INTRACELLULAR GAS SUPERSATURATION TOLERANCES OF ERYTHROCYTES AND RESEALED GHOSTS

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ABSTRACT Intact mammalian, avian, and amphibian erythrocytes were saturated with up to 300 atm nitrogen or argon gas and rapidly decompressed. Despite the profuse nucleation of gas bubbles in the suspending fluid, no evidence of intracellular gas bubble nucleation was found; all or most of the cells remained intact and little or no hemoglobin escaped. Internal bubbles were similarly absent from resealed ghosts of human erythrocytes as shown by lack of disintegration and by retention of an entrapped fluorescent compound. The absence of bubbles may indicate that much of the internal water does not have the same nucleation properties as external water.

INTRODUCTION

The ability of single-celled eucaryotic and procaryotic organisms to tolerate remarkably high gas supersaturations without the formation of intracellular bubbles has been shown only recently (Hemmingsen and Hemmingsen, 1978; 1979; 1983). For example, some microorganisms, such as bacteria without gas vesicles and yeast, are unaffected by decompression from 300 atm nitrogen saturation (Hemmingsen and Hemmingsen, 1978; 1979; 1980) even though the threshold for spontaneous homogeneous nucleation of bubbles in pure water or aqueous solutions contained in small glass tubes is only 180–190 atm (Hemmingsen, 1975; 1978). Others, such as *Tetrahymena* without food vacuoles and *Euglena*, are affected and damaged to various degrees by these extreme gas supersaturations. The damage appears to be caused by external bubbles or other factors rather than intracellular bubbles; however, the high concentration of dissolved gases and the pressures per se do not appear to be important (Hemmingsen and Hemmingsen, 1979; 1983). In only one special case is damage clearly caused by the formation of intracellular bubbles, namely, in *Tetrahymena* with food vacuoles (Hemmingsen and Hemmingsen, 1983). By using a direct cinephotomicrographic technique, decompression from 175 atm and higher nitrogen saturations can be seen to produce bubbles within the cells; their subsequent rapid expansion leads to cell disintegration. When the cells are treated to remove food vacuoles, no bubbles form within them even with nitrogen saturations ≥200 atm.

It appears then that resistance to the formation of gas bubbles is a property unique to the intracellular environment, of which 70–80% is water. This characteristic of living cells raises the possibility that cellular water may differ from external water or water in food vacuoles in its susceptibility to gas bubble nucleation. We have attempted to obtain more information about this phenomenon by examining erythrocytes, a type of cell that lends itself to osmotic manipulation and from which ghosts can be prepared. Such ghosts presumably contain fewer cellular components and more water than intact erythrocytes. Also, erythrocytes differ markedly from the types of cells we investigated earlier both with respect to the nature of the cell envelope and to the intracellular composition and structure.

The standard procedures that we used earlier (Hemmingsen and Hemmingsen, 1979) to induce gas supersaturations by rapid decompression were applied to human, rabbit, chicken, and toad erythrocytes, or to ghosts of human erythrocytes sealed in the presence of the fluorescent compound, dansyl-L-glutamate. Damage was assessed by postdecompression enumeration and by the escape of hemoglobin from the cells or of the fluorescent compound from the ghosts.

MATERIALS AND METHODS

Preparation of Erythrocytes

All solutions were prepared with distilled water and reagent grade chemicals and were filtered through a filter (1.2-μm pore size; Millipore/Continental Water Systems, Bedford, MA) to remove suspended particles. Unless stated otherwise, all centrifugations were at room temperature for 2 min at ~1,000 *g*.

Whole blood was drawn into EDTA (K_3) Vacutainers (Becton-Dickinson & Co., Rutherford, NJ) from adult human donors, 5–6 wk old New Hampshire chickens, and adult toads (*Bufo marinus*). Blood from an adult New Zealand rabbit was drawn with a 26 gauge needle into a 3-ml syringe containing 2 mg EDTA (Na_2) in 1 ml of saline (0.85% wt/vol NaCl) sterilized by filtration. Whole human blood was used fresh or was stored before use in Vacutainers for up to 1 d at room temperature (19–22°C) or for up to 1 wk at 0.2–5°C. Just before use, 2 ml of human

