PHYSICOCHEMICAL EFFECTS OF ALDEHYDES ON THE HUMAN ERYTHROCYTE

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ABSTRACT

The effects of formaldehyde, acetaldehyde, and glutaraldehyde on human red blood cells were investigated. It was found that (*a*) The surface negative charge of the erythrocytes at pH 7 was increased 10% by glutaraldehyde, but not by the other two aldehydes. (*b*) The effect of incomplete fixation of the red blood cells was demonstrated by hemoglobin leakage studies The leakage of hemoglobin subsequent to formaldehyde treatment was especially pronounced Acetaldehyde-fixed cells showed some leakage of hemoglobin after an hour of exposure to the fixative, whereas glutaraldehyde-fixed cells showed no hemoglobin leakage. (*c*) All three aldehydes caused K^+ leakage during fixation. The concentrations of K^+ in the fixing solutions all reached the same level, but whereas the leakage with glutaraldehyde was immediate, that with formaldehyde was more gradual and that with acetaldehyde reached a steady state only after 24 hr. (*d*) The effects of the aldehydes on red cell deformability and swelling revealed that glutaraldehyde hardened the cells within 15 min, formaldehyde within 5 hr, while acetaldehyde required at least 24 hr to produce appreciable fixation. (*e*) The hematocrit changes accompanying the fixation process depended upon cell volume changes and loss of deformability.

INTRODUCTION

The purpose of this communication is to describe some physicochemical features of the interaction of three aldehyde fixatives with human erythrocytes The aldehydes used were acetaldehyde, formaldehyde, and glutaraldehyde. The parameters of the red blood cell selected for study during aldehyde treatment were electrokinetic charge, potassium leakage, hemoglobin release, aldehyde consumption, deformability, and volume changes.

Although aldehyde-fixed cells have been used for rheological studies (5, 9), in electrophoretic (14) and serological investigations, and widely in electron microscope research on cells and tissues (7, 20), there is still a lack of information concerning the changes which occur in the physicochemical properties of cells during treatment with an aldehyde. Recently, some aspects of glutaraldehyde fixation were quantitated by Morel et al. (17). Fixation is generally assumed to mean a procedure that maintains cells in their original condition as regards size, morphology, and spatial relationship of organelles and macromolecules. In this work cellular deformability and hemoglobin leakage will be used as criteria of the extent of fixation.

MATERIALS

All reagents were of analytical quality. Standard saline consisted of 0.145 M aqueous sodium chloride solution with the pH adjusted to 7.2 ± 0.2 with added 0.5 M aqueous sodium bicarbonate solution. Isotonic aqueous solutions of hydrochloric acid or sodium hydroxide were added to standard saline for studies of variation of the electrophoretic mobility of the cells with pH. Isoosmotic phosphate-buffered saline, pH 7.2 ± 0.1, 285 ± 10 mosmols/kg, consisted of 50 parts of 0.15 M NaCl, 10 parts of 0.16 M NaH_2PO_4, and 40 parts of 0.13 M Na_2HPO_4.

Acetaldehyde

Samples of reagent grade acetaldehyde were obtained from J. T. Baker (Chemical Co., Phillipsburg, N. J.), Eastman Kodak Co. (Rochester, N. Y.), and Matheson, Coleman, and Bell (Cincinnati, Ohio). Examination of the spectrum of aqueous solutions of these acetaldehydes in the range 220–350 mμ indicated the main absorption to be at 278 mμ. The acetaldehyde samples were redistilled under nitrogen, and the fraction boiling at 21°–22° C was collected on ice and stored under nitrogen (3). 2 g% acetaldehyde was prepared by adding 2.5 ml of acetaldehyde at 0°C to 97.5 ml of isoosmotic phosphate-buffered saline on ice. The pH of the final solution was 7.2 ± 0.1, and the osmolarity 720 ± 20 mosmols/kg.

Formaldehyde

2 g% or 4 g% of formaldehyde was prepared by adding 2 or 4 g of paraformaldehyde (Fisher Scientific Company, Pittsburgh, Pa.) to isoosmotic phosphate-buffered saline and heating at 60°C for 30 min as described by Pease (20). The solutions were cooled and filtered. The final pH of the 2 g% and 4 g% solutions was 7.1 ± 0.1, and the osmolarities > 1000 mosmols/kg.

