

QUANTITATION OF HUMAN RED BLOOD CELL FIXATION BY GLUTARALDEHYDE

FRANCOIS M. M. MOREL, RICHARD F. BAKER, and
HAROLD WAYLAND

With the technical assistance of PAUL V. KNUST-GRAICHEN

From the Division of Engineering and Applied Science, California Institute of Technology, Pasadena, California 91109, and the Department of Microbiology, School of Medicine, University of Southern California, Los Angeles, California 90033

ABSTRACT

The uptake of glutaraldehyde by human red blood cells has been measured as a function of time by a freezing point osmometer. The rate of attachment of glutaraldehyde to the cell proteins is high over the first hour, declining to zero over a period of a few days. The number of glutaraldehyde molecules cross-linking with each hemoglobin molecule is of the order of 200, in reasonable agreement with the calculated number of attachment sites. The cell membrane is immediately highly permeable to glutaraldehyde. Selective permeability to ions is lost during fixation. Ionic equilibrium is obtained only after a few hours. An optimum fixation technique for shape preservation is suggested.

INTRODUCTION

Glutaraldehyde has been widely used as a fixative in electron microscopy since the demonstration by Sabatini et al. (14) that many enzymes retain activity (specificity) after glutaraldehyde treatment. Other advantages of glutaraldehyde include rapid cellular penetration and relative nontoxicity. Glutaraldehyde has also found use as a cross-linking agent in studies on crystalline enzymes (9, 10), and is being used by protein crystallographers for structure determinations (11).

This report uses the red blood cell (RBC) as a model object to study permeability changes to fixative and salts which occur during fixation. These results are used in an attempt to derive optimal conditions for preserving cell shape, in the hope that they will be applicable to other materials.

MATERIALS AND METHODS

Glutaraldehyde

Commercial glutaraldehyde was obtained from Polysciences, Inc., Rydal, Penn. This electron microscope grade glutaraldehyde is an aqueous unbuffered solution of 8% nominal concentration 10-cc volumes are packaged in glass vials under an atmosphere of nitrogen. Upon receipt, the vials were stored at 4°C. Although the manufacturer stresses the purity and the monomeric nature of the product, the first 10 ampoules were markedly and uniformly polymerized as shown by an absorption peak at 235 m μ in the UV spectra (1, 3, 8). They will be referred to as glutaraldehyde sample No. 1. The second box of 10 ampoules (graciously offered in replacement of the first one) had a much lesser degree of polymerization and will be referred to as glutaraldehyde sample No. 2.

Preparation of Fixative Solutions

The fixative solutions were prepared as mixtures of glutaraldehyde solutions and buffered saline. Typically a stock solution was made up by adding 10 cc of aqueous glutaraldehyde solution to 10 cc of 600 meq l^{-1} buffered saline (=13.40g NaCl + 1.35g KH_2PO_4 + 15.20g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of water). Other solutions were then obtained by diluting the stock solution with a 300 meq l^{-1} buffered saline (=4.40g NaCl + 1.35g KH_2PO_4 + 15.2g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of water). Such preparations all have a final electrolyte concentration of 300 meq l^{-1} , a pH of 7.4, and, of course, a variable concentration of glutaraldehyde.

Red Blood Cells (RBC)

NORMAL PACKED RBC: Blood from healthy donors was obtained from the blood bank in standard 500 cc bags with 75 cc of acid citrate dextrose (ACD) as anticoagulant. The cells were spun down at 3000g for 10 min. The plasma, the buffy coat, and, as a precaution, the top layer of cells were then removed. The remaining packed cells, used without any further washing, are called normal packed RBC. Except in some control experiments, the cells were used within 3 days after withdrawal from the donor, and for those experiments involving critical examination of the shape (Figs. 5 and 6) they were always used within 24 hr.

HEMOLYZED RBC: To obtain solutions of hemoglobin and cell membranes at the same concentration as found in packed RBC, normal packed RBC were frozen at $-80^\circ C$ by using a mixture of dry ice and ethanol, followed by thawing at room temperature. Phase-contrast microscopy demonstrated a high degree of hemolysis. The resulting solution will be referred to as hemolyzed RBC.

WASHED RBC: One control experiment was performed with cells that had been washed four times with isotonic buffered saline, with the use of approximately 4 volumes of saline per volume of red cells (packed as above).