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blood was diluted with 8 ml of saline, the cells collected by centrifugation, washed two or three times with 10 ml saline, and finally suspended in 10 ml saline or in one series of experiments the same volume of solutions of different NaCl concentrations. Chicken and rabbit erythrocytes, washed and diluted as above, were used immediately or were stored at 0.2–5°C for up to 2 d. Stored cells were washed and resuspended in saline immediately before use. The pH of a representative suspension was 7.2 to 7.4. Immediately after collection, toad blood was centrifuged for 20–30 min at ~160 *g* (2–8°C) in sealed 250 μl Natelson capillary tubes without anticoagulant or, if the volume exceeded 4 ml, in a single 15-ml centrifuge tube. After the erythrocytes were pooled, enough sterile Eagle's minimal essential medium (MEM) (modified Auto-POW, Flow Laboratories, McLean, VA) containing added sodium bicarbonate (0.28 mg/ml) and fetal calf serum (10% vol/vol) (freezing point depression −0.51°C) was added to replace the toad serum (freezing point depression about −0.55°) and the suspension held at ~5°C for up to 24 h. Before use, 1–2 ml of this suspension was diluted to 10 ml with saline, the cells washed two or three times with the same volume and finally suspended in 4 ml saline or solutions of different NaCl concentrations. Suspensions of the nucleated erythrocytes were mixed by gentle inversion of the tubes.

Preparation of Sealed Ghosts

The ghosts were prepared according to a modification of the method of Johnson (1975) from human erythrocytes within 24 h of collection. The erythrocytes were washed three times at room temperature in 5 mM Tris HCl, pH 7.4; 140 mM NaCl; and 1 mM EDTA, and then suspended in 10 or 80 times (Staros et al., 1974) the pellet volume in cold hemolyzing buffer (5 mM Tris HCl, pH 7.4; 7 mM NaCl; 1 mM EDTA). The ghosts were washed three times in the same volume of cold hemolyzing buffer and pelleted by centrifugation at 0°C for 30 min at 10,000 *g* or 20 min at 14,6000 *g*. The dense red button underneath the ghost pellet was removed after each wash (Johnson, 1975). Pellets were suspended by gentle inversion of the tubes to minimize trauma. The unsealed ghosts were then washed twice more in hemolyzing buffer without EDTA.

The pellet from the final wash was suspended in 9 ml of 5 mM Tris-HCl, pH 7.4; 140 mM NaCl; 10 mg/ml dansyl-L-glutamic acid (Sigma Chemical Co., St. Louis, MO) and incubated at 32.5–38°C for 3 h with gentle mixing by inversion four or five times. The sealed ghosts were washed in cold suspension buffer (5 mM Tris-HCl, pH 7.4; 140 mM NaCl) until the concentration of dansyl-L-glutamic acid in the supernatent was 0.05 to 0.1 μg/ml. This procedure yielded a pellet with a volume of ~0.5 ml that was suspended to 6–7 ml with fresh suspension buffer and stored on ice for up to 7 h before use.

Experimental Methods

All experiments were done at room temperature (19–22°C). For each experiment, 2 ml of washed and resuspended blood cells or 1 ml of the ghost preparation was placed into each of two 8 ml or 5 ml beakers, respectively, and stirred magnetically at ~230 rpm. Aliquots of blood cells were removed and diluted 1×10^{-3} to 8×10^{-5} in saline and numbers determined with a Coulter counter (model B; Coulter Electronics, Chicago, IL). The experimental suspension was rapidly compressed to elevated nitrogen or argon pressures and equilibrated for 30 min as previously described (Hemmingsen and Hemmingsen, 1979); this equilibration time is two to three times that necessary to saturate the suspending liquid, and hence the cells, with gas (see Appendix). The control suspension was maintained in the dark in room air and stirred at the same rate. Within 10 min after rapid (~0.7–1.5 s) decompression, cell counts were performed. The contents remaining in each beaker were divided approximately equally. One portion was centrifuged and the supernatant assayed for hemoglobin by the cyanmethemoglobin technique (Kit No. 525-A; Sigma Chemical Co., St. Louis, MO). The other was frozen and thawed at least twice (3–5 times for amphibian cells) to produce complete hemolysis, and total hemoglobin was determined. The amount of hemoglobin lost by control and experimental erythrocyte suspensions was calculated using a standard curve prepared with human hemoglobin for the mammalian cells, and one prepared with pigeon hemoglobin for the avian and amphibian cells.

