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ACTION OF HALOTHANE ON HUMAN ERYTHROCYTES
MECHANISMS OF CELL LYSIS AND PRODUCTION OF SEALED GHOSTS

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SUMMARY

1. Suspension of human erythrocytes in halothane-saturated physiological media induces major cell deformation.

2. Release of halothane from erythrocyte suspensions after equilibrium with the anaesthetic produces complete hemolysis.

3. The membrane fragments isolated after halothane release are in the form of biconcave ghosts, impermeable to macromolecules (lactoperoxidase) and small molecules (ATP).

4. The protein composition of the membranes differs from that of ghosts produced by hypotonic lysis in the lack of components previously shown to be adsorbed at low ionic strength.

5. A hypothesis is presented explaining the action of halothane in terms of both its action on membranes and its capacity to modify water structure.

6. Halothane-induced hemolysis constitutes a simple method for the large-scale production of hemoglobin-depleted, sealed erythrocyte ghosts under physiological ionic conditions.

INTRODUCTION

Numerous lipophilic and amphiphilic molecules, including diverse drugs and anaesthetics, at low concentrations protect erythrocytes against hypotonic lysis, but at high levels cause complete cell disruption [1, 2]. In the case of anaesthetics, the bulk concentrations which induce osmotic stabilization correspond rather well to pharmacological anaesthetic concentrations [3]. Also the stabilization phase has been ascribed to a reversible fluidization of membrane lipids [4] and there is evidence that such fluidization can be induced in lipid model membranes by anaesthetics such as halothane [5]. The labilization phases of the stabilization-labilization sequence appears to be due to denaturation of membrane proteins [6].

Recent data [7, 8] indicate that osmotic stabilization at low concentrations

may involve more than one mechanism. Thus, our own experiments [6] show that several nitroxide-lipid analogues at concentrations which reduce osmostic fragility in hypotonic media induce echinocytosis in iso-osmostic solutions. These studies also indicate that these membrane-active agents perturb the erythrocyte membrane focally and, at non-lytic levels, do not cause uniform membrane expansion. Moreover, related experiments show that some of the nitroxide-lipid analogues bind to membrane proteins when present at concentrations causing osmotic stabilization. On the other hand, substances such as chlorpromazine [7, 8] do not produce echinocytosis, but induce a variety of morphologic alterations, including "cup-shapes".

We have investigated the action of halothane in greater detail and here report on the unusual process by which this anaesthetic causes erythrocyte lysis. We also describe some important features of the membranes produced by halothane-induced lysis.

MATERIALS AND METHODS

Erythrocytes. We used freshly drawn, heparinized blood from healthy donors. This was centrifuged at 1500 $g \cdot$ min and the plasma and buffy coat discarded. The erythrocytes were then washed four times with 10 vols of Dulbecco's phosphate-buffered saline or Hanks balanced salt solution (Grand Island Biological Co.).

Halothane solutions. We prepared halothane-saturated isotonic saline, Dulbecco's phosphate-buffered saline and Hanks balanced salt solution by equilibrating these buffers with halothane liquid (Fluothane, Ayers Laboratory, Inc., New York) at 4, 25 or 37 °C.

Measurement of hemolysis. We used three methods. (a) Erythrocyte suspensions (approx. $1.5 \cdot 10^5$ cells/ml) were mixed with halothane-saturated buffer transferred to 16 mm × 100 mm glass tubes to occupy 20, 40, 60, 80 and 100 % of the tube volume. The tubes were then sealed with silicone-rubber stoppers and hemolysis monitored turbimetrically as a function of time. (b) Samples from (a) were examined by phase-contrast and scanning electron microscopy. (c) 3.5-ml aliquots of halothane-saturated cell suspensions were transferred to 1 × 1 × 4 cm fluorescence cuvettes, either sealed or unsealed. Light scattering at 400 nm was then measured as a function of time at 25 °C, using a Perkin-Elmer MPF3 spectrofluorometer.