Glutaraldehyde

Glutaraldehyde was obtained as a 70 g% aqueous solution (Ladd Research Industries Inc., Burlington, Vt.) stored under Freon. The glutaraldehyde was usually found to be sufficiently pure on the basis of spectral analysis to require no redistillation. An 8 g% aqueous glutaraldehyde solution (Polysciences Inc., Rydal, Pa.) required vacuum distillation to reduce the absorbing impurities at 235 mμ. The criteria suggested by Anderson (1) were used in evaluating the purity of the glutaraldehyde. The glutaraldehyde (1.65 g%) was made up from 2 ml of 70 g% glutaraldehyde, 4.25 ml of 1.5 M NaCl, 8.5 ml of 0.16 M NaH_2PO_4, 34 ml of 0.13 M Na_2HPO_4, and 36.25 ml of water. The 3.3 g% solution was made up in a similar way. Both glutaraldehyde solutions had a pH of 7.2 ± 0.1 and osmolarities of 500 ± 20 mosmols/kg and 700 ± 20 mosmols/kg, respectively.

Neuraminidase

Neuraminidase (Behringwerke AG, (Marburg-Lahn, Germany) had an activity of 500 units/ml, where 1 unit of activity is that quantity of enzyme which would liberate 1 mg of sialic acid from a glycoprotein substrate in 15 min at 37°C.

Blood

Blood was obtained fresh by venipuncture from normal healthy donors of various blood groups, using either 1 volume of a 5 g% aqueous solution of disodium ethylenediaminetetraacetate (EDTA) to 40 volumes of blood or 1 volume of a 3.8 g% aqueous trisodium citrate to 10 volumes of blood as the anticoagulant. There was no detectable difference between these two anticoagulants as regards any of the results subsequently obtained.

METHODS

Assay of Aldehydes

The UV spectra on the 70 g% glutaraldehyde and acetaldehyde distillates were measured on a Beckman DB recording spectrophotometer. Both distillates gave a single absorption maximum at 280 mμ (1, 3). The amount of free aldehyde present in each fixative solution was estimated by the sodium sulfite method (24). This procedure was also used to estimate the amount of aldehyde remaining in supernatant fluids from red blood cell suspensions undergoing fixation for various periods of time. The assay is based on the reaction:

$$RCHO\ (aqueous) + Na_2SO_3 + H_2O \rightarrow NaOH + RCH\ (NaSO_3)\ OH.$$

The NaOH liberated is titrated with either 0.1 or 1.0 N HCl, using thymolphthalein as an indicator.
Percentage Yield

$$= \frac{\text{Acid titer} \times \text{HCl normality} \times \text{aldehyde mol wt}}{\text{Sample wt} \times 10} \times \text{aldehyde functional groups per molecule}$$

The percentage yields obtained for the various aldehyde solutions were as follows. 2 and 4 g% formaldehyde and 2 g% acetaldehyde, 90–95% and 1.65 and 3.3 g% glutaraldehyde, 100–110%.

Osmometry

The osmotic pressure measurements were made with a freezing point depression osmometer (Pre-

cision Systems, Waltham, Mass., Osmette Model 2007). Measurements were carried out on 0.2 ml samples at 21°C. The instrument measures from 0 to 1000 mosmols/kg with a precision in the range used of ± 2 mosmols/kg. The instrument was calibrated with standard aqueous sodium chloride solutions of 100 and 500 mosmols/kg.

Aldehyde Fixation

Red blood cells (1 volume) were washed three times in standard saline (50 volumes) and centrifuged at 1200 *g*, 15 min, 21°C (designated standard washed cells). 1 volume of packed standard washed cells was added to 2 volumes of the appropriate aldehyde fixative solution. Fixation was performed in stoppered glass tubes rotated on a Fisher Roto-Rack at 21°C. Samples were obtained at suitable time intervals for the various physicochemical studies. The formaldehyde- and acetaldehyde-fixed cells used for the electrokinetic studies were prepared as described by Heard and Seaman (14). Glutaraldehyde-fixed cells were examined by electrophoresis from 1 hr to several months after initial fixation.

The amount of aldehyde used up during the fixation of red blood cells over a period of 7 days was monitored. The control system consisted of 2 ml of standard washed and packed red cells suspended in 4 ml of isoosmotic phosphate buffer. The test samples consisted of 18 ml of packed cells suspended in 36 ml of the appropriate fixative solution. The suspensions were incubated at 21°C on a rotator, and aliquots were removed at varying times for the estimation of aldehyde consumption. The samples were spun down and a 1 ml aliquot of the supernatant fluid was used used in the sodium sulfite assay with 0.1 N HCl added to titrate the NaOH released.

Measurement of Electrophoretic Mobility

Electrophoretic mobility measurements were performed in a cylindrical chamber appartus at 25°C as described previously (2). The electrophoretic mobilities expressed as μ/sec per v per cm were obtained from the times of migration of at least ten cells per run. The pH versus mobility relationships and examination of the electrokinetic reversibility of the cells were conducted as described previously (13).

Neuraminadase Treatment

Standard washed and centrifugally packed normal or aldehyde-fixed red blood cells were treated with neuraminidase as outlined by Seaman and Uhlenbruck (22).

