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STABILITY OF CELLS FIXED WITH GLUTARALDEHYDE AND ACROLEIN

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Dielectric techniques have been shown to be useful in studies of the effects of osmium-fixing procedures on the stability of erythrocytes (Carstensen and Smearing, 1967; Carstensen et al., 1969). In this report a similar approach has been used to investigate calf erythrocytes fixed with glutaraldehyde and acrolein. Both agents can produce stable cells and preserve as an insulating barrier all but a fraction of a per cent of the membrane.

METHODS AND MATERIALS

Calf blood collected with sodium citrate as an anticoagulant was washed twice in 0.145 M saline to remove plasma and the buffy coat.

Glutaraldehyde Fixation

Two forms[1] of glutaraldehyde were used in these studies, the first, a highly purified monomeric form, and the second, a condensed form:

(*a*) TAAB[2] glutaraldehyde was purified by shaking with charcoal and distillation (Fahimi and Drochmans, 1965). As received, this material always has considerable spectrophotometric absorption at 235 nm, which was completely removed by this purification procedure. Immediately before use, the stock solution (24.4%) was filtered through a Diaflo UM-05 membrane (Amicon Corp., Lexington, Mass.) at 30 psi and then diluted to obtain the final fixing solution containing 2% glutaraldehyde, in 0.14 M NaCl, 0.005 M sodium acetate, and 0.005 M $CaCl_2$ (pH 7).

(*b*) Polysciences[3] EM grade glutaraldehyde as supplied (8% aqueous solution, pH 7) had a large peak in the absorption spectrum at 235 nm. From ultracentrifugation it appears that more than 90% of this glutaraldehyde is condensed to dimer or higher species. A 2% fixing solution was made by diluting this reagent directly with isotonic (1/15 M) phosphate buffer (pH 7).

For fixing, one part of packed cells (hematocrit ~0.80) was mixed with 10 parts of fixing solution and held for 10 min. The cells were then centrifuged and washed two times in 0.145 M NaCl to remove excess glutaraldehyde.

Acrolein Fixation

Acrolein was purchased from the Shell Oil Co., New York. As supplied, the raw acrolein contained approximately 0.1% hydroquinone as a stabilizer, and was partially polymerized. Before use in these experiments, the acrolein was distilled at 55°C at ambient pressure in a rotary evaporator using a constant flow of dried nitrogen or helium gas. The distillate was collected in a flask, cooled by an acetone–dry ice mixture (Aldridge and Wickens, 1971). Two acrolein preparations were used in these studies; one was a 2% solution without stabilizer, and the other was a 10% solution with stabilizer: (*a*) The unstabilized acrolein in these preparations was stored (no longer than 3 weeks) at −37°C. The final fixing solutions, containing 2% acrolein in 0.14 M NaCl, 0.005 M sodium acetate, and 0.0005 M $CaCl_2$ (pH 7), were prepared immediately before use. One part of packed red cells was mixed with 10 parts of the fixative and held at 0°C for 10 min., at which time they were

[1] In addition, a single check was made using a technical grade (Baker) glutaraldehyde. Immediately after fixing, cells from this preparation had a low frequency effective dielectric constant of between 200 and 300. Thus, even though the cells were superficially stable and appeared normal in the light microscope, from a dielectric point of view their membranes had almost vanished.

[2] TAAB Laboratories, Emmer Green, Reading, England.

[3] Polysciences, Inc., Rydal, Pa.

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centrifuged and washed two times in 0.145 M NaCl to remove excess fixative. (*b*) For the second preparation, the distilled acrolein, with 0.1% hydroquinone as stabilizer, was stored at 4°C. The fixing solutions, containing 10% acrolein with stabilizer in 0.145 M NaCl plus 0.01 M sodium acetate adjusted to pH 7, were prepared immediately before use. One part of cells was mixed with four parts of fixing solution and held at 0°C for 1 hr with occasional stirring. The cells were centrifuged and washed in acetate-saline two times to remove excess acrolein.

Immediately after washing to remove excess fixing agent, the cells were suspended in 0.145 M NaCl and the effective dielectric constant and conductivity were measured over a frequency range from 1 to 200 MHz using a Hewlett-Packard Model 250A (Berkeley Heights, N.J.) RX Meter. The cells were subsequently "stressed" by two washes in distilled water, and on the following day were again measured in 0.145 M NaCl. All measuring and analytical techniques were carried out as described earlier (Carstensen and Smearing, 1967; Carstensen et al., 1969).