Scanning electron microscopy. 1 vol. of suspended erythrocytes or ghosts was mixed with 10 ml of 2 % glutaraldehyde (w/v) in Millonig's buffer (pH 7.3). The fixed erythrocytes or ghosts were then deposited on a trapping layer of African green monkey kidney fibroblast cells or a Nucleopore membrane (General Electric Co.). After dehydration, using ethanol followed by two washings in amyl acetate, the samples were dried by Anderson's critical point method, with CO_2 as transition fluid. The specimens were then coated with a thin layer of gold palladium (60 : 40) at room temperature and $1 \cdot 10^{-4}$–$5 \cdot 10^{-5}$ mm of Hg, using a JEOL 4B vacuum evaporator. The specimens were placed on a tilted rotary evaporator during coating. Electron microscopy was with a JEOL-U3 instrument operated at 25 kV and at 0° tilting angle.

Membrane isolation. 10–20 vols of halothane-saturated medium were decanted into a centrifuge tube or centrifuge bottle containing 1 vol. of packed, washed erythrocytes. The cell suspension was then processed by one of two methods. (a) The

suspension was mixed gently and intermittently until it became non-turbid. The membranes were then pelleted at 400 000 g · min (International Centrifuge B-20; rotor 870). (b) After mixing for 1–2 min, the cells were pelleted at 1500 g · min, the halothane-saturated medium removed and the cells resuspended to the original volume in halothane-free medium. After the suspension had become clear, the membranes were pelleted at 400 000 g · min. The membranes isolated by either method were washed 1–8 times in halothane-free Dulbecco's phosphate buffered saline, Hanks balanced salt solution or isotonic saline, pelleting at 400 000 g · min each time.

Membranes obtained by hypotonic lysis according to Dodge et al. [9] were used as controls.

Lactoperoxidase labelling. Both ghosts prepared by the method of Dodge et al. [9] and halothane-prepared ghosts were labelled with ^{125}I using lactoperoxidase and H_2O_2 as in refs 10 and 11: 2 ml of phosphate-buffered saline containing 10^{10} ghosts were chilled in an ice-bath and 100 μg lactoperoxidase (Sigman Chemical Co., 1 mg/ml) and 5 μCi $Na^{125}I$ (carrier-free; New England Nuclear; 500 μCi/ml) were added. To avoid lipid peroxidation, 100 μg butylated hydroxytoluene (Sigma Chemical Co., 10 mg/ml) was added. The samples were then warmed to 25 °C and the iodination was initiated by adding first 20 μl of 0.003 % H_2O_2 followed by 2 aliquots of 10 μl at 60-s intervals. After 3 min the iodination reaction was stopped by cooling the mixture at 0 °C and diluting with 30 ml ice-cold phosphate-buffered saline, containing 5 mM KI. Excess of ^{125}I was removed by two washing steps in 30 ml buffer. The washing buffers for ghosts prepared by hypotonic lysis or with halothane were 5 mM sodium phosphate, 5 mM KI, pH 8.0, and phosphate-buffered saline containing 5 mM KI, respectively.

Electrophoretic analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the membranes were solubilized in 2 % dodecyl sulfate, 40 mM dithiothreitol at a protein concentration of 2–4 mg/ml and heated for 5 min at 100 °C [12]. 100 μg of membrane protein was separated on 5 % acrylamide, cross-linked with 2.5 % *N,N'*-methylene-bisacrylamide (Bio-Rad). The gels were 5 mm in diameter. The electrophoresis buffer was 40 mM Tris, 20 mM acetate and 2 mM EDTA, at pH 7.4, containing 1 % sodium dodecyl sulfate. A current of 3 mA/gel was used for separation. To estimate protein concentration, gels were stained with Coomassie Blue [12] and scanned at 620 nm (Gilford spectrophotometer, Model 240). To detect glycoproteins, gels were stained with the periodate-Schiff reagent and scanned at 560 mM. To monitor the ^{125}I distribution, the gels were frozen after electrophoresis, cut into 1.5-mm slices and counted in a Nuclear-Chicago Autogamma Scintillation Spectrometer.

($Na^+ + K^+$)-ATPase activity. Membranes were washed three times in 20 mM Tris · HCl, pH 7.4, 80 mM NaCl, 40 mM KCl, 0.05 mM EDTA, 1.0 mM $MgCl_2$, 0.5 mM ATP before assaying for ($Na^+ + K^+$)-ATPase activity using γ-labelled [^{32}P]-ATP (New England Nuclear) as in ref. 13.