Ghosts numbers were found by diluting aliquots 1×10^{-4} in fresh suspension buffer before counting with the Coulter counter (Coulter Electronics). At the end of the experiments, the contents of the control and experimental beakers were divided in half. One set was centrifuged as before and the amount of fluorescent label in the supernatant found by placing 200 μl in virgin borosilicate glass tubes (12 × 75 mm) containing 4.8 ml 95% ethanol, exciting at 350 nm, and measuring fluorescence at 500 nm with a Turner 430 spectrofluorometer (AMSCO Instrument Company, Carpenteria, CA). The other set was frozen and thawed five times to release all of the fluorescent label. The concentration of dansyl-L-glutamic acid released was determined by reference to standard curves prepared for each batch of resealing solution. Release by the ghosts exposed to gas supersaturations and the control ghosts was calculated as percentage of the total label in the freeze/thaw lysate.

RESULTS

All of the erythrocytes were unaffected by decompression from nitrogen saturations of 175–200 atm (Fig. 1). With higher saturations, some of the chicken and toad erythrocytes sustained damage as indicated by a decrease in cell number and loss of hemoglobin to the suspending fluid, while little or no damage of the mammalian erythrocytes was apparent. Most experiments were conducted with 300 atm nitrogen saturations, where the differences between the two erythrocyte types were most pronounced. The larger, nucleated erythrocytes from chickens and toads were somewhat less tolerant to the higher supersaturations than were the mammalian ones; ~20% of the cells disappeared and the amount of hemoglobin that appeared in the suspending fluid reflected the degree of cell disintegration. The mammalian erythrocytes were not obviously altered in appearance in the phase-contrast microscope by exposure to any of the nitrogen supersaturations. However, the surface of some of the nucleated cells appeared wrinkled, particularly after decompression from 300 atm nitrogen, and some debris, chiefly cell-free nuclei and nuclei surrounded by membranes, was present.

Because argon is more soluble in water than nitrogen and has a lower bubble-nucleation threshold (Hemmingsen, 1977), we also exposed human and toad erythrocytes to high argon supersaturations. Human cells were as tolerant as when exposed to the same nitrogen supersaturations; freshly collected toad erythrocytes showed about the same range of stability as in the experiments with nitrogen (data not shown). In separate experiments, toad and human erythrocytes were saturated with nitrogen using a slow, stepwise compression procedure (Hemmingsen and Hemmingsen, 1979) with 8 min pauses for equilibration over 2.5 h. After 20 min at the final saturation pressure of 200 atm, the cells were rapidly decompressed; little or no damage was noted (data not shown).

In a series of experiments, human and toad erythrocytes were suspended in hypotonic saline solutions just before equilibration at 300 atm nitrogen. With decreasing NaCl concentration there was little release of hemoglobin from human erythrocytes in both the experimental and control