RESULTS

Fixing with either glutaraldehyde or acrolein results in a 5–10% reduction in cell volume. This may be compared to a shrinkage of nearly 25% with osmium fixation (Carstensen et al., 1969). After fixing, the cells are stable in size, e.g. a decrease in ionic strength of the environment by a factor of 10 caused no observable change in volume of the fixed cells.

A summary of the dielectric data is presented in Table I. Some preparations have a fairly wide range of values. This simply means that the cells were observed at various stages of membrane breakdown. The method for calculating membrane resistance has been described previously (Carstensen and Smearing, 1967). In addition to the data in Table I, it was assumed that the internal dielectric constant for the cells is 100. This assumption has very little influence on the results of the calculations. The membrane capacitance was taken to be 0.01 F/m^2 (Fricke, 1953). In the case of cells freshly fixed in 2% acrolein, the effective, homogeneous dielectric constants are so high that all we can say with assurance is that the membrane resistance is very large. In all other cases the low frequency dielectric constant is significantly smaller than would be expected for an insulating membrane even at the time of the first measurement. After washing in distilled water the low frequency dielectric constant decreases, corresponding in each case to a drop in membrane resistance by roughly a factor of two.

The effective conductivity of the cell at 50 MHz is approximately equal to its internal conductivity (Carstensen and Smearing, 1967). When the environmental conductivity is low, the internal conductivity is determined largely by counterions of fixed charges in the cell. The 50 MHz conductivities for glutaraldehyde- and acrolein-fixed cells are presented in Table II. These values can be compared to internal conductivities ranging from 0.07 to 0.3 mho/m for cells fixed in osmium tetroxide solutions at low and high pH, respectively (Carstensen and Smearing, 1967). From the data in Table II alone, it is not clear whether the very low values of internal conductivity for acrolein-fixed cells implies that the total or simply the net charge on the hemoglobin is very small, i.e. whether acrolein fixation binds most of the charge groups or just that the isoelectric point for the fixed hemoglobin is near pH 7. Tooze (1964) has shown that osmium tetroxide fixation lowers the isoelectric point of hemoglobin. To differentiate between these two possibilities, cells fixed in a 2% acrolein solution were measured at pH 5 and pH 9 and found to have internal conductivities of 0.07 and 0.12 mho/m, respectively. Since the conductivity is much greater on either side of pH 7, it appears that the values in Table II reflect net charge rather than total charge.

DISCUSSION

Membrane resistance is a very sensitive measure of changes in the condition of the membrane. If one assumes that the resistance is controlled by small holes which have the same conductivity as the environment, a membrane resistance of 10^{-5} ohm m^2 requires on the order of 0.1% of the membrane to be holes. Thus, even though membrane resistances of fixed cells are generally much lower than those of normal cells, all but a fraction of a per cent of the fixed membrane still acts as an insulating barrier. If we use membrane resistance as a criterion, the data in Table I indicate that the condensed form of glutaraldehyde is more effective in maintaining the insulating properties of the membrane than the highly purified monomer. This supports the observations of Robertson and Schultz (1970). Of all the fixing agents studied, one of the most effective is purified acrolein at low concentrations ($\sim$2%). It is interesting in this connection to mention observations which have been made recently on lipid loss during acrolein fixation of dog erythrocytes (Aldridge, unpublished data). At acrolein concentrations of up to 3%,

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Table I

Membrane Resistance Values for Calf Erythrocytes Fixed with Glutaraldehyde and Acrolein

Low frequency limits for the effective, homogeneous dielectric constants of the cells were calculated from measurements of suspensions of cells in 0.145 M NaCl immediately after fixing and again in saline after having been stressed by two washes with distilled water and overnight storage at 4°C (in distilled H_2O). Measurements were made at 29°C, pH 7. Mean radius of the fixed cells is 1.9 μ. Internal conductivity of the cells in 0.145 M NaCl is 0.40 mho/m.