Hemoglobin Release During Fixation

Standard washed samples of human red blood cells were mixed with fixative solutions, as described under aldehyde fixation, with the cells rotated at 21°C and sampled at regular intervals by removal of 1.0 ml aliquots taken in duplicate; centrifuged at 1200 *g*, 10 min; supernatant aldehyde fixative was removed and 9.5 ml standard saline were added to the red cell pellet, the cells were resuspended and left for 30 min; recentrifuged at 1200 *g*, 10 min, 1 ml of supernatant saline containing any hemoglobin which may have leaked was added to 4 ml of Drabkin's reagent and assayed by the cyanmethemoglobin method. The absorbance was measured spectrophotometrically at 540 mμ (15). The results were expressed as a percentage of the total hemoglobin content, obtained from complete lysis of an equivalent sample of untreated control red blood cells.

Potassium Ion Leakage During Fixation

Aliquots of cells undergoing fixation were removed for analysis of the potassium ion content of the supernatant fluid The potassium ion content was determined by means of an EEL Flame Photometer (Evans Electroselenium Ltd , Halstead, Essex, England) after standard calibration with aqueous potassium chloride solutions. The results were expressed as milliequivalents of K^+ per kilogram of wet cell weight.

Red Blood Cell Packing Experiments

Standard washed and packed erythrocytes (1 volume) were added to the appropriate aldehyde fixative solution (2 volumes). In addition to the isoosmotic aqueous phosphate buffer, a hypotonic aqueous phosphate buffer (220 mosmols/kg) and a hypertonic aqueous phosphate buffer (400 mosmols/kg) were prepared by suitable adjustment of the NaCl content of the buffer. These three buffer systems were used in the preparation of aldehyde fixative solutions, each with the same aldehyde content. Microhematocrit values were determined on the control cell suspension in isoomsotic buffer and at various time intervals on cell suspensions undergoing fixation in the hypo-, iso-, and hyper-osmotic buffer aldehyde-fixing solutions. The microhematocrit tubes were filled in duplicate and spun at 13,000 *g* for 5 min at 21°C The hematocrits were read on a IEC microcapillary reader (International Equipment Company, Needham Heights, Mass.) and the results were expressed as percentage changes over the original isoosmotic buffer control hematocrit for the same concentration of cells in buffer.

RESULTS

Uptake of Aldehyde During Fixation

The amount of aldehyde consumed during fixation of red cells over a period of 8 days is

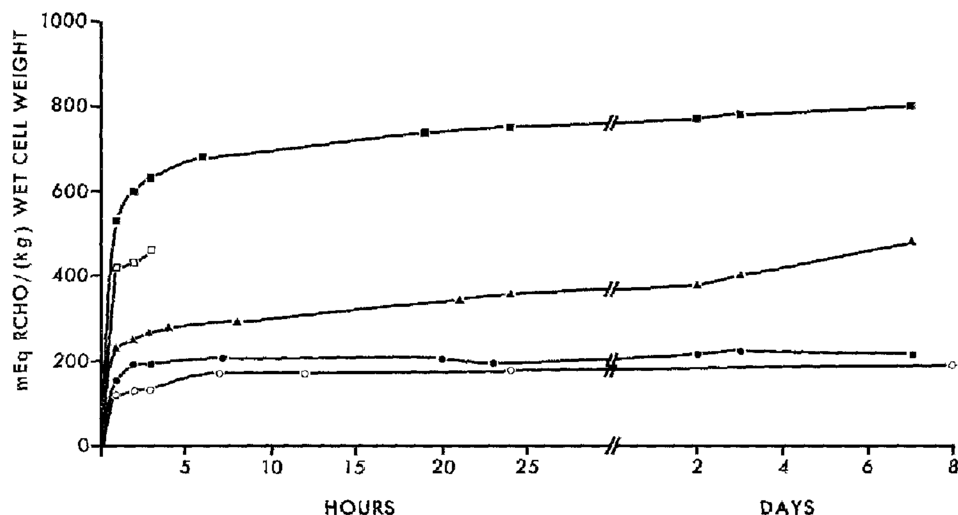


FIGURE 1 Aldehyde consumption expressed as milliequivalents of aldehyde consumed per kilogram wet cell weight. Circles indicate the amount of aldehyde consumed by formaldehyde-treated red cells: open for 2 g% initial concentration, closed for 4 g% initial concentration. Glutaraldehyde uptakes are indicated by square symbols: open for 1.65 g% initial concentration, closed for 3.3 g%. Acetaldehyde fixation at 2 g% results in the uptake of aldehyde represented by triangles.

presented in Fig. 1. The sodium sulfite assay showed that the concentration of aldehyde decreased with time. This decrease arises from the entry of aldehyde into the cells where it reacts with the intracellular proteins, principally hemoglobin. Assuming facile entry of aldehydes, and correcting for the dilution of aldehyde by entry into the cell water, the amount consumed by fixation of the cells can be calculated and expressed as milliequivalents of aldehyde consumed per kilogram of wet cell weight.

