

STABILITY OF ERYTHROCYTES FIXED
IN OSMIUM TETROXIDE SOLUTIONS

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INTRODUCTION

In a dielectric study of osmium tetroxide-fixed erythrocytes (Carstensen and Smearing, 1967), it was observed that after multiple washing in distilled water the membranes of the cells suffered severe deterioration as indicated by a marked drop in membrane resistance and an increase in permeability of the cells to small solute molecules. Whereas the previous study was concerned primarily with the phenomenon of red blood cells with highly permeable membranes, the present investigation has sought to determine which of several fixing techniques produces the most stable cells. The criteria used in evaluating stability are (1) cell volume and (2) membrane resistance. The constancy of cell volume under a variety of osmotic conditions over a period of 2 wk after fixation depends upon the action of the fixing agent, not only at the surface but very likely throughout the cell. The dielectric data, on the other hand, provide information specifically about the condition of the cell membrane.

The previous study, together with the work of Tooze (1964), clearly demonstrates the importance for stability of fixing solutions of pH somewhat below 7.0. Tooze also found that the stability of the fixed cell was enhanced when the fixing solution contained calcium. Whereas in our previous work the osmolarity of the fixing solutions was determined largely by sodium chloride, Tooze and others (Davies and Spencer, 1962; Caulfield, 1957) have used sucrose for this purpose.

Three fixing solutions, all having pH 6.5, are compared in this investigation. In these solutions,

the osmotic environment of the cells is established by (A) sucrose, (B) physiological NaCl solution, or (C) NaCl solution containing 0.01 M CaCl_2 . In this study, it is demonstrated that striking advantages accrue from the use of saline as opposed to sucrose. There are also indications that the addition of calcium to the fixing solution may improve the stability of the fixed cells.

METHODS AND MATERIALS

Cal^f red blood cells were collected, sodium citrate being used to prevent coagulation. After centrifugation of the blood, the plasma and buffy coat were removed. The cells were washed twice in 0.145 M phosphate-buffered saline (pH 7).

The three fixing solutions investigated contained final reagent concentrations as follows: A, 1% osmium tetroxide, 0.24 M sucrose, 0.015 M sodium barbital, 0.015 M sodium acetate. B, 1% osmium tetroxide, 0.11 M NaCl, 0.015 M sodium barbital, 0.015 M sodium acetate. C, 1% osmium tetroxide, 0.11 M NaCl, 0.015 M sodium barbital, 0.015 M sodium acetate, 0.01 M calcium chloride. In each case, cells occupied approximately 9% (by volume) of the fixing solution.

After the reagents were combined, the fixing solutions were adjusted to pH 6.5 with HCl and cooled in an ice bath. The cells were added, held in the bath for 10 min, then removed to the bench and allowed to warm for an additional 20 min before centrifugation. The total time during which the cells were exposed to osmium tetroxide was approximately 45 min. The cells were then washed twice so as to remove

¹ A preliminary study with sheep erythrocytes showed results which are qualitatively similar to those reported here for beef cells.

free OsO_4 . Washing solutions were identical with fixing solutions except that no OsO_4 was added.

The shape of the cells before and after fixation was observed by microscopic examination of unstained wet preparations (phase microscope) and stained smears.

For testing the stability of the cells to osmotic stress and with time after fixation, the following sequence of treatments was carried out for each preparation of fixed cells: 1. After fixation, on the day they were collected, the cells were washed once with distilled water. 2. The cells were washed three additional times with distilled water after overnight storage (2nd day) at 5°C . 3. The cells were then stored at 5°C for approximately 2 wk and given a final distilled water wash at that time.

The volume of fixed cells in suspension was determined from the space in the suspension which was impermeable to a large (mol wt 150,000) dextran molecule (Carstensen and Smearing, 1967). Two independent estimates of mean cell volume (MCV) were obtained. In one method, the dextran-impermeable volume of a suspension was divided by the total cell count. In the other method, the MCV was computed from the observed frequency distribution of cell volumes as measured by means of a Coulter Counter which had been calibrated with erythrocytes or polystyrene spheres of known volume. In the first method, cells were suspended in 0.01 M NaCl. All Coulter Counter measurements were made on cells suspended in isotonic phosphate-buffered saline (300 milliosmols/liter).² No significant differences were found among cell volumes determined by the two methods.