UV Spectrophotometry

Ultraviolet absorption was recorded at $24^\circ C$ with a Beckman Ratio Recording Spectrophotometer Model DKA (Beckman Instruments, Inc., Fullerton, Calif.) with matched 1 cm quartz cuvettes (Carl Zeiss, Inc., New York). The reference solutions were distilled water or phosphate-buffered saline solutions corresponding to those of the glutaraldehyde solutions. The samples and the references were diluted 10 times with distilled water.

Scanning Electron Microscopy

Fixed and water-washed red blood cells were prepared for scanning electron microscopy by air drying on an aluminum specimen mount, followed by the evaporation in vacuum of a conducting coating. A layer of carbon was first uniformly evaporated over the cell surface. Gold was then evaporated over the carbon. The carbon formed a thin, continuous conducting film over the cell surface, while the gold yielded a high secondary emission ratio. A Cambridge scanning microscope was used at 20 kv. Images were photographed on Polaroid 55-PN film.

Ion Measurements

A Na-sensitive electrode (Corning No. 476210) and a K-sensitive electrode (Orion No. 92-19) were used to measure the activities of sodium and potassium, respectively. The electrodes, together with a single junction reference electrode (Orion No. 20-01), were coupled to a digital pH/mv meter (Orion model 801) through a switching box. With adequate control of temperature and good grounding, precision better than 0.2 mv was obtained. The pH and the selectivity constants were such that no correction for interference was necessary. Under such conditions, relative activities of the ions are readily obtained by using the Nernst equation, and the accuracy achieved is of the order of 1%.¹

Osmometry

The osmotic measurements were made with a freezing point depression osmometer.² Both 2 cc and 0.2 cc samples were used without any appreciable influence on precision or on absolute values. Commercial standards of 100 and 500 milliosmols (respectively, $-0.1858^\circ C$ and $-0.9291^\circ C$) were systematically used for calibration before each series of measurements. The instrument is directly graduated in milliosmols. Although better precision can be achieved with special care, most measurements were reproducible to about ± 2 milliosmols ($\pm 0.0037^\circ C$).

Tests showed that the presence of suspended RBC had no effects on osmotic readings. Therefore the red cells were left in the samples for osmotic measurements made during fixation.

In a typical experiment 1 ml of normal packed RBC was mixed rapidly in a test tube with 9 ml of fixative solution. The freezing point depression of the mixture was then recorded as a function of time by removing 0.2 cc samples for measurement at pre-

¹ For theoretical and practical considerations on ion-sensitive electrodes, see *Ion Selective Electrodes*. R. A. Durst, editor. N.B.S. Special Publication 314, Nv. 1969.

² Osmette 2007 precision systems.

determined time intervals. The zero time was taken as the time of initial mixing, and the time of the measurement was recorded when pressing the seeding button. To avoid any sedimentation of the cells, the tubes were closed with neoprene stoppers and kept in constant rotation. The experiments were carried out at a constant temperature of 24°C ($\pm 1^\circ\text{C}$). A sample of fixative solution was kept under similar conditions as the cell preparations and was measured as a reference.

RESULTS

Characterization and Calibration of the Solutions

The absorption spectra of glutaraldehyde samples No. 1 and No. 2 are shown in Fig. 1 and demonstrate peaks at 235 $m\mu$ and 280 $m\mu$. In each sample all the vials gave reasonably similar results. It is seen that sample No. 1 absorbs more than sample No. 2 at 235 $m\mu$, whereas the opposite occurs at 280 $m\mu$. The spectra of the fixative solutions in which the buffered saline was added were also recorded and are similar to those shown in Fig. 1, the only difference being a slight upward shift of the 235 $m\mu$ peak.

Fig. 2 shows the result of a calibration curve for osmotic measurements of glutaraldehyde solutions. 10 different solutions were obtained by dilution of a common glutaraldehyde solution with distilled water. These solutions were then mixed with equal volumes of: (a) water for the lower curve; (b) 600 meq l^{-1} buffered saline for the upper one, and then measured. As can be seen, both series of measurements lie on straight lines, but the lines are not parallel. Since the pure aqueous solutions of glutaraldehyde can be considered as nearly ideal, the corresponding freezing point depressions were taken as a direct measure of their

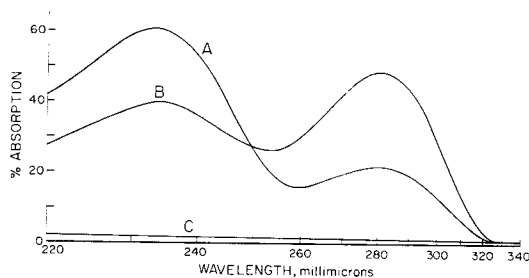


FIGURE 1 UV absorption spectra of glutaraldehyde samples. *A*, glutaraldehyde sample No. 1; *B*, glutaraldehyde sample No. 2; *C*, reference solutions.

concentrations: concentration in moles $l^{-1} = 0.5382 \times$ freezing point depressions in $^\circ\text{C}$. To obtain glutaraldehyde concentrations from osmolarity measurements of solutions containing the salts, one has to make a systematic correction given by the calibration curve.