The results of hemoglobin leakage from red cells are presented in Fig. 2. The hemoglobin leakage is expressed as a percentage of cells lysed after they have been exposed to fixative solution for a period of 0–24 hr and then washed and resuspended in standard saline for 30 min. 100% leakage corresponds to the amount of hemoglobin released from the unfixed control sample after exposure to an equivalent volume of distilled water as opposed to standard saline. It can be seen that there is a drastic leakage of hemoglobin from red cells treated for short periods of time with formaldehyde. Acetaldehyde produces minor and variable leakage of hemoglobin, whereas glutaraldehyde treatment of red cells leads to no leakage of hemoglobin under any of the conditions tested.

The leakage of intracellular potassium ions

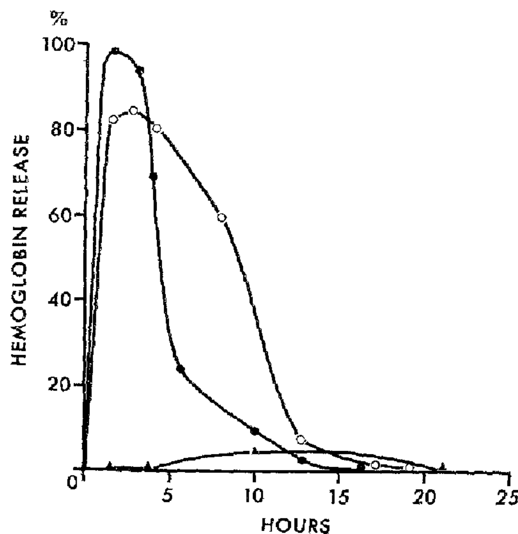


FIGURE 2 Hemoglobin leakage from red cells. The time scale indicates the length of time that the cells have been in fixative. The hemoglobin leakage is expressed as a percentage of cells lysed after they have been washed and resuspended in standard saline for 30 min. Circles represent hemoglobin released from red cells during formaldehyde fixation: open for 2 g% formaldehyde, and closed for 4 g%. Triangles show leakage from cells exposed to 2 g% acetaldehyde.

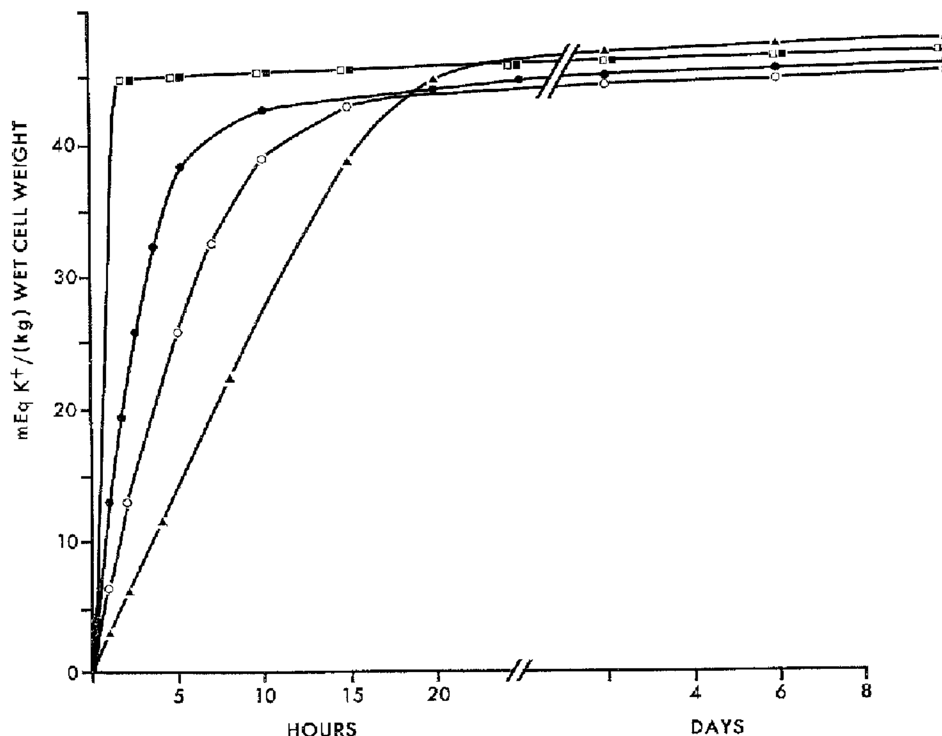


FIGURE 3 The release of intracellular potassium during aldehyde treatment of cells is shown. The release of K^+ has been calculated in milliequivalents per kilogram wet cell weight and was monitored for 8 days. Symbols as in Fig. 1.