Frequency distribution histograms of cell volumes for fixed and normal cells are shown in Fig. 1. Although there is some shrinkage of cells as the result of fixing, the distributions for normal and fixed cells are similar. Mean cell volume measurements were carried out before distilled water washing, after the first and fourth washes, and after storage for 2 wk. Observed differences in MCV are discussed under Results.

Measurements of dielectric constant and conductivity of the fixed cells over the frequency range from 100 Hz to 2.10^8 Hz were performed after the first and fourth distilled water washes and after storage for 2 wk. After distilled water washing, the cells were re-

² It appears reasonably safe to assume that the conductivity of fresh red blood cells is low enough relative to buffered saline to be considered zero (Carstensen et al., 1968). This is not true for OsO_4 -fixed cells. Errors in Coulter volumes which arise from finite conductivity of the cells may be corrected as indicated in the Appendix. The magnitude of the correction is minimized, however, if the conductivity of the suspending medium is high.

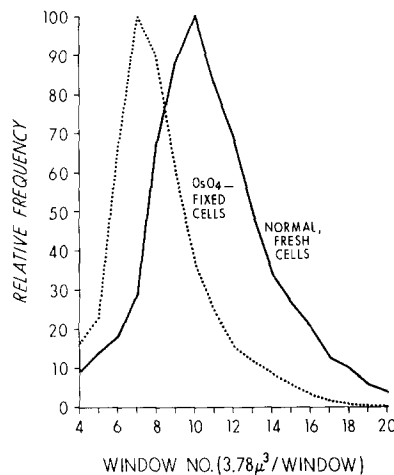


FIGURE 1 Relative cell volume distribution for normal and OsO_4 -fixed bovine erythrocytes.

suspended in 0.01 M NaCl for measurements. The techniques used in the dielectric studies have been described previously (Einolf and Carstensen, 1967; Carstensen and Smearing, 1967; Carstensen et al., 1968).

EXPERIMENTAL RESULTS

Measurements as described in the previous section were carried out for two completely independent preparations of OsO_4 -fixed cells with each of the fixing solutions.³

MCV

The mean cell volume of fresh, unfixed, calf erythrocytes in phosphate-buffered saline is $39.0 \mu^3$ with a standard error of $1.5 \mu^3$ (samples taken from six animals). The MCV of OsO_4 -fixed erythrocytes is $30.0 \mu^3$ with a standard error of $1.1 \mu^3$. There was no significant difference in cell size among the three methods of fixing. Although fixation resulted in an immediate decrease in MCV, there was no significant further change in cell volume throughout the 2-wk period of observation. A reduction in ionic strength of the environ-

³ Hemoglobin leakage from red blood cells fixed in sucrose is an order of magnitude greater than that from cells fixed in saline. Hemoglobin leakage studies which were carried out as a part of this investigation have been described in an internal report which can be made available to the interested reader, but are eliminated from the present paper so as to conserve space.

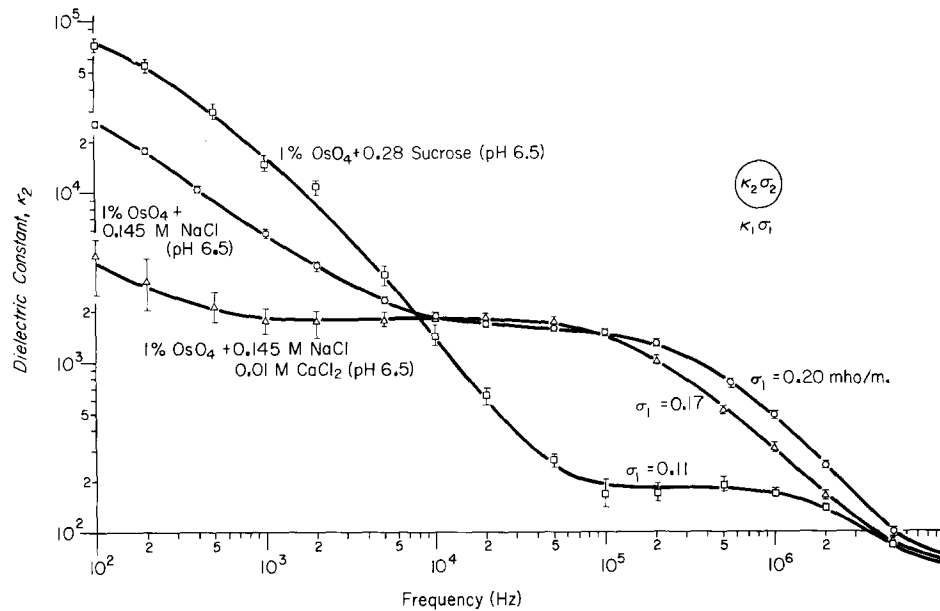


FIGURE 2 Effective homogeneous dielectric constants of OsO_4 -fixed bovine erythrocytes after one distilled water wash. Typical data are compared for each of three fixing methods: (A) sucrose, (B) saline, (C) saline plus calcium. Conductivity σ_1 of the environment of the cells during measurement is indicated in the figure. Temperature 25°C .