Osmolarity as a Function of Time for Mixtures of Cells and Glutaraldehyde

NORMAL PACKED RBC: Fig. 3 shows the results of three typical experiments in which various dilution factors and glutaraldehyde concentrations were used. An initial rapid drop in osmolarity over the first 30 min was followed by a slower rate of decrease in osmolarity over a period of 5–8 days. The influence of various parameters can be summarized as follows:

(a) Mixing did not seem to be a critical factor although it is clear that a good definition of the reference time depends on mixing. Similar curves were obtained whether continuous agitation was used or the tubes were shaken just before measuring.

(b) Experiments carried out with 10 day old blood gave identical results.

(c) Glutaraldehyde samples No. 1 and No. 2 gave results that were qualitatively similar but, as shall be seen, quantitatively different.

(d) The concentration of glutaraldehyde was an important factor; the most reproducible results were obtained with concentrations of the fixative solutions of 1.5% and over. Smaller concentrations gave results which were less reproducible.

(e) The dilution factor $f = \text{volume of fixative} / \text{volume of RBC} = V_f/V_c$, was also an important parameter. The most consistent results were obtained with f values greater than 5. Values between 8 and 20 were usually used.

HEMOLYZED RBC: Parallel experiments were carried out with the same fixative, the same dilution factor, and the same volume of RBC, but in one case the RBC were normal packed cells and in the other they were hemolyzed cells. The results of such parallel experiments are shown on the middle curve of Fig. 3 and can be said to be identical within experimental precision. It is worth noting that fixation of hemolyzed cells leads to the formation of a gel. For dilution factors up to 10, the entire mixture of cells and fixative solution is involved in the formation of this gel; gelation appears to be complete after about 10 min.

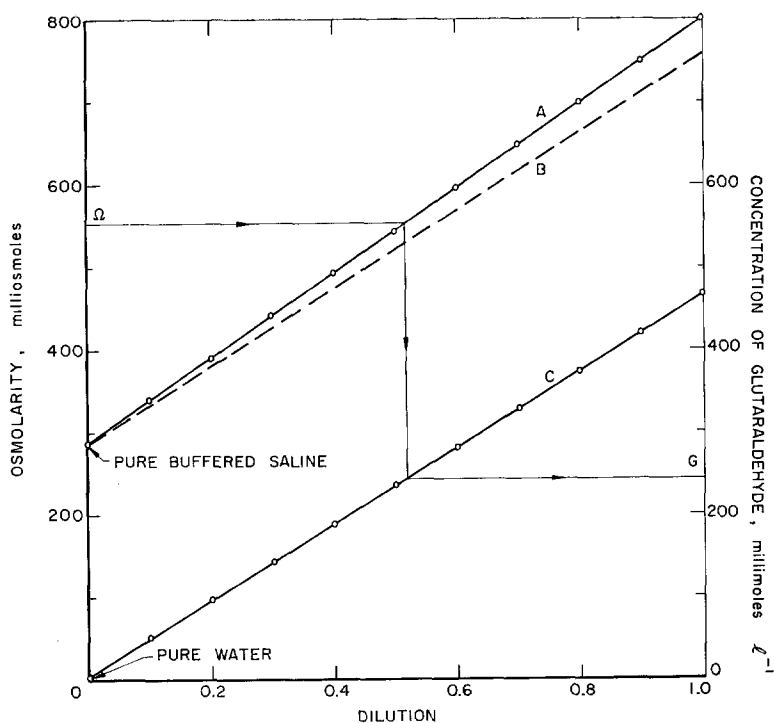


FIGURE 2 Osmometry of glutaraldehyde solutions. 10 different solutions were obtained by dilution of a common glutaraldehyde solution with distilled water. *A*, osmolarities of the 10 solutions mixed 1:1 with 600 meq l^{-1} of buffered saline; *C*, osmolarities of the 10 solutions mixed 1:1 with distilled water; *B* has been drawn parallel to *C* in order to display the difference in slope between *A* and *C*. Arrows indicate the steps involved in deriving the glutaraldehyde concentrations (*G*) from the osmotic measurements of mixtures of glutaraldehyde and buffered saline (Ω).

