

INFLUENCE OF GLUTARALDEHYDE AND/OR OSMIUM
TETROXIDE ON CELL VOLUME, ION CONTENT,
MECHANICAL STABILITY, AND MEMBRANE
PERMEABILITY OF EHRlich ASCITES TUMOR CELLS

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ABSTRACT

Effects of fixation with glutaraldehyde (GA), glutaraldehyde-osmium tetroxide (GA-OsO₄), and osmium tetroxide (OsO₄) on ion and ATP content, cell volume, vital dye staining, and stability to mechanical and thermal stress were studied in Ehrlich ascites tumor cells (EATC). Among variables investigated were fixation time, fixative concentration, temperature, osmolality of the fixative agent and buffer, total osmolality of the fixative solution, osmolality of the postfixation buffer, and time of postfixation treatment in buffer (Sutherland, R. M., et al. 1967. *J. Cell Physiol.* **69**:185.).

Rapid loss of potassium, exchangeable magnesium, and ATP, and increase of vital dye uptake and electrical conductivity occurred with all fixatives studied. These changes were virtually immediate with GA-OsO₄ or OsO₄ but slower with GA (in the latter case they were dependent on fixative temperature and concentration) (Foot, N. C. 1950. *In McClung's Handbook of Microscopical Technique*. 3rd edition. 564.).

Total fixative osmolality had a marked effect on cell volume with OsO₄ but little or no effect with GA or GA-OsO₄. Osmolality of the buffer had a marked effect on cell volume with OsO₄, whereas with GA or GA-OsO₄ it was only significant at very hypotonic buffer osmolalities. Concentration of GA had no effect on cell volume. Osmolality of the postfixation buffer had little effect on cell volume, and duration of fixation or postfixation treatment had no effect with all fixatives.

Freezing and thawing or centrifugal stress (up to 100,000 g) had little or no effect on cell volume after all fixatives studied. Mechanical stress obtained by sonication showed that OsO₄ alone produced poor stabilization and that GA fixation alone produced the greatest stabilization.

The results indicate that rapid membrane permeability changes of EATC follow fixative action. The results are consistent with known greater stabilizing

effects of GA on model protein systems since cells were also rendered relatively stable to osmotic stress during fixation, an effect not noted with OsO_4 . After fixation with GA and/or OsO_4 cells were stable to osmotic, thermal, or mechanical stress; this is inconsistent with several earlier reports that GA-fixed cells retain their osmotic properties.

One purpose of fixation is to maintain cells as close as possible to their original *in vivo* condition with regard to volume, morphology, and spatial relationships of organelles and macromolecules. It is clear that when various kinds of tissues or larger groups of cells are fixed, the conditions will be more complicated than they will be for individual cells. This may be one reason why there are several conflicting opinions about the properties of fixatives, fixative vehicles, and fixation techniques (16, 18) used for electron microscope studies.

The main emphasis in fixation studies has been concentrated on the effects of fixatives on the morphological appearance of cells and tissues at light and electron microscope levels (29, 37, 39). There are, however, several methods other than microscope appearance that have been adapted for studies on the mechanisms of fixation. These methods include measurement of cellular and subcellular volume changes (3, 7, 23, 36, 40), determination of accumulation (5, 22) and loss (1, 8, 15, 22, 36, 40) of material, consumption of fixatives (22, 40), measurement of changes in reflectivity of tissue components (6), characterization of conformation of cell membrane proteins (25), and electrophysiological properties of fixed cells (9, 10, 11, 13).