ment from 0.14 to 0.01 had no observable effect on the volume of the fixed cell. Microscopic observations of suspensions of OsO_4 -fixed cells showed that the cells are intact although their contour is somewhat angular in contrast to the round outline of unfixed cells.

Dielectric Data

Dielectric data show marked differences among the three fixing techniques. Fig. 2 gives typical values for the effective homogeneous dielectric constant of bovine red blood cells fixed with solutions A (sucrose), B (saline), or C (saline plus calcium) after one distilled water wash. Note in particular the plateau in the dielectric constant values near 10^5 Hz. Normal bovine erythrocytes have an effective, homogeneous dielectric constant of approximately 1500 at this frequency. As illustrated by Fig. 2, which compares cells soon after fixing, cells fixed in saline have dielectric constants almost equal to those of normal cells. In contrast, even at the time of the first observation, dielectric constants of cells fixed in sucrose were found to be lower than those of normal cells by nearly a factor of ten.

Although shortly after fixation the 10^5 Hz dielectric constants of cells fixed in saline solution were nearly the same as those of unfixed normal cells, continued washing and aging resulted in decreased dielectric constants (Fig. 3). As discussed previously (Carstensen and Smearing, 1967), the observed decrease in dielectric constants results from a decrease in the membrane resistance of the cells. Independent estimates of membrane resistance values have been calculated from the dielectric constant and conductivity data for all conditions of fixing, washing, and aging and are summarized in Fig. 4. The sensitivity of the dielectric data to changes in membrane resistance in the range above 10^{-4} ohm m^2 is poor. For this reason values of membrane resistance in excess of $2 \cdot 10^{-4}$ ohm m^2 may be considered effectively infinite, insofar as can be determined by these observations. As indicated in Fig. 4, the membrane resistance of cells fixed in a sucrose medium is very low from the time of the first observation. On the other hand, cells fixed in saline have very high membrane resistance values on the 1st day of observation and show marked decreases only after 2 wk, when the final measurements were made. There is a suggestion in these data that the addition of calcium to

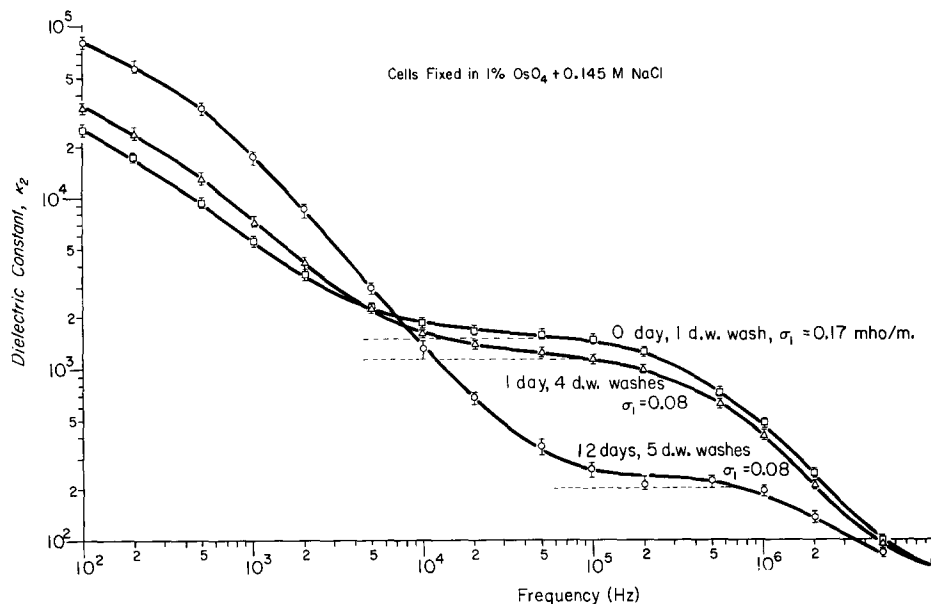


FIGURE 3 Changes in the effective homogeneous dielectric constant of OsO_4 -fixed bovine erythrocytes with aging. Cells fixed in Solution B are used to illustrate the phenomena. Conductivity σ_1 of the environment during measurement is indicated in the figure. Temperature 25°C . *d. w.*, distilled water.

the fixing medium has a stabilizing effect on the cell membrane.