WASHED RBC: Washing the cells did not qualitatively change the results. However, the recorded osmolarities were systematically lower (by 10–20 milliosmoles) than the corresponding values for unwashed cells. The results of the experiments at low glutaraldehyde concentrations (< 100 millimoles l^{-1}) were also more consistent when the washed RBC were used instead of the normal packed RBC. This can be seen in Fig. 4. (Fig. 4 is explained in the Discussion.)

Scanning Electron Microscopy

Fig. 5 illustrates the osmotic effect on the cells of a high percentage glutaraldehyde concentration in the fixative. The flat aspect of the cells is indicative of water loss. In contrast, the normal shapes of RBC in Fig. 6 have been preserved by minimizing the osmotic imbalance due to glutaraldehyde during fixation. This has been achieved by adding a 1% glutaraldehyde fixative solution,

over a period of 15 min, to RBC's in isotonic buffered saline.

Ion Measurements

28 cc of packed RBC were suspended in 47 cc of isotonic buffered saline and then fixed by addition of 30 cc of a 6% glutaraldehyde fixative solution (total volume = 105 cc). The temperature was maintained constant at 37.5°C ($\pm 0.1^\circ\text{C}$) and the activity of potassium and sodium ions continuously recorded. Table I gives results at some of the observed times, showing that leakage of ions took place during fixation and that an equilibrium was attained after some 2 hr.

Permeability of the Fixed RBC

Experiments were carried out on fixed cells (more than 5 days of fixation) to test the permeability of the membrane to solutes (see Table II).

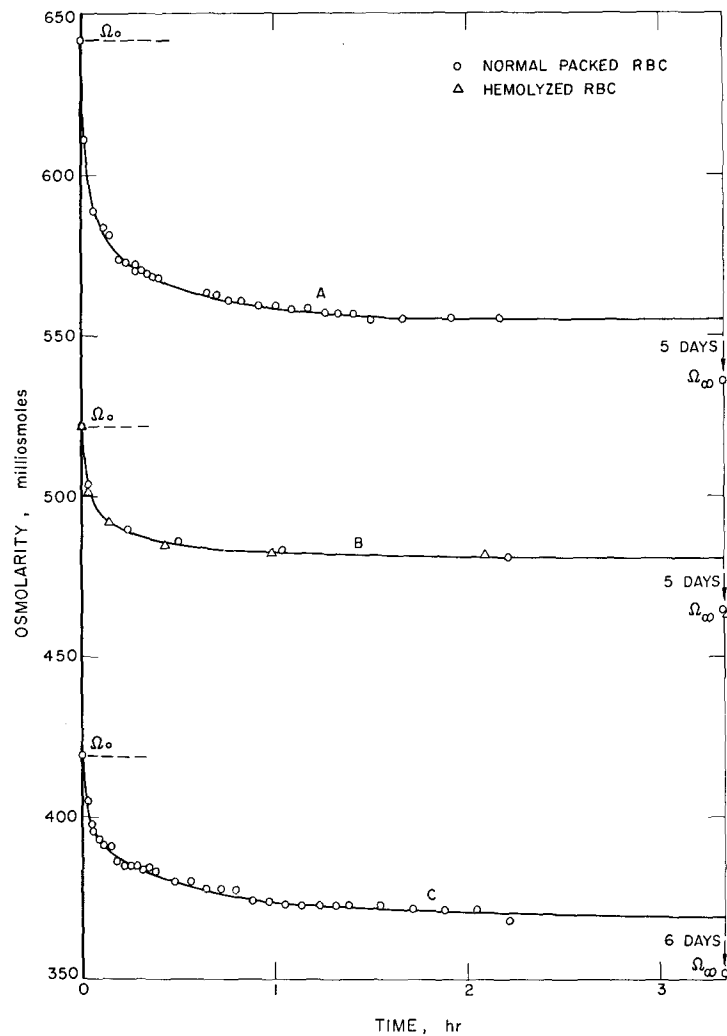


FIGURE 3 Osmolarity as a function of time for mixtures of cells and glutaraldehyde. *A*, 1 cc of normal packed RBC mixed with 9 cc of fixative solution (3.4% glutaraldehyde); *B*, 1 cc of normal packed and hemolyzed RBC mixed with 19 cc of fixative solution (2.2% glutaraldehyde); *C*, identical to *A* but 1.2% glutaraldehyde. Ω_0 are the osmolarities of the fixative solutions; Ω_∞ are the osmolarities of the mixtures after 5-6 days.