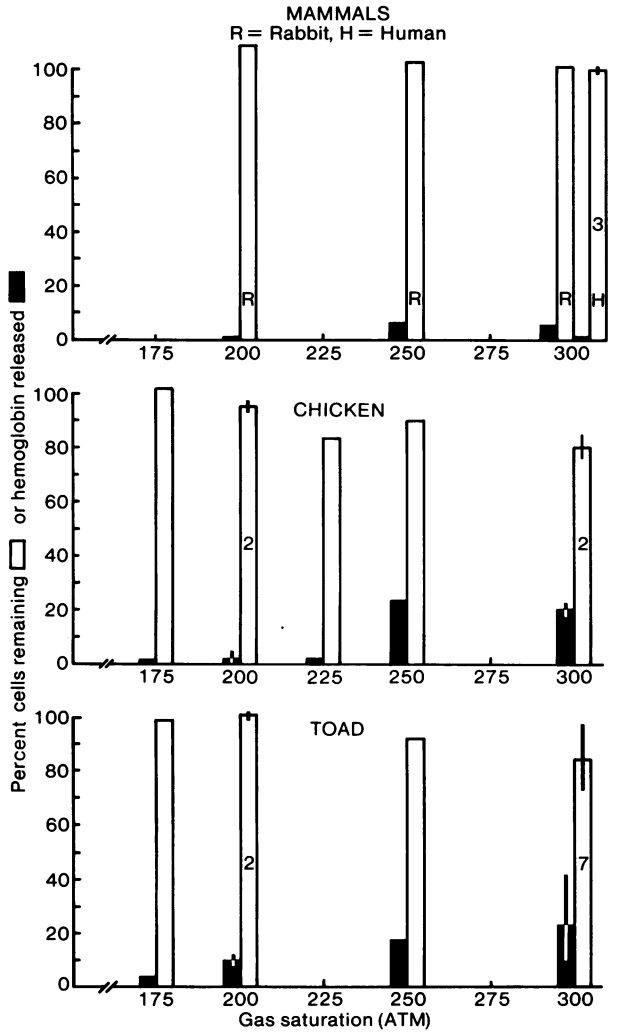

FIGURE 1 Exposure of mammalian, avian, and amphibian erythrocytes to various nitrogen saturations followed by rapid decompression. Uncompressed control suspensions showed little or no decrease in cell number or release of hemoglobin. The number of experiments is given on the bars, and the range of data is shown by the lines. All bars in a cluster represent results obtained with the gas saturation given below. Cell numbers ranged from 7 to 11 $\times$ 10^8/ml for anucleated erythrocytes and 1 to 4 $\times$ 10^8/ml for nucleated ones.

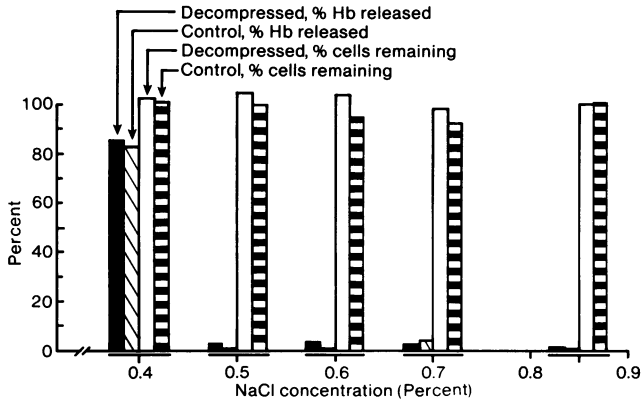

FIGURE 2 Suspension of human erythrocytes in hypotonic solutions and exposure to 300 atm nitrogen saturation followed by rapid decompression. All bars in a cluster represent results obtained at the NaCl concentration given below. Cell concentrations ranged from 7.7 to 21 $\times$ 10^8/ml.

suspensions until the NaCl concentration reached 0.4% (Fig. 2). Fragmentation of the cells was not observed until the NaCl concentration dropped to 0.3% (data not shown); the same degree of cell disappearance was found in both the control and experimental suspensions. Toad erythrocytes exposed to hypotonic solutions were generally no more affected by exposure to 300 atm nitrogen supersaturation than cells suspended in 0.85% NaCl (data not shown).

Washed ghosts prepared by lysing human erythrocytes in 10 vol of hypotonic buffer with EDTA and sealed in the presence of dansyl-L-glutamate, were not affected by any of the nitrogen or argon supersaturations used (Table I). In the first series of experiments, the ghosts were mostly spheres (4–5.5 μm in diameter) with some disk and cup shapes. In the second series, more disk- and cup-shaped ghosts were present and the spheres were larger (~80% were 5–6.8 μm in diameter, with a few 8 μm in diameter). The pellets were always white, indicating the depletion of hemoglobin. Ghosts from both preparations became increasingly leaky with time but release of the fluorescent label was essentially the same in the compressed and uncompressed preparations. After decompression, most of the ghosts remained and could be seen in the microscope and counted by the Coulter counter. A few experiments were performed using washed ghosts prepared by lysing human erythrocytes in 80 vol of the hypotonic buffer, a procedure that is supposed to remove more of the cytoplasm (Staros et al., 1974). The results were essentially the same as those reported in Table I. The threshold for spontaneous nucleation of argon bubbles in the saline and in the suspension buffer containing dansyl-L-glutamate, was checked by a method using glass tubing (Hemmingsen, 1975), and was found to be identical to the threshold for pure water and other aqueous solutions (data not shown).