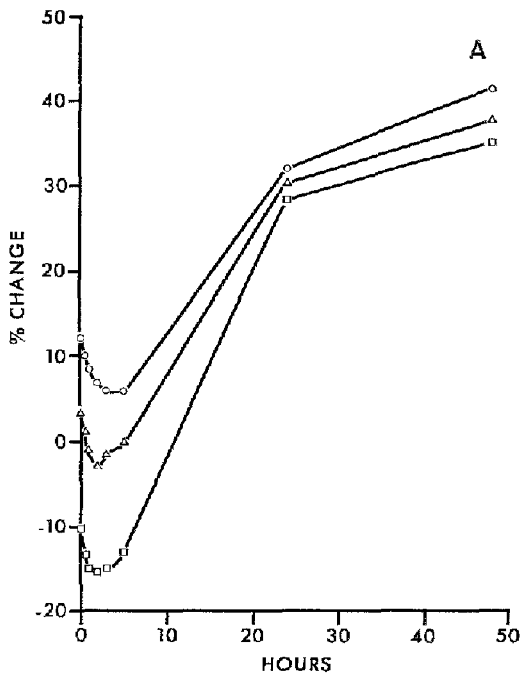
during treatment of cells with the three aldehyde fixatives is shown in Fig. 3. The release of K^+ was monitored for 8 days and has been calculated in milliequivalents per kilogram of wet cell weight.

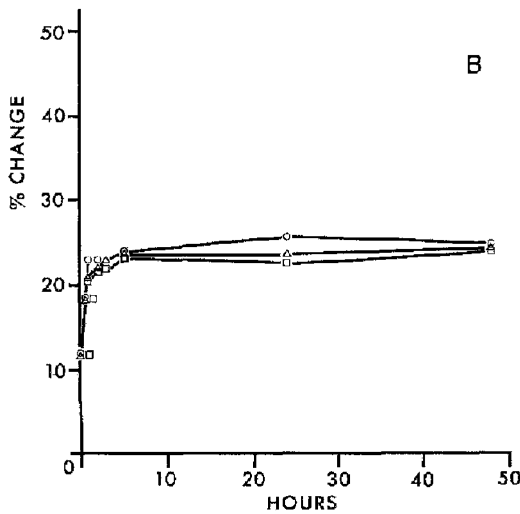
In Fig. 4 a, 4 b, and 4 c are presented the changes which occur in the hematocrit with time for the three aldehyde fixatives made up in hypo-osmotic, isoosmotic, and hyper-osmotic phosphate buffer systems The hematocrits were followed for 48 hr and reflect the combined effects of changes in cellular volume and deformability.

The electrokinetic results are summarized in Fig. 5. The upper dotted line indicates the previously found results for the fixation of human red cells with formaldehyde and acetaldehyde. Such fixation produces no change between pH 5 and pH 9 in the pH versus mobility relationship as compared with the normal untreated human red blood cell (13) In addition, neuraminidase treatment of the red cells before and after fixation resulted in about a 60% decrease in the electrophoretic mobility. On the other hand, glutaraldehyde fixation (Fig 5) results in about a 10% increase in the electrophoretic mobility above pH 6 Neuraminidase treatment before or after fixation with glutaraldehyde results in about a 60% decrease in the electrophoretic mobility at pH 7 The increase in mobility which occurs at pH 6 is still present in the glutaraldehyde-fixed cells treated with neuraminidase.

DISCUSSION

As stated in the Introduction, the purpose of this study was to investigate changes in some physicochemical parameters which occur during the interaction of erythrocytes with aldehydes. This was felt to be necessary because of gaps in our knowledge concerning cell fixation and because of the use of aldehydes to block positive charges in the peripheral zone of cells in electrokinetic studies. It was thought desirable to use purified aldehydes so that the observed phenomena could be ascribed unambiguously to the effects of the aldehydes per se. Implicit in the results is the achievement of a





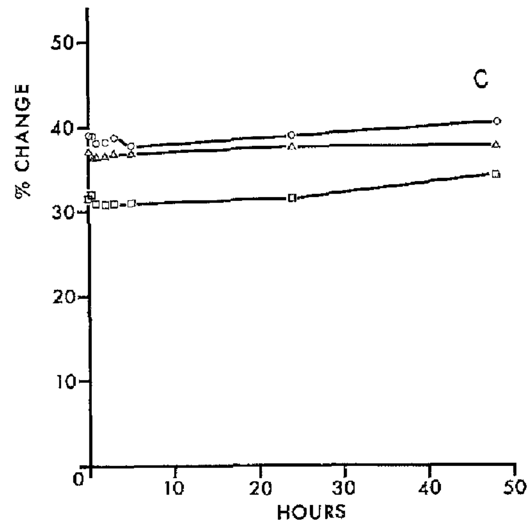


FIGURE 4 Three graphs representing the percentage changes which occur in hematocrit with time for the three aldehyde fixatives: (A) 2% acetaldehyde, (B) 4% formaldehyde, and (C) 3.3% glutaraldehyde. Squares represent hyperosmolar phosphate buffer (400 mosmols/kg), triangles represent iso-osmolar phosphate buffer (285 mosmols/kg), and circles represent hypo-osmolar phosphate buffer (220 mosmols/kg).