Fixed RBC that had been suspended in media of various compositions and osmolarities were mixed with solutions of various compositions and osmolarities. Assuming free exchange of solutes between the inside and outside of the cells and taking the fluid volume of the cell to be 70% and the packing efficiency of the hardened cells to be 60% (2), one can compute the expected osmolarities of the mixtures (Table II, footnote *). These computed values and the experimental osmolarities

are shown in Table II and are seen to be within a few per cent.

DISCUSSION

In comparing the results of UV absorption of the glutaraldehyde samples with similar curves published in the literature (1, 3, 8), it appears that both samples either are partially polymerized or contain some impurities as demonstrated by the existence of the 235 μ peak. However, in contrast

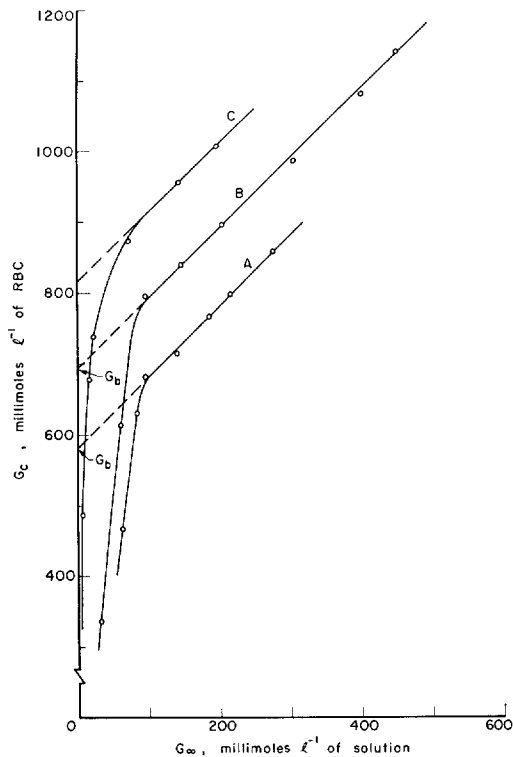


FIGURE 4 Equilibrium curves G_c versus G_∞ . These curves are obtained by plotting the number of moles of glutaraldehyde G_c used up per liter of RBC versus the final glutaraldehyde concentration G_∞ in the solutions. *A*, normal packed RBC with glutaraldehyde sample No. 1; *B*, normal packed RBC with glutaraldehyde sample No. 2; *C*, washed RBC with glutaraldehyde sample No. 2. G_b is a measure of glutaraldehyde bound to red cell proteins.

to the pH of untreated commercial solutions which is usually about 2 or 3 (1, 3), the pH of both samples was neutral without added buffer. Therefore, the 235 $m\mu$ absorption peaks observed with our samples are probably due to polymerization of the glutaraldehyde solutions after their purification and not to impurities, in agreement with Robertson and Schultz (13). In other words, both samples can be considered as fairly pure mixtures of monoglutaraldehyde and various oligomers, sample No. 1 having a greater degree of polymerization than sample No. 2.

The two straight lines obtained for the calibration curves by plotting concentration against osmolarity are not parallel; the osmolarity of the solution containing both glutaraldehyde and buffered saline is more than the sum of the

osmolarity of the individual solutions. This is theoretically predicted by cross effects between the salts and the organic solution because of their different polar characteristics with respect to water (reference 7, p. 531). The buffered saline "salts out" the glutaraldehyde. It decreases its solubility, i.e., increases its chemical activity. Reciprocally, glutaraldehyde salts out salt. This cross effect between the solutes can also be described with an osmotic coefficient different from 1.

Such an increase in the activity of glutaraldehyde can be expected to increase its polymerization. This is probably the reason for the observed upward shift of the 235 $m\mu$ absorption peak. However, this effect is certainly small compared to the direct effect on the freezing point depression and does not appreciably change the osmotic values observed.

The information given by the curves of Fig. 3, osmolarity versus time, can be divided into three parts related to (a) the first osmotic drop; (b) the overall shape of the curves; (c) the final osmotic values reached after a few days.