In all of the experiments with erythrocytes, both unstressed and osmotically stressed, and with ghosts, experimental preparations were examined at a magnification of 1,000 with a phase contrast microscope within 1.5 min after decompression; under these conditions a bubble ~0.5 μm in diameter can be resolved. In no case were internal gas bubbles observed; no bubbles were associated with any of the debris.

DISCUSSION

Our results show that the intracellular environment of anucleate mammalian erythrocytes is resistant to gas bubble formation even when subjected to such extreme nitrogen and argon supersaturations that profuse, spontaneous nucleation of bubbles occurs in the external water. Had bubbles nucleated intracellularly, their inevitable expansion would have produced damage, i.e., leakage of hemoglobin into the suspending fluid and destruction of

TABLE I

HUMAN ERYTHROCYTE GHOSTS REMAINING AND RELEASE OF INCORPORATED DANSYL-L-GLUTAMIC ACID AFTER SATURATION WITH GAS AND RAPID DECOMPRESSION

Gas	Equilibration pressure	Time since last wash	Percent of ghosts remaining after decompression*	Percent of dansyl-L-glutamic acid released‡	
				after stirring without compression	after decompression
	atm	*h*			
Series No. 1					
N_2	300	0.5	96	19	20
N_2	250	5.0	96	28	30
N_2	300	7.0	96	42	37
Series No. 2					
Ar	300	1.0	82	35	44
Ar	250	3.0	88	50	50
N_2	300	6.0	92	52	66

*The concentration of ghosts ranged from 2.6 to 4.9 $\times$ 10^8/ml. There was no decrease in number of ghosts after stirring in air at 1 atm ambient pressure.

‡Dansyl-L-glutamic acid released is given as a percent of the total present in the sample. In series No. 1, the total amount of dansyl-L-glutamic acid present ranged from 1.18 to 1.31 μg/ml. In series No. 2, the total ranged from 2.29 to 2.40 μg/ml.

the cells, as has been shown with ciliates (Hemmingsen and Hemmingsen, 1983).

The absence of both homogeneous and heterogeneous nucleation of bubbles in the erythrocytes strengthens our earlier suggestion, based on results with other types of cells, that bubbles do not nucleate because of the particular physical conditions that exist in the intracellular environment. The lack of heterogeneous nucleation is striking given the large number of interfaces, both hydrophilic and hydrophobic, that exist in cytoplasm. In simpler aqueous systems, the presence of solid interfaces tends to facilitate the heterogeneous nucleation of bubbles, particularly if these surfaces are hydrophobic in character (Gerth and Hemmingsen, 1980). Yet, in cytoplasm, the bubble nucleation threshold appears to exceed that of homogeneous nucleation in solutions. Perhaps such interfaces are in the intracellular environment coated with surfactants that provide a transition layer between the interface and water. These layers would prevent direct contact of the water with the hydrophobic surfaces and thereby prevent bubble nucleation.

The mere presence of dissolved solutes in the cellular water, and the resulting decreased gas solubility, increased viscosity, and decreased surface tension of the liquid would not markedly affect the threshold for homogeneous bubble nucleation as studies with aqueous solutions of electrolytes and surfactants have shown (Hemmingsen, 1978). The lack of bubbles strongly indicates that the quantity of intracellular water with normal nucleation properties is insufficient to allow the nucleation process to occur. Such insufficiency may come about in three ways: (*a*) The total volume of intracellular water may be too small to permit bubble nucleation. (*b*) The total volume may be large enough but it is so subdivided by the various components of the cytoplasmic matrix (Weinstein, 1974; Wolosewick and Porter, 1979; Brinkley, 1981) that only subcritical pools are present. (*c*) The water present is mostly associated with macromolecules and such bound or ordered water may be less likely to undergo the spontaneous structural rupture that must occur for bubble nucleation.