RESULTS

When erythrocytes are suspended in halothane-saturated, isotonic media and evaporation of the anaesthetic is prevented, the cells do not lyse even after hours

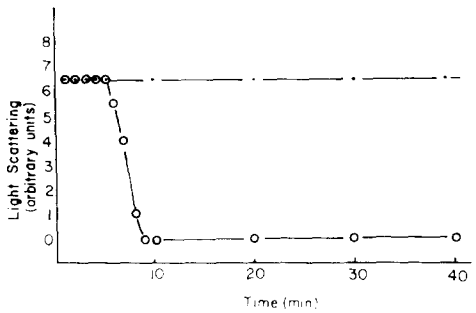

Fig. 1. Light-scattering changes subsequent to halothane release from erythrocyte suspension. (- . - . -) cuvette sealed; (-○-○-) cuvette unsealed.

of incubation. However, if the anaesthetic concentration in the suspension is reduced by equilibrating the suspension with a gas phase, lysis occurs at a rate and to a degree depending on the volume of the gas phase and the rate of halothane release.

The process of hemolysis induced by halothane release is illustrated in Fig. 1,

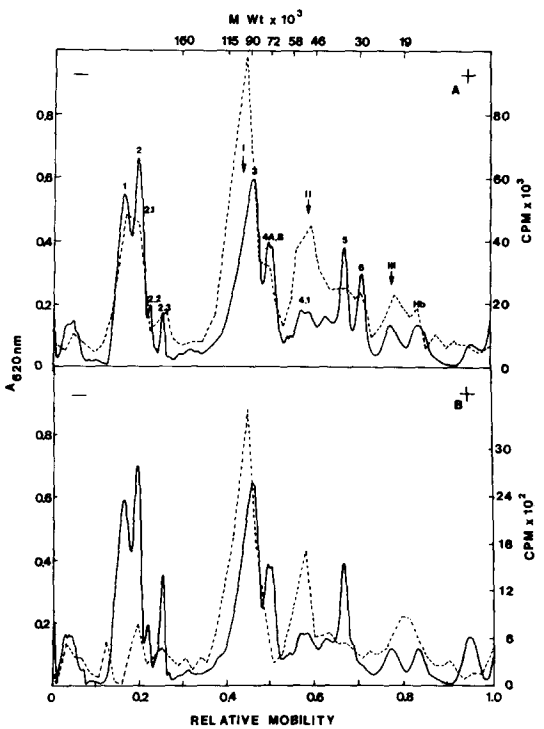

Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of ^{125}I-labelled erythrocyte ghosts prepared by the Dodge procedure [9] (Panel A) and the halothane procedure (Panel B). Relative mobilities and molecular weights are given on the abscissa. Absorption for Coomassie Blue at 620 nm and ^{125}I counts are presented on the ordinate. The proteins are assigned the numbers 1–6 (upper panel). Hb = hemoglobin. Numbers I–III (arrows) refer to glycosylated protein visualized by periodate-Schiff staining.

comparing the light scattering of a suspension of erythrocytes in halothane-saturated phosphate-buffered saline kept in a sealed cuvette lacking a gas phase, with that of an identical sample after halothane release was initiated by unsealing. Light scattering from the sealed cuvette is invariant, whereas scattering decreases abruptly after unsealing.

Cell lysis is complete within 10 min after initiation of halothane evaporation at 25–37 °C. Moreover, after 1–2 washes with halothane-free medium, the membranes are essentially free of hemoglobin, as judged by sodium dodecyl sulfate-polyacrylamide electrophoresis (Fig. 2).

Lysis is much slower at 4 °C and the membranes isolated at this temperature contain appreciable amounts of hemoglobin.

Suspending erythrocytes in isotonic media saturated with halothane leads to pronounced shape transformations. In less than 1 min, a variety of bizarre folded shapes, spherical cells as well as some crenated cells, become apparent by phase-contrast microscopy. Scanning electron microscopy illustrates these transformations in greater detail (Fig. 3). The morphological sequence leading to hemolysis is similar to that found with cationic substances and nonpenetrating anions [7]. The biconcave discs change into cup-shaped cells, which then form spheres with multiple invaginations, and finally become smooth spheres before lysing. An exceptional spherical cell type is often seen with approx. 15 % of its surface aggregated into a "cap" (Fig. 3).