(a) The results obtained with normal packed RBC suggest that the initial drop in osmolarity is inversely proportional to the dilution factor. This initial drop could be due to a rapid entrance of glutaraldehyde into the cell and its immediate partition between the fluid inside the RBC and the fixative solution. Confirmation of this hypothesis is given by the results of the parallel fixation of normal packed cells and hemolyzed cells. Whether the hemoglobin is enclosed in the membrane or freely available for reaction with the fixative makes no difference in the osmotic drop. The dilution of the fixative solution by the cell volume as well as the rate of the reaction are the same in both cases; thus glutaraldehyde enters the cells readily. This explains why glutaraldehyde shows little or no osmotic activity with respect to the RBC and, thus, why it is necessary to use an isotonic concentration of electrolytes in the fixative solution. Any other concentration creates an osmotic imbalance that changes the volume of the cells while they are being fixed. It is important to note that the diffusion of glutaraldehyde across the membrane, although fast, is not so fast as water diffusion and, in using very high concentrations of glutaraldehyde, one can demonstrate in the final fixed shape of the RBC the transient osmotic imbalance. This is illustrated in Fig. 5 where one

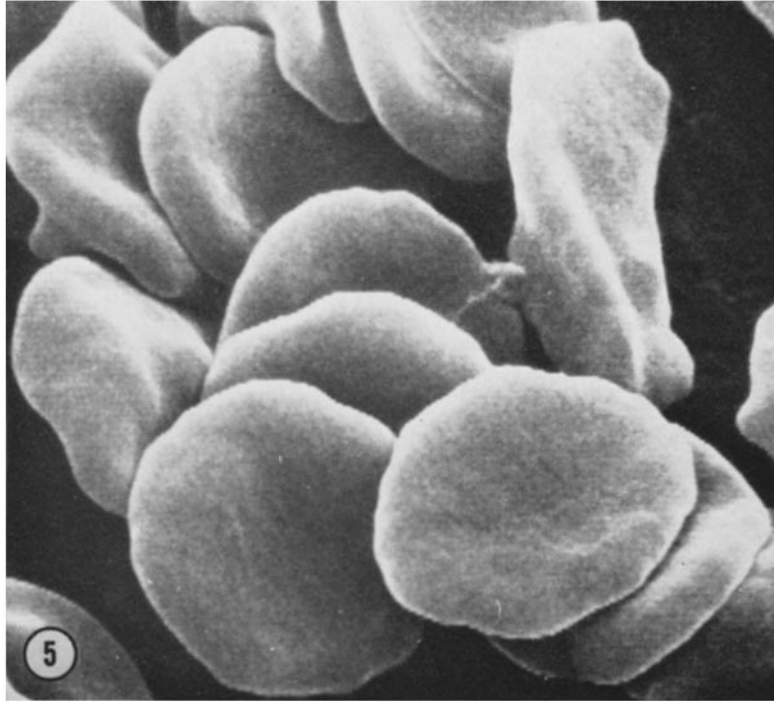


FIGURE 5 Scanning electron micrograph of RBC fixed for 2 hr by mixing the cells with a solution of high glutaraldehyde concentration (8%) and isotonic buffered saline.

can see the dehydrated aspect of the cells despite the salt's isotonicity in the fixative solution.

(b) The kinetics of the reaction of glutaraldehyde with the RBC do not appear to be simple. However, curves like those of Fig. 3 can be quite precisely fitted up to a few hours by equations of the type: $1/(\Omega - \Omega_1) = k \cdot t + \text{constant}$, where Ω is the osmolarity of the solution at time t and Ω_1 is a reference value corresponding to the osmolarity of the solution after 5–10 hr of fixation. Since Ω is a linear function of the glutaraldehyde concentration, it seems that such a fit implies a pseudo-second-order type of reaction. The later part of the reaction, from a few hours to a few days, cannot be fitted in the same way and might correspond to a different mechanism. It could be polymerization of the glutaraldehyde, although the control fixative solution did not show such a decrease in osmolarity.

(c) An interesting equilibrium curve is obtained by plotting the number of moles of glutaraldehyde G_c used up per liter of RBC versus the final glutaraldehyde concentration G_∞ in the solution. The osmolarity Ω_0 of the fixative solution before mixing

with the cells allows the glutaraldehyde concentration G_0 of the fixative to be computed by using the calibration curves (Fig. 2). In the same way, the osmolarity Ω_∞ of the suspending solution after 5–8 days gives G_∞ . Then: $G_c = \text{number of moles of glutaraldehyde used up per liter of cell} = (G_0 - G_\infty)V_f/V_c$. ($V_f = \text{volume of fixative}$, $V_c = \text{volume of RBC}$.)