high degree of aldehyde purity and lack of detectable contaminants as evidenced by the UV spectra and osmolarities of standard aqueous solutions of the aldehydes. However, it must be realized that artefacts may arise from deposited aldehydic polymers or aldehyde groups which may be introduced (4)

It is apparent from Fig. 1 that there is a considerable uptake of aldehydes by human erythrocytes during fixation. The criteria used for adequate fixation included the following: (*a*) stability of the fixed red cells suspended in either standard saline or distilled water (no change in cell geometry nor leakage of any hemoglobin); (*b*) increase in cell rigidity as evidenced by marked hematocrit increases.

Morel et al. (17) have shown that glutaraldehyde enters the human red blood cell very rapidly; thus the uptakes observed in Fig. 1 are likely to reflect the binding of the aldehydes to the intra-

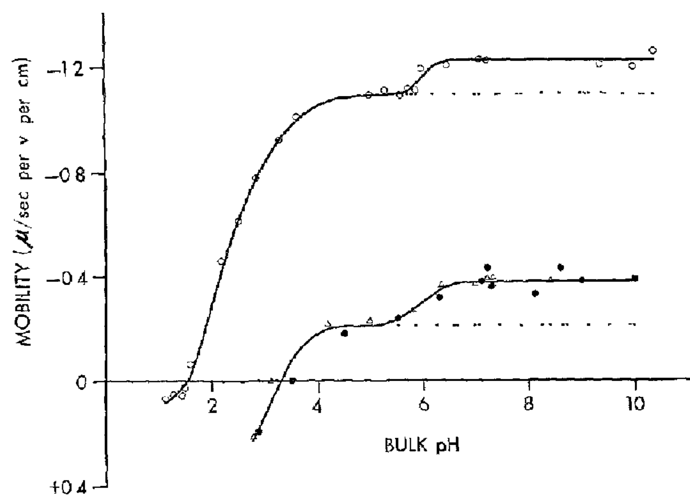


FIGURE 5 pH versus electrophoretic mobility relationships for red cells fixed in 1.65% glutaraldehyde. The upper dotted line represents the previously found results for red cells fixed in 2% acetaldehyde or 2% formaldehyde. The lower dotted line indicates the earlier results obtained by acetaldehyde or formaldehyde fixation of neuraminidase-treated cells. Open circles indicate glutaraldehyde-treated cells; closed circles indicate neuraminidase-treated cells subsequently fixed with glutaraldehyde; triangles represent glutaraldehyde-treated cells subsequently treated with neuraminidase

cellular proteins with perhaps a small contribution from the membrane proteins, rather than their dilution by cell water which would have taken place before any uptake measurements With the exception of 1 65 g% glutaraldehyde, there was excess aldehyde present at the conclusion of the experiment after 8 days of fixation It is of interest that cells fixed in 1 65 g% glutaraldehyde consumed the aldehyde within a few hours, nevertheless, these cells appeared completely fixed by our previously stated criteria for fixation The similarity between the 2 g% and 4 g% formaldehyde uptake curves implies a considerable excess of the aldehyde throughout the entire period of fixation As was found by Morel et al. (17), the uptake of glutaraldehyde corresponds to about 150 molecules of the aldehyde involved in the interaction with each hemoglobin molecule. The binding of formaldehyde and acetaldehyde is much less and is probably related to the inability of monoaldehydes to participate effectively in crosslinking reactions, although bridging by methylene bridge formation via amides or phenolic indole or imidazole rings has been suggested as a possibility for formaldehyde (12).