For clarifying the calcium effect, additional measurements were made comparing the stability of cells which had (a) no calcium added to the fixing medium (Solution B); (b) 0.01 M CaCl_2 added to the fixing medium (Solution C); and (c) 0.03 M CaCl_2 added. Effective homogeneous conductivities of these cells were observed after the first and fourth distilled water wash and after 2 wk of storage. The results of one experiment after 2 wk of aging are presented in Table I. It is apparent that calcium in the fixing medium has very little influence on the internal conductivity, but significantly lowers the effective homogeneous conductivity of the cell as observed after distilled water washing and after aging.

Normal erythrocytes show no significant dispersion in their dielectric properties at frequencies below 10^5 Hz (Schwan, 1957). In contrast, the dielectric constant data of Figs. 3 and 4 show very high dielectric constants at low frequencies—in some cases approaching effective, homogeneous dielectric constants of the order of 10^5 . This phenomenon was observed and reported earlier (Carstensen *et al.*, 1968). In the present study, however,

it is possible to follow the transition from the case of no dispersion, as seen in the normal cell, to very large low-frequency dispersion as the cell membrane deteriorates.

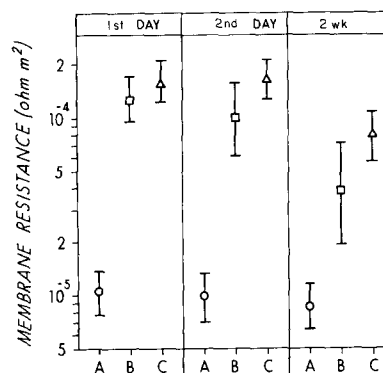


FIGURE 4 Membrane resistance values for OsO_4 -fixed erythrocytes. Measured values of the effective homogeneous dielectric constants and conductivities have been used to compute membrane resistances of cells fixed in solutions A (sucrose), B (saline), C (saline plus calcium). Measurements were made after the first distilled water wash on the day the cells were fixed, after the fourth wash (2nd day), and after approximately 2 wk (fifth wash).

TABLE I
Effect of Calcium in the Fixing Medium upon the
Stability of OsO₄-Fixed Bovine Erythrocytes

	Calcium in fixing medium		
	—	0.01	0.03 M
σ_t	0.010	0.010	0.010 mho/m.
σ_i	0.13	0.12	0.11 mho/m.
σ_2	0.042	0.032	0.022 mho/m.
R_m	$3 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	$7 \cdot 10^{-5}$ ohm m. ²

These cells had received five distilled water washes and were observed after 2 wk of storage at 5°C. The environmental conductivity σ_1 is the same for all cells. The internal conductivity σ_i is determined from measurements at $5 \cdot 10^7$ Hz where the effects of the membrane are negligible. Note that internal conductivity is greater by a factor of 10 than the environmental conductivity. The effective, homogeneous conductivity σ_2 is determined from measurements at 1500 Hz where the membrane effects are dominant. Membrane resistance R_m is calculated by using a value of 1.8μ for the radius of the cell.

DISCUSSION

The mean cell volume of OsO₄-fixed erythrocytes appears to be stable for all environments investigated. In each case, the pH of the fixing solutions was 6.5. Tooze (1964) found some swelling of amphibian erythrocytes and their nuclei after fixation at pH 7.4. Our own work with sheep erythrocytes showed evidence of swelling of cells that had been fixed at higher pH values. Although none of the cells fixed at pH 6.5 in this study showed signs of swelling, there appear to be great differences in the properties of the cell membranes depending upon the fixing medium and the subsequent treatment of the fixed cells.