Fig. 4 shows the equilibrium curves G_c versus G_∞ , obtained with normal packed RBC for glutaraldehyde samples No. 1 and No. 2. It is seen that, for concentrations greater than 100 millimoles l^{-1} , the curves are straight lines of slope 1. Such a slope suggests that the glutaraldehyde taken up by the cells is made up of two parts: (a) a fixed amount corresponding to the saturation of the cell proteins by the glutaraldehyde. Its value G_b is the intercept of the straight line with the vertical axis; (b) an amount of free glutaraldehyde in solution inside the cell and equal in concentration to the glutaraldehyde in the external medium.

This interpretation has been corroborated by the experiments testing the permeability of the membranes of the fixed cells. In fact it has been shown

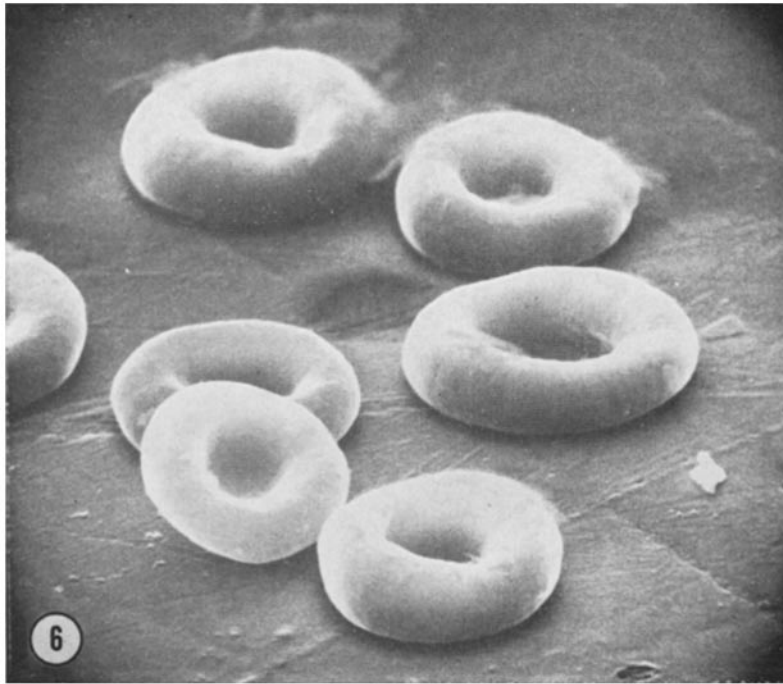


FIGURE 6 Scanning electron micrograph of RBC fixed for 2 hr in low glutaraldehyde concentration. A solution of glutaraldehyde (1%) and isotonic buffered saline was added to an isotonic suspension of cells over a period of 15 min.

that, after 2 days of fixation, electrolytes as well as glutaraldehyde are freely exchangeable between the cells and their bathing solution, and that the concentrations inside the cells are identical to those in the solution. The results obtained on the ionic leakage across the membrane show that this increase in membrane permeability to salt occurs progressively over a period of approximately 2 hr.

The values G_b of bound glutaraldehyde are found to be of the order of 600 and 700 millimoles l^{-1} for the samples No. 1 and No. 2, respectively. Since the amount of hemoglobin available for reaction is much larger than the amount of membrane proteins or of plasma proteins trapped between the cells, one can consider that the glutaraldehyde reacts effectively with a 5 millimoles l^{-1} hemoglobin solution, i.e., 120–140 molecules of glutaraldehyde saturate 1 molecule of Hb. If both aldehyde groups of each glutaraldehyd molecule react with a hemoglobin site, then there are 240–280 such sites. If, on the contrary, glutaraldehyde molecules react at one end with a hemoglobin site and at the other end with another glutaraldehyde molecule, then there might be 120

or even fewer sites. Thus, depending upon the glutaraldehyde polymerization that accompanies such a reaction, it can be considered that between 100 and 300 sites in each hemoglobin molecule react with glutaraldehyde molecules. This result is in agreement with the structure of hemoglobin and the known sites of reaction of glutaraldehyde with proteins (6, 12).

The difference observed between the samples in the value of G_b is thought to be qualitatively significant. It is probably due to a greater efficiency of the longer oligomers in cross-linking the proteins. Monomers might often be too short to be able to react at both ends, and the cross-linking is achieved by polymerization of those monomers. Thus more molecules of the less polymerized sample No. 2 are needed to saturate the proteins than of sample No. 1. This result is to be compared with that of Robertson and Schultz (13) who claimed that fixatives containing a high proportion of dimer fixed brain tissue better than purified glutaraldehyde containing a high proportion of the monomer.