Hemoglobin release studies are illustrated in Fig. 2, where severe hemoglobin loss from red cells occurs after short term formaldehyde treatment, on the other hand, no hemoglobin loss was detectable after glutaraldehyde treatment. Leakage of hemoglobin after acetaldehyde treatment was minimal and somewhat variable. The leakage of hemoglobin from formaldehyde-treated cells may reflect either total hemolysis of a part of the cell population or partial hemolysis of essentially all of the cell population. Examination of thin films of cell suspensions by phase contrast microscopy indicated complete hemolysis of a part of the cell population. Since the reaction of the formaldehyde with the constituents of the red cells appears to be appreciably slower than their interaction with glutaraldehyde, hemolysis of partially fixed formaldehyde-treated red cells when placed in standard saline probably arises from the rapid entry of water into the red cells which contain effectively > 1000 mosmols/kg formaldehyde. The osmolarities of the acetaldehyde and glutaraldehyde fixatives are appreciably less, and therefore the entry of water from the extracellular compartment is likely to be much less pronounced Furthermore, in the case of the glutaraldehyde fixative the crosslinking reactions are rapid and the cell is probably essentially fixed before the possibility of any cellular disruption by an osmotic mechanism could occur; in addition, the rapidity of the reaction would also quickly lower the intracellular

osmolarity of the glutaraldehyde solution. Thus, the observed leakages of hemoglobin may be rationalized in terms of depression of the intracellular water activity by the presence of aldehyde, the greatest depression in activity occurring in the case of the formaldehyde with resultant rapid entry of water and disruption of the incompletely fixed cells.

It should also be noted that the technique used in generating the formaldehyde may also result in the presence of oxidation products which are lytic at low concentrations. Similarly, there is some possibility that the acetaldehyde may undergo oxidation during the manipulations involved in the fixation of the red cells; the variability in the degree of hemoglobin leakage from the acetaldehyde-treated cells may, for example, arise from differences in the oxygenation of the cell suspension. Acetaldehyde will absorb oxygen from the air rapidly to form peroxyacetic acid which will react in the presence of some catalysts to form acetic acid which is lytic (18).

Although the aldehydes apparently penetrate the cell at different rates, the membrane is permeable to all species studied. Their osmotic effects are, therefore, transient, and the principal osmotically active molecules in the fixation media are the cations; as a consequence, the aldehydes are less osmotically active than would be expected on the basis of their measured osmolarities. Thus, the isotonicity of aldehyde fixative solutions has to be maintained by the presence of appropriate buffer salts. It is unclear what role (if any) the pH shifts during fixation, changes in the volume of aldehyde-modified hemoglobin molecules or changes in the activity of ion transport systems of the membrane surface pump play in the observed physicochemical changes during fixation.

The results presented in Fig. 3 for the leakage of K^+ during fixation indicate appreciable loss of impermeability of the membrane to K^+ over the course of a few hours for glutaraldehyde and formaldehyde. Acetaldehyde required about 1 day before the cells undergoing fixation became largely permeable to K^+. If a mean value of 44–45 mEq/kg wet cell weight is taken for potassium ions released at the later stages of cellular fixation when presumably equilibrium has been reached with the intracellular compartment, this would indicate an original intracellular K^+ concentration of 65–67 mEq/kg wet cell weight (10) and suggests that the leakage of potassium ions is incomplete even though a constant level has been reached in the supernatant fluid. There is evidence for the role of positive groups in the membrane (21) controlling cation permeation. These groups are believed to be amino groups with a pK of ~ 9 (19) and as such would react with the aldehyde fixatives. Blocking of these groups would probably facilitate passive diffusion of K^+ out of the cells and thus lead to the leakage phenomena which we have observed.

Red blood cells made rigid by fixation cannot be packed completely by centrifugation, unlike normal deformable red blood cells. Burton (6) calculated that nondeformable, fixed cells retain about 40% dead space (trapped fluid volume) when centrifuged. Complete fixation of red blood cells without change in the volume of individual cells should thus result in about a 40% increase in hematocrit. Chien et al. (8) have confirmed experimentally by an isotope dilution method the marked decrease which occurs in the cellular packing of red blood cells upon fixation.

Figs. 4 a, 4 b, and 4 c, which show the percentage change in hematocrit of red blood cell suspensions with time, during treatment with acetaldehyde, formaldehyde and glutaraldehyde, give an indication of the relative importance of buffer osmolarity, entry of aldehyde, efflux of cell water, loss of cation impermeability, and rate of cellular fixation in determining the final geometry of the fixed cells. The percentage changes in hematocrit will reflect the combined effects of changes in cell volume and cell deformability. Immediately before treatment with an aldehyde, red blood cells in the hyperosmolar buffer show a significant decrease in cell volume, and red blood cells in the hypoosmolar buffer show a significant increase in cell volume with respect to the isoosmotic buffer control system. These initial differences in cell volume result from cation impermeability of the cells and their behavior as osmometers. However, as is seen in the K^+ leakage experiments, red cells undergoing fixation eventually lose their cation impermeability and the osmolarity of the buffer system will then no longer play a role in the regulation of cellular volume. Elimination of the osmotic pressure differential as a factor in the maintenance of a certain cell volume results in the cell membrane taking up its natural geometry, presumably that found in an isoosmotic buffer (11). Thus, the percentage changes in hematocrit during aldehyde treatment were all calculated with respect to the isoosmotic buffer control rather than each system with respect to its