Although basic cell membrane changes during and after fixation have been studied (for reviews see references 16 and 18), there are many conflicting views. It is known that fixation alters the permeability of the cell membrane and probably the osmotic behavior of the cell (16, 18). Therefore, in electron microscopy attention has been paid to the concentrations of fixation agents and their vehicle solutions, while for light microscopy the osmotic effects are still mostly ignored (6, 7, 16, 18, 27, 32, 41). The view that the osmolality of the fixation agent is not as important as that of the vehicle has gained acceptance. There is general agreement that osmium tetroxide (OsO_4) destroys the semipermeable properties of the cell membrane, making them permeable to low molecular weight substances (6, 10, 16, 36). In addition, some workers are of the opinion that proper fixation rapidly stabilizes the cell, after which the cell is

fairly resistant to osmotic stress. Carstensen et al. (9), for example, used membrane resistance of fixed human erythrocytes to electric current as a criterion and noted that glutaraldehyde (GA) fixation rendered the cells osmotically inactive. Bone and Denton (6), and Bone and Ryan (7), however, have recently reported that after GA-fixation the cells remain osmotically active. They arrived at this conclusion using volume changes of reflecting cells of fish scales or crab axons as models. These two conclusions regarding osmotic properties of fixed cells are therefore in conflict and the differences are difficult to explain on the basis of different characteristics of the cell membranes involved.

The chemical mechanism of fixation with GA is different from that with OsO_4 . GA acts mainly as a protein cross-linking agent, whereas OsO_4 reacts mainly with lipids (16, 26, 31), and this difference may result in differing stability of the tissues. The sensitivity of GA- or OsO_4 -fixed cells to osmotic stress has been studied to some extent (6, 7), but little attention has been paid to the resistance of fixed cells to other physical stresses (9, 10). The question of the resistance to osmotic, mechanical, or thermal stresses becomes even more important in the case of injured cells, which may be more vulnerable than normal fixed cells to many kinds of stresses during their processing for electron microscopy.

The following questions were asked in the present study. What are the effects of GA and/or OsO_4 fixation on the permeability properties of the cell membrane, and are there any differences induced by fixative agents? What are the effects of fixatives on the osmotic properties of the cells and are the changes irreversible? What are the effects of fixatives on the resistance of cells to mechanical, thermal, or osmotic stress during and after fixation?

MATERIALS AND METHODS

General

Experiments were performed with Ehrlich ascites tumor cells which were grown in the peritoneal cavity of

white mice by weekly transplantation. The cells were harvested 7 days after inoculation and washed twice with modified Krebs-Ringer phosphate buffer (KRP) (20) to remove the ascitic fluid. The KRP contained 150 mM Na, 3.5 mM K, 1.0 mM Ca, 0.67 mM Mg, 130 mM Cl, 25 mM PO₄, and 0.67 mM SO₄, pH 7.45. The osmolality of isotonic KRP was adjusted to 310 mosmol later with additional NaCl if necessary. The appropriate hypo- or hypertonic solutions were prepared by multiplying the above mentioned concentrations by 0.33–3.0. The osmolalities of the solutions were determined with a freezing point depression osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). The pH of all incubation, fixation, and postfixation solutions was adjusted to pH 7.45 (room temperature), except those prepared in deionized water.

After washing, the cells were collected by centrifugation at 71 g for 5 min at room temperature, and thereafter, the loosely packed cells were resuspended in 4 vol of fresh isotonic KRP. Before all experiments the cells were preincubated in isotonic KRP for 10 min at 37°C, with aeration by gentle manual shaking for about 5–10 seconds every 2 min. After preincubation the viability of the cells was examined by treating an aliquot with 0.1% nigrosin in isotonic KRP. Nigrosin stains dead cells dark blue, leaving the viable cells unstained (19). Only samples essentially free of red blood cells and in which the number of nigrosin-stained cells was less than 1% of the total were used.

Parameters of Fixation and Postfixation Treatment

The following parameters of fixation were varied and their effects studied.

VITAL DYE PERMEABILITY: The cells were fixed for 2 min–24 h at 37°C, or 0°C in 3% GA, 1% OsO₄, or 3% GA-1% OsO₄ in isotonic KRP, and effects of fixation agents on the permeability properties of the cell membrane for vital dyes were studied.