Ghosts isolated from halothane-containing media exhibit the same mor-

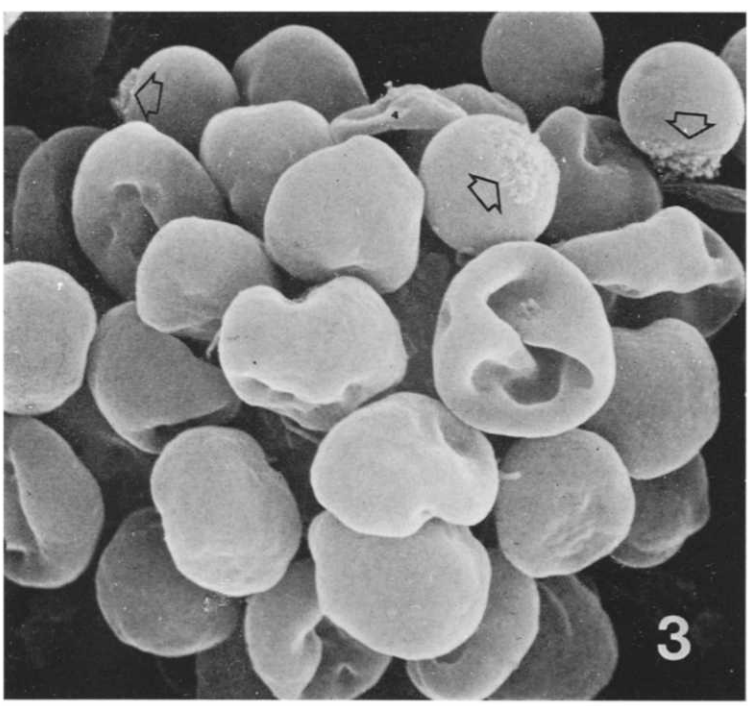

Fig. 3. Effects of halothane on erythrocyte morphology. Arrows indicate capped spherocytes. (4000 ×.)

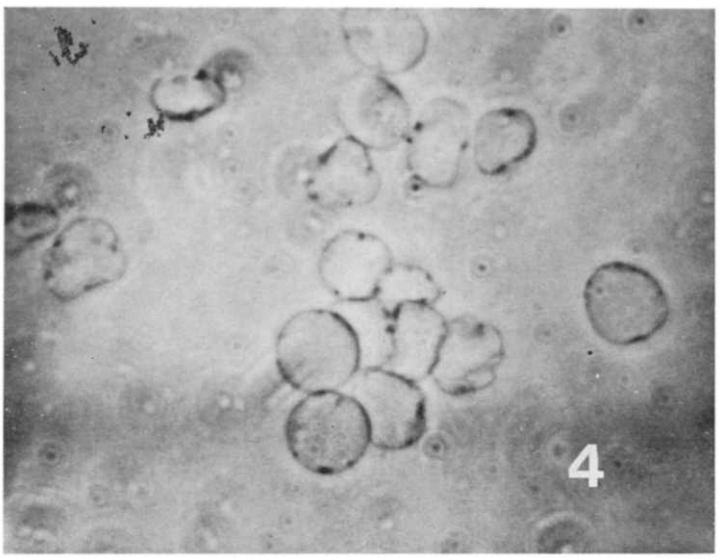

Fig. 4. Phase-contrast photomicrograph of erythrocyte ghosts prepared in halothane-saturated medium.

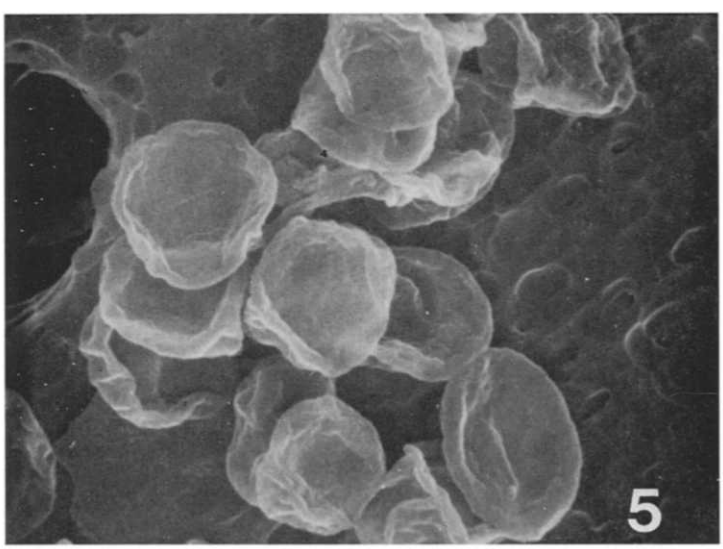

Fig. 5. Erythrocyte ghosts prepared in halothane-saturated medium. Scanning electron photomicrograph of 2 % glutaraldehyde-fixed ghosts. (1400 × and 5000 ×, respectively.)