own hyperosmotic, isoosmotic, or hypoosmotic control buffer

Red blood cells undergoing acetaldehyde fixation show an initial decrease in cell volume in all three buffer systems (Fig. 4 a), probably because of efflux of cell water into the markedly hyperosmolar buffer solutions. The initial differences in the volume of the red blood cells suspended in hyperosmotic, isoosmotic, and hypoosmotic acetaldehyde-buffer systems continue to exist for many hours and indicate only a very gradual loss of cation impermeability. The rate of cellular fixation with acetaldehyde appears to be relatively slow as evidenced by (*a*) the slow increase in hematocrit which is still evident even after 50 hr of exposure to acetaldehyde, although the increase does approach the theoretical increase of 40% for rigid cells, (*b*) loss of hemoglobin from acetaldehyde-treated cells when placed in distilled water, at least up until 24 hr of previous exposure to the acetaldehyde This loss of hemoglobin should not be confused with the small and variable losses which occur when the aldehyde-treated cells are suspended in standard saline (Fig. 2), (*c*) the persistence of appreciable impermeability of the acetaldehyde-treated red blood cells to potassium ions, at least for the first 15–20 hr of exposure to the aldehyde.

The rate of fixation with formaldehyde appears to be faster than with acetaldehyde in some respects. There is a more rapid loss of impermeability to potassium ions, with a concomitant disappearance of differences in cellular volume between the cells suspended in hyperosmotic, isoosmotic, or hypotonic buffers (Fig. 4 b). The hematocrit increases by only 25% as opposed to the 35–40% expected on the basis of loss of cell deformability. The cells appear to have been fixed in a shrunken condition. Although fixation is apparently complete after about 5 hr on the basis of hematocrit changes, the hemoglobin release (Fig. 2) and potassium ion leakage studies (Fig. 3) suggest that 15–20 hr are necessary for fixation by these criteria. These observations are consistent with the idea of a more rapid fixation of surface membrane and hence loss of deformability, followed by slower cross-linking reactions involving the intracellular contents.

With glutaraldehyde treatment (Fig. 4 c) the fixation and the loss of cation impermeability are very rapid It is inferred that fixation is more rapid than the loss of cation impermeability since cells in the hypoosmotic buffer system are apparently still somewhat swollen and the cells in the hyperosmotic buffer system are somewhat shrunken after fixation. The increase in the hematocrit of around 40% for the isoosmotic case is close to that expected for loss of cell deformability at constant volume.

Fig. 5 illustrates changes in surface electrokinetic charge after exposure of erythrocytes to glutaraldehyde. The electrokinetic behavior of human blood cells after formaldehyde and acetaldehyde treatment has already been well documented (13, 14, 23). In contrast to this previous work the unexpected finding of an increase in negative surface charge of human red cells treated with glutaraldehyde requires some comment. A reasonable explanation might appear to be that the bifunctional glutaraldehyde may act at some sites in the peripheral zone of the cell as a unifunctional agent. In fact, glutaraldehyde-treated red cells have a marked Schiff reaction (16), presumably because of involvement of only one of the aldehydic functional groups of the glutaraldehyde molecule in reactions at the red cell surface. The remaining group could be oxidized to the corresponding carboxylic acid and thereby increase the net negative surface charge. However, the pK 6.0 is high for carboxyl groups, and the sites for glutaraldehyde reaction are likely to be amino groups which are not present in sufficient numbers to influence the electrophoretic mobility Furthermore, both formaldehyde and acetaldehyde would interact with amino groups and should, therefore, be expected to also give a change in electrophoretic mobility. Posttreatment of acetaldehyde-fixed cells with glutaraldehyde also produces an equivalent increase in the electrophoretic mobility. The increase cannot be removed by washing the cells with standard saline a large number of times, and thus appears to result from the appearance of additional ionogenic groups in the peripheral zone of the glutaraldehyde-treated red cell. Treatment of the glutaraldehyde-fixed cells with neuraminidase does not eliminate the increase in mobility which occurs at about pH 6 5. This implies that the *N*-acetyl–neuraminic acid moiety is not involved in the increase in surface charge which occurs upon treatment of the cells with glutaraldehyde The pH versus mobility relationship for glutaraldehyde-fixed red cells is identical below pH 5.5 with those for formaldehyde and acetaldehyde. There is no significant positive branch to the curve at low pH confirming the absence of a significant

number of positive groups in the peripheral zone of the aldehyde-fixed cells and implying the absence of significant adsorption of hydrogen ions at low pH. Caution should be exercised in the interpretation of electrokinetic data obtained after short treatments with formaldehyde and acetaldehyde, since leakage of intracellular proteins followed by their coupling to the surface may occur (25).

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