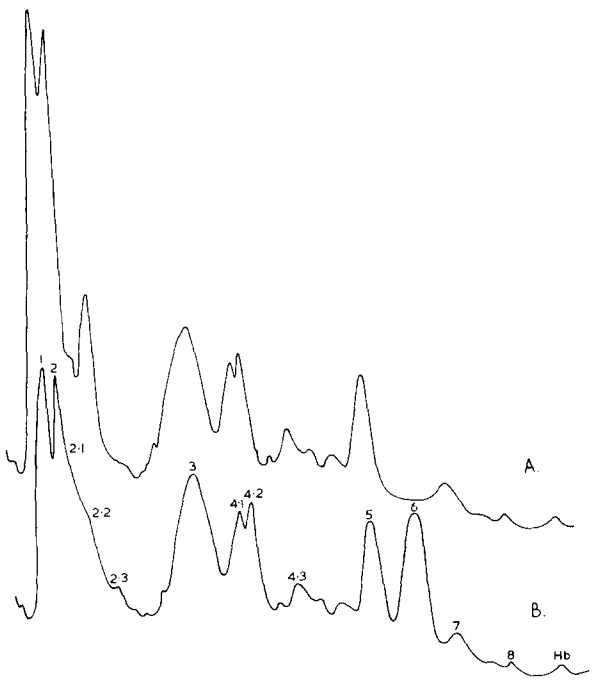


Fig. 6. Spectrophotometric scans (at 570 nm) of 0.1 % sodium dodecyl sulphate/6 % polyacrylamide gels of haemoglobin-free erythrocyte ghosts. A, prepared by glycol-induced osmotic lysis under isoionic conditions. B, prepared by hypo-ionic haemolysis in 15 imosM sodium bicarbonate buffer, pH 7.4 at 0 °C. Band designations are as in Fig. 5.

## DISCUSSION

The procedure outlined in this paper provides haemoglobin-free erythrocyte ghosts which are of uniform spherical shape (Fig. 3a) and which appear to be impermeable to large molecules (Fig. 3b). In this respect and also in their relative stability they appear to offer considerable advantages for use in studies of membrane structure and function as compared with haemoglobin-free ghosts prepared by osmotic lysis in media of low ionic strength [5]. Furthermore, it should be noted that the re-sealing process following lysis induced by glycol or glycerol gradients is spontaneous and rapid, requiring neither the change of medium nor the prolonged incubation which are necessary for re-sealing "pink" ghosts prepared by lysis under moderate hypo-ionic conditions [5.] In addition, impermeability even to small molecules such as Mg-ATP, [$^3$H]-inositol and [$^{14}$C]choline is substantially preserved in these ghosts prepared under isoionic conditions. (A paper describing more extensive observations on permeabilities both to macromolecules and to small molecules and ions, is in preparation.)

The maintenance at the inner surface of the membrane of an ionic environment closely approximating that of normal cytoplasm would be expected to minimise modifications to this surface during lysis and re-sealing. As the outer surface appears not to be sensitive to small modifications of ionic environment this choice of medium should ensure that the composition of the isolated membrane reflects as closely as

possible that in the intact cell. The possibility of specific interaction between the non-electrolyte molecule and the membrane cannot be ruled out completely. However, both glycol and glycerol have been used extensively in permeability studies and in tissue preservation and there have been no indications either of their incorporation into, or of their damaging effects on membranes. Their role in the preparative procedure is simply to create an osmotic gradient of sufficient magnitude to produce lysis. In this respect the approach is significantly different from that involving the use of halothane which interacts directly with the lipids of the membrane to produce lysis.

The polypeptide profile (by sodium dodecyl sulphate-gel electrophoresis) of erythrocyte membranes (Fig. 5) prepared by the present procedure shows significant differences when compared with those of ghosts prepared under a variety of hypo-ionic conditions (Fig. 6). In particular, band 6 which has previously been identified with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [15–18], is absent from this isoionic preparation. This observation thus supports the suggestion [15, 19] that the presence of glyceraldehyde-3-phosphate dehydrogenase in ghosts prepared under hypo-ionic conditions is an artefact arising from specific adsorption of this cytoplasmic component under conditions of low ionic strength. Band 8 has been suggested to be associated with maintaining the impermeability of the membrane to ATP [20]. This suggestion is not supported by our finding that the isoionically-prepared ghosts are impermeable to ATP (unpublished data) and band 8 is absent. Observations made in the present studies are more consistent with the identification of this component as a cytoplasmic constituent whose behaviour closely parallels that of haemoglobin. The behaviour of bands 4.3 and 7 during the preparative procedure would seem to suggest that these bands initially include both membrane and cytoplasmic components; neither these bands nor the 2.2 band have yet been identified in functional terms.

This erythrocyte ghost preparation appears to have considerable potential for use both in the studies of membrane structure and functions. It may be particularly useful for the study of functions involving the cytoplasmic face of the membrane since it offers an opportunity for introducing new components into the cell interior during the brief period of membrane permeability. It should also be noted that this approach may have a general applicability in the preparation of plasma membranes from a variety of cells. Barber and Jamieson [21] have, in fact, used glycerol to generate an osmotic gradient to lyse platelets but only under hypo-ionic conditions.

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