INTRACELLULAR POTASSIUM, MAGNESIUM, AND ATP: The cells were fixed for 2 min–24 h at 37° or 0°C in 0.1, 0.5, 1.0, 3.0, or 5.0% GA, in 1% OsO₄, or in 3% GA-1% OsO₄, all in isotonic KRP. Effects of fixatives on cellular potassium and magnesium concentrations were determined as described below. Cellular ATP was determined (see below) after fixation of the cells for 2–60 min in 3% GA, 1% OsO₄, or 3% GA-1% OsO₄, all in isotonic KRP at 37°C.

VOLUME OF THE NORMAL CELLS: The cell diameters of the nonfixed cells were determined after a 10-min preincubation. These mean diameters of nonfixed cell populations were used as control values and plotted as 100%. The diameters of the nonfixed cells and, in most cases, after fixation and postfixation treatment, were determined in duplicate and in some cases in quadruplicate. The variability between different determinations on the same sample averaged 2.2%.

EFFECTS OF FIXATION ON CELL VOLUME: The following effects of fixation on the cell volume were studied: (a) effects of osmolality of the fixative vehicle on the cell diameter. The osmolality of the fixative vehicle was varied by preparing the fixative in the KRP buffer of 32, 103, 207, 310, or 413 mosmol/liter. The concentration of the fixative agent was kept constant, i.e., either 3% GA or 1% OsO₄, or 3% GA-1% OsO₄. The fixation times were 1 h, 24 h, or 7 days at 37°C. The experiment is presented schematically in Fig. 1; (b) effects of the fixation agent in deionized water on the cell diameter. The following agents were used for 1 or 24 h at 37°C: 3% GA in water, pH 4.45 and osmolality 331 mosmol/liter, 1% OsO₄ in water pH 5.40 and osmolality 37, and combined 3% GA-1% OsO₄ in water, pH 4.53 and osmolality 365; (c) effects of different concentrations of GA in isotonic KRP on the cell diameter. The cells were fixed for 1 h at 37°C in 0.1, 0.5, 1.0, 3.0, or 5.0% GA buffered by isotonic KRP.

EFFECTS OF POSTFIXATION TREATMENT ON CELL VOLUME: Cells were fixed in fixatives of different osmolalities as described above. Postfixation treatments with the following solutions were applied: (a) samples of the cells which were primarily fixed in 3% GA in KRP (310 mosmol) for 60 min at 37°C were postfixated with 1% OsO₄ in the same KRP for 60 min at 37°C (this treatment corresponds to one common procedure used in electron microscopy); (b) the cells were primarily fixed in 3% GA, 1% OsO₄, or 3% GA-1% OsO₄ buffered by KRP of different osmolalities (103, 207, 310, 413 mosmol). Samples of fixed cells were subjected to postfixation treatment with KRP of 103, 207, 310, or 413 mosmol for 60 min, 24 h, or 7 days; (c) the cells were fixed for 1 h at 37°C either in 3% GA, 1% OsO₄, or 3% GA-1% OsO₄, buffered with isotonic KRP, and after fixation were transferred to deionized water for 24 h. The experiment is presented schematically in Fig. 1.

EFFECTS OF SONICATION ON FIXED CELLS: The cells were fixed for 10 s or 1 h at 37°C in 3% GA, 1% OsO₄, or 3% GA-1% OsO₄ buffered with isotonic KRP. After fixation the cells were treated by sonication in isotonic KRP. A model W 185 sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) with a 0.5-in diameter disruptor horn was used. 7 ml of the cell suspension were treated for 1 s–5 min in a plastic tube with an inner diameter of 0.63 in and a length of 3.75 in; the sonic pressure was 9.7 g. Temperature in the cell suspension was not allowed to exceed 37°C. An ice-salt water bath was used and exposures were performed in 1-min periods, interrupted by cooling of the tube and the horn. Samples were taken at serial time intervals, and cell counts and photomicrographs were taken in the same area of the hemocytometer. Counts of the cells with intact cell membrane and the cell diameters were determined at a magnification of 200.