phology under phase-contrast microscopy as ghosts prepared by hypotonic lysis (Fig. 4). They appear disk shaped, free of hemoglobin and have similar diameter as intact erythrocytes. After glutaraldehyde fixation, the ghosts remain in their disk shapes but exhibit some wrinkles (Fig. 5).

Halothane-prepared ghosts and ghosts from osmotic lysis show similar

TABLE I

$(Na^+ + K^+)$-ATPase ACTIVITIES OF INTACT ERYTHROCYTES AND GHOSTS PREPARED BY TREATMENT WITH HALOTHANE AND BY HYPOTONIC LYSIS

Sample	ATPase activity (nmol P_i released/mg protein per h)
Erythrocytes	1.3
Halothane ghosts	12.0
Halothane ghosts after osmotic lysis [9]	202.0
Ghosts prepared by osmotic lysis [9]	200.0

qualitative and quantitative protein patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), except for two major differences: in the halothane ghosts Component 2.3 (M_r 190 000) is present at high concentrations and Component 6 (M_r 37 000) is lacking. Periodate-Schiff staining (PAS bands I–III) is identical in both ghost preparations.

Iodination of halothane ghosts using the lactoperoxidase method yields only 2.5 % of the ^{125}I incorporation obtained with osmotically lysed ghosts under identical reaction conditions. Moreover, the distribution of ^{125}I in electrophoretograms of halothane ghosts resembles that obtained after iodination of intact erythrocytes, rather than after labelling of ghosts prepared by osmotic lysis [11]. Most of the label is located in the region of the major glycoprotein (PAS-I band);(apparent M_r 90 000) but the PAS-II (apparent M_r 60 000) and PAS-III (apparent M_r 25 000) bands are also iodinated. Traces of label are also found in the region of Components 1 and 2, as well as in the hemoglobin zone (Fig. 2).

The $(Na^+ + K^+)$-ATPase activities of the two ghosts preparations are summarized in Table I. Halothane ghost preparations hydrolyze externally added ATP at only 5 % the rate observed with ghosts lysed according to Dodge et al. [9]. However, if these same halothane ghosts are cycled through the hypo-osmotic procedure [9], they show a $(Na^+ + K^+)$-ATPase activity identical to that of ghosts prepared solely by hypotonic lysis.

DISCUSSION

We note three unusual aspects in the interaction of halothane-saturated physiological salt solutions and human erythrocytes; the morphological transformation in the presence of the anaesthetic, the cell lysis induced by the release of the anaesthetic and the character of the membrane fragments produced by halothane-induced lysis.

Unlike other membrane-active agents, halothane does not cause cell lysis at high concentrations, but only when its concentration is reduced after equilibration with the cells. That equilibration is necessary to prime the cells for lysis is shown by the fact that no hemolysis occurs during addition of the anaesthetic. At concentrations known to be anaesthetically effective halothane causes expansion of model

lipid bilayers [4] and increases the hydraulic water permeability of erythrocytes [14], but these facts explain neither the abnormal erythrocyte morphology seen in halothane-saturated media, nor the hemolysis induced by halothane release.

Our morphologic studies cannot be explained by simple, overall membrane expansion, nor with the type of focal membrane perturbation induced by substances such as nitroxide-lipid analogues, although such processes may be taking place. We, therefore, consider two alternative lytic mechanisms.

First, it is conceivable that halothane somehow interferes with the postulated contractile properties of cytoplasmic, membrane-associated proteins, such as "spectrin" [15–17] thereby producing erythrocyte deformation. We cannot exclude this possibility, but note that sodium dodecyl sulfate-polyacrylamide gel electrophoresis of halothane ghosts reveals the same proportion of spectrin found in ghosts from hypotonic lysis and that the spectrin components of the two types of membrane isolated exhibit identical apparent molecular sizes. Also, we cannot evolve a coherent hypothesis which implicates spectrin in both erythrocyte deformation and the cell lysis induced by halothane release.