These membrane effects are shown most directly by the dielectric data. In dielectric terms, the normal blood cell may be described as a highly conducting core (the cytoplasm) surrounded by a very thin insulating shell (the membrane). At low frequencies (of the order of 10^5 Hz and below) where the reactance of the membrane is much greater than the impedance of the interior, the effective dielectric properties of the cell are determined by the membrane. At 10^5 Hz, normal beef erythrocytes appear to be insulators and have effective dielectric constants of the order of 1500. At high frequencies (of the order of 10^8 Hz), the

reactance of the membrane is negligible and the apparent dielectric properties of the cell are just those of the cytoplasm. At 10^8 Hz, the conductivity of a normal bovine erythrocyte is approximately 0.7 reciprocal ohm/m. and the dielectric constant is about 60. The transition from domination of the effective dielectric constant of the cell by membrane at low frequencies to domination by cytoplasm at high frequencies has been called the β -relaxation by Schwan (1957), and occurs at around $4 \cdot 10^6$ Hz for normal erythrocytes. In contrast with the normal cell, the conductivity of the core of OsO₄-fixed erythrocytes in 0.01 M NaCl at pH 6.5 is of the order of 0.1 mho/m. Because of this low internal conductivity, β -relaxation for OsO₄-fixed cells occurs at frequencies of the order of $5 \cdot 10^5$ Hz. If the membrane resistance decreases to the point at which it is comparable to the reactance of the membrane at low frequencies, the apparent dielectric constant of the cell may decrease. A more quantitative discussion of this phenomenon has been presented earlier (Carstensen and Smearing, 1967).

Figs. 2 and 4 show the striking difference in stability between the membranes of cells fixed in saline and those of cells fixed in sucrose. From Table I, it can be seen that the addition of calcium to the fixing medium significantly increases the stability of the membrane. It might have been postulated (Tooze, 1964) that Ca⁺⁺ ions would bind to fixed hemoglobin with a consequent reduction in the net fixed charge in the interior of the cell. As indicated by the internal conductivities in Table I, this effect must be small. However, the effects of Ca⁺⁺ on the membrane stability are clear.

Although a detailed explanation of the low-frequency dispersion shown in Figs. 2 and 3 cannot be given at this time, the present study supports the postulate that the high dielectric constants observed at low frequencies for some OsO₄-fixed cells are associated with the fixed charge—counterion array in the interior of the cell. It can be seen in Fig. 3 that as the membrane resistance drops, the low-frequency dielectric constant increases. For example, the low frequency dispersion for cells with high membrane resistance (cells fixed in Solution C, Fig. 2) is extremely small. It appears that when the membrane impedance is high and the electric field inside the cell is very small, the fixed charge-counterion array in the interior of the cell cannot contribute to the dielec-

tric polarization. As the membrane opens up, however, the interior of the cell is subjected to the electric field with the resulting high, effective, dielectric constants shown in Figs. 2 and 3. One may conjecture that changes in the low frequency dielectric constants are a more sensitive, although indirect, indication of the conditions of the membrane than are the 10^5 Hz dielectric constant and conductivity data.

Although the membranes of cells fixed in saline are comparatively intact by the criteria used in this study, they probably begin to degenerate even during the fixing process and quickly become permeable to small solute molecules. For this reason, sucrose has little value as a stabilizing agent for erythrocytes during OsO_4 fixation. Sodium chloride may act by reducing the electrostatic repulsion of fixed charge sites, thus relieving some of the stress on the cells.

APPENDIX: CELL CONDUCTIVITY IN COULTER SIZING

If a spherical particle with a volume v and conductivity σ_2 is suspended in a large volume V of a medium with conductivity σ_1 , the apparent conductivity σ of the inhomogeneous medium which contains the particle can be written (Maxwell, 1881)

$$\sigma = \sigma_1 \frac{\sigma_2 + 2\sigma_1 + 2 \frac{v}{V} (\sigma_2 - \sigma_1)}{\sigma_1 + 2\sigma_1 - \frac{v}{V} (\sigma_2 - \sigma_1)} \quad (1)$$

or

$$v = \frac{2V(1 - \sigma/\sigma_1)}{2 + \sigma/\sigma_1} \left[\frac{1}{2} \frac{\sigma_2/\sigma_1 + 2}{1 - \sigma_2/\sigma_1} \right] \quad (2)$$

If the conductivity of the particle is zero as is normally assumed in Coulter sizing, the bracketed term goes to unity. If the Coulter Counter is used to size conducting particles, the indicated volume should be multiplied by the correction factor

$$\frac{1}{2} \frac{\sigma_2/\sigma_1 + 2}{1 - \sigma_2/\sigma_1}$$

As an example, when $\sigma_2/\sigma_1 = 0.1$, the correction factor is 1.17.

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