Although a definitive assessment of each of these possibilities cannot yet be made, it appears that at the highest gas supersaturations used in our experiments the total volume of water within the erythrocytes is well above the critical volume necessary for homogeneous nucleation of bubbles. Based in part on data published elsewhere (Hemmingsen, 1977; 1978), the nucleation density for bubbles in bulk water may be estimated from cinemicrographic recordings made during decompression from argon saturation pressures of up to 202 atm. Bubbles begin to nucleate spontaneously in the water at ~150 atm and the number of bubbles per volume unit increases with increasing gas supersaturations. From 170 to 200 atm argon supersaturations, the number increases gradually by about a factor of 10 per 10 atm increase in supersaturation, reaching ~10^7 bubbles/cm^3 at 200 atm. Assuming that a similar rate of increase also occurs in a pressure range above 200 atm, we may extrapolate that 10^{10} bubbles/cm^3 or 1 bubble/100 μm^3 will nucleate at 230 atm argon. Thus, with argon supersaturations of 230 atm, at least 1 bubble can be expected to nucleate in a volume of water corresponding to the volume of the human erythrocyte (94 μm^3) and more bubbles in the larger (Andrew, 1965) nucleated erythrocytes. Elevated saturation pressures such as the 250 and 300 atm argon we have tested with human erythrocytes would dramatically increase the number of bubbles nucleating in these volumes; yet no significant release of hemoglobin was apparent. At equivalent nitrogen supersaturations, the number of bubbles formed per volume unit would be smaller because this gas has a higher nucleation

threshold in water and solutions (Hemmingsen, 1978). Nevertheless, the volumes of the erythrocytes and the procaryotic and eucaryotic cells we have examined are within the minimum volumes required for homogeneous nucleation of nitrogen and argon bubbles.

The extent of the increase in intracellular gas supersaturation tolerance relative to that of bulk water cannot be estimated due to the drainage of some gas from the cells after the nucleation of extracellular bubbles in the later phases of decompression (Hemmingsen and Hemmingsen, 1979; 1980). The half-time for gas diffusion equilibrium to be established between the erythrocytes and the exterior water is similar (Roughton, 1959; Forster, 1964) to the half-time for the fastest decompressions used by us, ~0.1 s. Therefore, a gas supersaturation of at least tens of atmospheres higher than the nucleation threshold for water is generated intracellularly during decompressions from the highest equilibration pressures used.

The intracellular resistance of erythrocytes to bubble nucleation is maintained when some water is added osmotically and even when sealed ghosts are prepared. Thus, it appears that ghosts may not be simple membrane-bound vesicles that contain water and solutes but may retain enough of their original internal macromolecular constituents to affect the nucleation properties of the entrapped solution.

Far too little is known about the critical volume of liquid required for gas bubble nucleation or about the internal organization of erythrocytes or other cells to decide whether the observed stability of the intracellular environment is the result of the subdivision of the water into subcritical pools or is attributable to the association of most of the water with cell constituents. While all investigators agree that some water is captured by macromolecules as water of hydration, some (e.g., Ling, 1979; Fulton, 1982) argue that much of the cellular water is more ordered than is bulk water; other investigators argue against this view (e.g., Finch, 1979). We do not know if water structured by hydration or by association with cell constituents has altered nucleation properties, but in view of the fact that the nucleation behavior of bulk water is altered near its freezing point (Hemmingsen, 1975) and in the presence of high concentrations of water structuring solutes (e.g., Mg and Na) (Hemmingsen, 1978), we wish to speculate that this is the case and that our present results (as well as those from earlier studies, e.g., (Hemmingsen and Hemmingsen, 1979; 1980; 1981; 1983) may reflect extensive structuring and stabilization of cell water.

A fraction of the nucleated avian and amphibian erythrocytes were damaged upon exposure to the highest gas supersaturations used in our experiments. Although for this cell type it cannot be excluded that bubbles may have nucleated intracellularly, this seems unlikely because, after decompression, bubbles were conspicuously absent in the remaining intact cells, in cells with various degrees of damage, and in cell fragments. These observations are in contrast to those made on *Tetrahymena* after decompression from gas saturation pressures of 250 atm and above, a procedure that produces internal bubbles, provided the cells contain food vacuoles (Hemmingsen and Hemmingsen, 1983). In neither system is the damage due to factors inherent in the experimental procedure other than extreme gas supersaturation, because the cells are not affected by a slow, stepwise release of pressure (data not shown), a procedure that prevents the formation and growth of external bubbles and potential internal bubbles. Direct photomicrographic evidence for the absence of internal bubbles in the nucleated erythrocytes cannot be obtained because at the damaging saturation pressures large numbers of external bubbles obscure the events that may be taking place within the cells. We consider it most likely that the damage is due to forces exerted by the external bubbles on the plasma membranes. The large size of the nucleated erythrocytes and the greater rigidity of their plasma membranes (Waugh and Evans, 1976) may play a role in their susceptibility to external bubbles. The anu-