The second hypothesis invokes, in addition to membrane changes, the fact that halothane disrupts water structure [18], the tendency of compounds such as halothane to form microhydrate crystals which are stabilized at ambient temperatures by macromolecules [18] such as hemoglobin, the very high intracellular concentration of hemoglobin (approx. 34 g/100 ml erythrocytes) and the very large proportion (approx. 20 %) of intracellular water associated with hemoglobin. We propose the following sequence of events.

(a) Halothane diffuses into the erythrocytes, alters the structure of intracellular water, causing hemoglobin precipitation and cell deformation. The bizarre membrane morphology in halothane-saturated media is thus attributed to hemoglobin precipitation, in analogy to the sickle shapes caused by the polymerization of hemoglobin S. We cannot now produce conclusive proof that halothane can induce intracellular hemoglobin precipitation, but have found that the anaesthetic can cause reversible gelling of concentrated hemoglobin solutions (Wallach, D. F. H. and Lin, P. S., unpulished). Freeze-fracture electron microscopy and freeze-etch electron microscopy of glutaraldehyde-fixed halothane-saturated erythrocytes, shows no conspicuous differences in the cytoplasmic space compared with normal erythrocytes (Weinstein, R. S., Wallach, D. F. H. and Lin, P. S., unpublished). However, such experiments are not conclusive, since they require fixation and since fixation itself precipitates hemoglobin.

(b) Water enters into the cell due to membrane expansion, i.e. increased intracellular volume, and/or halothane-induced lowering intracellular water activity. This process is facilitated by the increased water permeability of erythrocyte membranes, but lysis does not occur due to halothane-induced membrane stabilization [1].

(c) Return to normal (lower) membrane water permeability and lower osmotic stability produces cell rupture.

Turning to the properties of the membranes isolated after halothane-induced lysis, we note that these exhibit sodium dodecyl sulfate-polyacrylamide electrophoretograms identical to those of ghosts from the hypotonic lysis method [12], in terms of both proteins and glycoproteins except for the lack of Component 6 in the halothane membranes. We attribute this difference to the fact that halothane ghosts

are generated at physiological ionic strengths. Component 6, identified as glyceraldehyde-3-phosphate dehydrogenase [19], can be eluted from ghosts as prepared by Dodge et al. [9] at physiological ionic strengths [20, 21] and our data are consistent, indicating that this protein is not tightly associated with the erythrocyte membrane interior under physiologic ionic conditions.

The electrophoretograms of ^{125}I-labelled halothane ghosts resemble those of ghosts osmotically isolated from iodinated erythrocytes, rather than those of iodinated hypotonically lysed ghosts. The labelling of halothane ghosts is largely limited to the protein and glycoproteins accessible to lactoperoxidase in the case of intact erythrocytes. These data indicate the halothane ghosts, in contrast to membranes produced by hypo-osmotic lysis, are not permeable to molecules the size of lactoperoxidase (80 000).

The trace labelling of Components 1, 2 and hemoglobin can be attributed to the presence of a small proportion of unsealed ghosts.

Our data on the hydrolysis of external ATP by halothane membranes shows that these ghosts are also poorly permeable to small molecules. In isotonic media, the halothane ghosts show only approx. 5 % of the $(Na^+ + K^+)$-ATPase specific activity of membranes prepared according to Dodge et al. [9]. Exposure of halothane ghosts to low ionic strengths equivalent to those used in hypo-osmotic lysis produces the same ATPase activity as found in osmotically lysed ghosts. The anaesthetic thus does not influence ATPase activity per se, only the permeability of the ghost membranes to ATP.

The use of halothane to isolate erythrocyte ghosts offers interesting advantages. (a) Membrane isolation is simple, rapid and suitable for large-scale preparations. (b) Ghosts are prepared under isotonic conditions, using physiological ionic strength and ionic composition; this avoids the need of special steps to re-establish physiological conditions and eliminates artefactual adsorption of soluble proteins. (c) Halothane ghosts, while morphologically similar to hypotonically produced ghosts are "sealed" to molecules the size of ATP and thus highly suited for transport studies.

We are currently exploring the mechanisms of halothane-induced cell lysis in greater detail utilizing both erythrocytes and other cell types.

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