Fixative	Concn	Fixing temp.	No. of experi-ments	Relative dielectric constant Mean ± SE	Membrane resistance Mean ± SE
	%	°C			*ohm* $m^2 \times 10^6$
Glutaraldehyde (purified TAAB)					
Freshly fixed	2	0	2	800 ± 50	8 ± 1
Stressed	2	0	2	420 ± 80	4 ± 1
Freshly fixed	2	25	2	660 ± 50	6 ± 1
Stressed	2	25	2	300 ± 50	3 ± 1
Glutaraldehyde (Polyscience)					
Freshly fixed	2	25	4	1200 ± 250	21 ± 10
Stressed	2	25	4	800 ± 150	10 ± 2
Acrolein					
(*a*) Unstabilized					
Freshly fixed	2	0	3	1600 ± 100	60 ± 20
Stressed	2	0	3	1300 ± 100	27 ± 7
(*b*) Stabilized					
Freshly fixed	10	0	8	800 ± 100	9 ± 1
Stressed	10	0	5	450 ± 50	5 ± 1

Table II

Internal Conductivity of Calf Erythrocytes Fixed with Glutaldehyde and Acrolein

The 50 MHz effective, homogeneous conductivities of the cells are listed in the columns labeled "Internal conductivity." Measurements were made at 29°C, pH 7. Since with 2% acrolein environmental conductivity is equal to cell conductivity, the internal conductivity includes the contribution of environmental ions. Thus, the contribution from counterions of fixed charge sites is <0.03 mho/m.

Fixative	Concn	Fixing temp.	No. of experi-ments	Environmental conductivity	Internal conductivity Mean ± SE
	%	°C		*mho/m*	*mho/m*
Glutaraldehyde (TAAB purified)	2	0	2	0.01	0.05 ± 0.01
	2	25	2	0.02	0.06 ± 0.005
Glutaraldehyde (Polyscience)	2	25	4	0.02	0.12 ± 0.02
Acrolein					
Unstabilized	2	0	3	0.03	0.03 ± 0.005
Stabilized	10	0	8	0.02	0.05 ± 0.01

there is no detectable (i.e. <5%) loss in cell phospholipids. At 10% acrolein concentrations, 18% of the phospholipid is extracted during fixing. With hydroquinone stabilizer present the phospholipid loss increases to 28%. This supports the view that membrane lipids are responsible for their insulating properties. Acrolein appears to react readily with the membrane phospholipid. The resulting, new, active site on the phospholipid may then undergo a reaction with a nearby site of structural protein and achieve cross-linking. At higher acrolein concentrations the chances improve that the active site will react with a second molecule of acrolein rather than structural protein

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forming derivatives that are soluble and extractable (Feustel and Geyer, 1966; Schmid and Takahashi, 1968; Jones, 1968).

The size stability of fixed cells depends on the strength of the bonds achieved by the fixing agent and on the stresses imposed upon the cell. Dielectric data give information on both osmotic and electrostatic stresses. Normal erythrocytes are good osmometers. From the membrane resistances in Table I, it appears likely that the membranes of glutaraldehyde- and acrolein-fixed cells cease to be osmotic barriers immediately after fixing. Cells with membrane resistances less than 10^{-4} ohm m^2 are readily permeable to sucrose and raffinose (Carstensen and Smearing, 1967). Electrostatic repulsion of charges on the macromolecules of the cell would tend to cause swelling of the cells at low ionic strengths, exerting what we refer to as "electrostatic stress." This phenomenon is observed in ion exchange resins, in erythrocytes fixed in osmium at high pH (Carstensen and Smearing, 1967), and in bacterial cell walls (Carstensen and Marquis, 1968; Marquis, 1968). When cells are suspended in low ionic strength media their internal conductivities are determined primarily by the counterions of fixed charges on the macromolecular solutes in the cytoplasm (Carstensen and Smearing, 1967). As seen from Table II, acrolein-fixed cells have the lowest fixed charge concentration of any of the preparations studied thus far.

SUMMARY

From dielectric observations it appears that both glutaraldehyde and acrolein can produce stable cells with a large portion of the membrane intact. These fixed cells are not subject to osmotic stress. Cells fixed in unstabilized acrolein at low concentrations (2%) have highly stable membranes and are subject to minimal electrostatic stress. These are the most favorable conditions for the preservation of morphology.

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