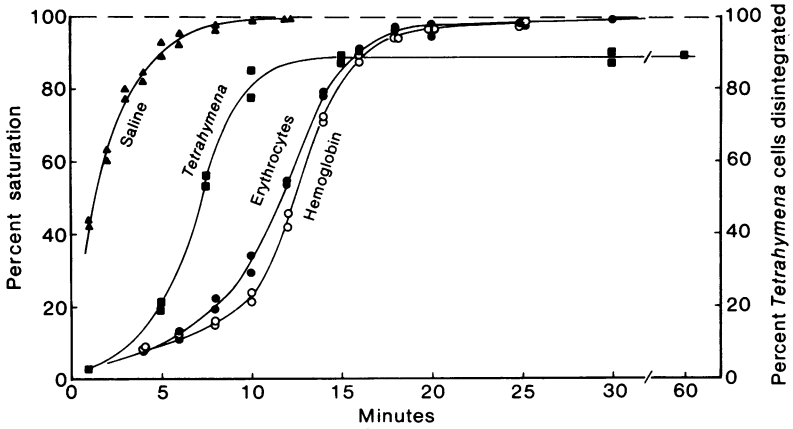

FIGURE 3 Equilibration with air of oxygen-depleted solutions and erythrocyte suspensions as a function of time. The cells of *Tetrahymera pyriformis* were suspended in 2 ml LL salts (Hemmingsen and Hemmingsen, 1983), compressed to 200 atm with nitrogen, and decompressed at the times indicated.

cleated erythrocytes appear to be more resistant to damage by external bubbles than the nucleated erythrocytes and also many of the species without cell walls we have examined previously. The human erythrocyte membrane skeleton (Gratzer, 1981; Marchesi, 1983) probably enables the cell surface to withstand the mechanical and surface tension forces that are likely generated by the formation and growth of bubbles adjacent to it; certainly, recent studies suggest that this skeleton aids in protecting the erythrocytes against shear forces within the circulatory system (Mohandas et al., 1982).

APPENDIX

At the request of the reviewers, we are presenting data (Fig. 3) to show that under our experimental procedures the erythrocytes and ghosts are fully gas equilibrated in the time we allow. Saline (0.85% NaCl), suspensions of erythrocytes in saline (oxygen capacity 10.5 vol%), and hemolyzed erythrocytes in saline (same oxygen capacity) were deoxygenated by equilibration with helium at atmospheric pressure for at least 1 h. Deoxygenation was verified by measuring polarographically the oxygen tension of the liquid with a microoxygen electrode. Two milliliters of liquid were then placed in our experimental beakers and equilibrated with air at 1 atm using our standard stirring speed. The oxygen tension of the liquids was measured as a function of time after start of air equilibration. The curves are drawn through the means of two sets of experiments with each liquid. The presence of hemoglobin causes a delay in the equilibration due to the added quantity of gas that must be taken up. At full air saturation, the content of the oxygen bound to hemoglobin is twenty times that in physical solution in all of the liquid. This high oxygen capacity was used to dramatize the effect of a large gas sink on the equilibration. The difference between the suspensions of intact cells and hemolyzed cells reflects the diffusion resistance across the cell boundary (Forster, 1964*a*), and probably a viscosity effect.

About 5×10^5 cells per ml of the ciliate *Tetrahymena pyriformis* strain GL, which are much larger than human erythrocytes, were equilibrated with nitrogen. Gas bubbles will form within these cells when they are equilibrated with at least 175 atm nitrogen and rapidly decompressed; the expanding gas bubbles disintegrate the cells (Hemmingsen, 1982; Hemmingsen and Hemmingsen, 1983). The ciliates appear to be fully nitrogen equilibrated after 15 min, because exposure for 30 or 60 min does not significantly change the numbers of disintegrated cells.

As would be expected from earlier studies (e.g., Forster, 1964*a*, *b*), these experiments show that cells in contact with gas saturated solutions are fully gas equilibrated in the time allowed by our experimental protocol.

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