Fig. 4 also shows an equilibrium curve obtained

TABLE I
Ion Activities in the Supernatant as a Function of Time

Time	K		Na	
	emf	Relative activity*	emf	Relative activity*
<i>min</i>	<i>mv</i>	%	<i>mv</i>	%
0	-6.0	100	+4.5	100
10	+2.7	138	+3.6	96.7
20	+11.5	192	+2.2	91.8
30	+17.2	238	+1.2	90.4
60	+23.5	302	+0.3	85.4
120	+25.2	322	+0.2	85.1
180	+25.7	327	+0.3	85.4

* The Nernst equation can be written: $E = E_0 + k \log(x)$ where E is the measured emf, E_0 is a reference value, x is the activity of the ion, and k is a constant ($k \approx 61.5$ at 37.5°C). Then one obtains a relative value with respect to any activity (x_1) by:

$$\frac{E-E_1}{k} = \log \frac{(x)}{(x_1)}$$

for washed RBC. It is seen that the curve is higher and follows the vertical axis more closely than the curves obtained for unwashed cells. The presence or absence of plasma proteins probably plays a key role in these differences, mainly in introducing a systematic bias in the osmometry of unwashed cell solutions. Nonetheless, the overall shape of the curve is the same and the differences are small enough not to modify the above discussion.

Some optimal methods for cell fixation by glutaraldehyde have been published in the literature (4, 5). It is clear from the preceding discussion that the presence of an electrolyte at isotonic concentration is a *sine qua non* condition for shape preservation during the fixation of the red cells. The problem, then, is to determine the glutaraldehyde concentration and the dilution factor that will produce minimal distortion while allowing complete fixation of the cells. The curves of Fig. 4 give the minimum amount of glutaraldehyde necessary to saturate the binding sites. A large dilution factor (10 or greater) will minimize the

TABLE II
Permeability of Fixed RBC

Fixed RBC				Solutions			Mixtures	
Volume	Hct†	Supernatant		Volume	Composition§	Osmolarity	Experimental osmolarity	Computed* osmolarity
		Composition§	Osmolarity					
<i>cc</i>			<i>milliosmols</i>	<i>cc</i>		<i>milliosmols</i>	<i>milliosmols</i>	<i>milliosmols</i>
1.05	0.92	BS and G	363	1.00	W	7	168	172
1.05	0.92	BS and G	363	1.00	BS	289	327	324
1.05	0.92	BS and G	363	1.00	BS and G	492	431	431
1.05	0.92	BS and G	363	1.00	BS	582	492	480
1.13	0.92	W	7	1.00	BS and G	492	264	257
1.00	0.80	W	7	1.00	KCl	385	231	222
1.00	0.80	W	7	1.00	NaCl	500	234	230
1.00	0.80	W	7	1.00	E	348	196	192

* These values are obtained in assuming (a) free exchange of solute; (b) fluid volume of the cells = 70%; (c) packing efficiency for Hct measurement = 60%. Then the solid volume of the cells is 18% of the packed cell volume (volume of cell \times Hct).

Computed osmolarity of mixture

$$= \frac{\text{vol. RBC} \times (1 - \text{Hct} \times 0.18) \times \text{osmolarity of supernatant} + \text{vol. soln.} \times \text{osmolarity of soln.}}{\text{vol. RBC} \times (1 - \text{Hct} \times 0.18) + \text{vol. soln.}}$$

† Hct, hematocrit. It is here defined experimentally as the ratio of observed packed cell volume and total volume using a microcapillary centrifuge (6 min, 13,000 g).

§ BS, buffered saline; G, glutaraldehyde; W, water; E, ethanol.

osmotic stress due to glutaraldehyde during the initial mixing. A still better way is to use a step-wise technique: the cells are suspended in an isotonic buffer, and drops of fixative solution (glutaraldehyde in isotonic buffer) are slowly added to the system. Fig. 6 illustrates this method of fixation.

The authors would like to thank Mr. John Devaney and the Jet Propulsion Laboratory for their assistance in scanning microscopy, and Dr. H. J. Meiselman and Dr. S. Lang for helpful discussions and criticisms.

This work was supported in part by grants HE-08977 and HE-07976 of the National Heart Institute.

Received for publication 9 April 1970, and in revised form 10 July 1970.

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