EFFECTS OF FREEZING AND THAWING ON FIXED CELLS: The cells were fixed for 1 h as in sonication experiments and thereafter exposed five times

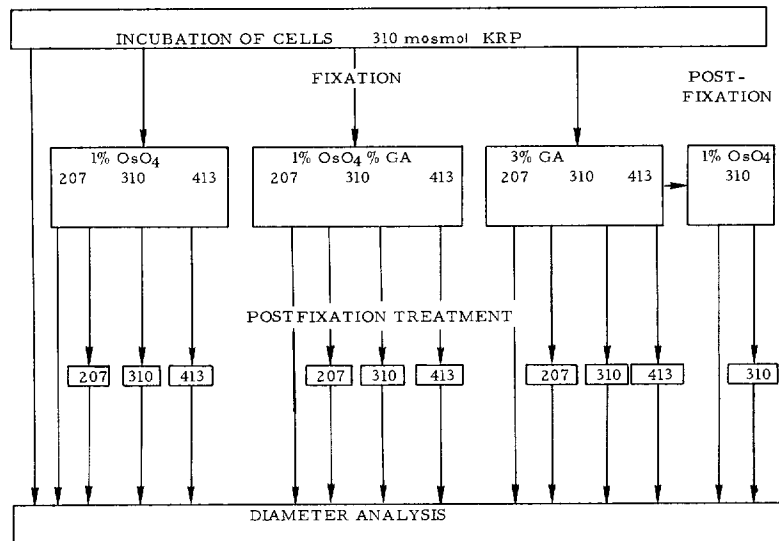


FIGURE 1 Schema showing the diameter analysis of the cells fixed, postfixed, and treated after fixation in various modifications of the KRP buffer.

to freezing and thawing in isotonic KRP, using liquid nitrogen and a water bath at 37°C. The cell diameters were determined.

EFFECTS OF CENTRIFUGAL FORCE ON FIXED CELLS: The cells were fixed as in freezing and thawing experiments. After a 1-h fixation the cells were exposed at room temperature for 10 min to centrifugal forces of 10,000 or 100,000 *g* using a Beckman Model 12-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The cell diameters were determined.

Purified GA manufactured by Ladd Research Industries, Inc., Burlington, Vt., as 70% concentrate was used to exclude the effects of different impurities (1); OsO₄ was supplied by Polysciences, Inc., Warrington, Pa. The GA was stored in a glass bottle with nitrogen atmosphere in a refrigerator and the fixatives were always prepared in the required KRP buffers immediately before use.

Analytical Methods

The cellular volumes were determined by photographing them at a standard magnification in the same area of a hemocytometer with a depth of 0.1 mm. From 70 to 170 cells were included in each micrograph and the diameters of the cells were determined by the same person at a total enlargement of 648. Unfixed cells were used as controls in each series of fixation and postfixation experiments. The mean and standard deviations of the diameters were calculated with a laboratory computer. Some comparative measurements were done with a model B Coulter Counter particle size analyzer or Coulter counter model ZBI analyzer combined with a Coulter Channelyzer model C-1,000 (Coulter Electronics, Inc., Hialeah, Fla.). The aperture was 100 μm in

diameter and 75 μm in depth and the cell suspension was adjusted to approximately 25,000 cells/ml. Ragweed pollen and latex particles were used for calibration (Fig. 9).

The vital dye uptake of differently fixed cells was studied using 0.1% nigrosin, 0.1% trypan blue, or 0.1% methylene blue in isotonic KRP. The nonfixed but stained, and the fixed but nonstained cells were used as a reference when estimating the vital dye uptake. The cells were allowed to take up nigrosin and trypan blue for 3 min and methylene blue for 30 s before the counting procedure was begun.

The release of intracellular potassium and magnesium as a consequence of fixation was determined as follows: the cells suspended in the fixation solution were centrifuged at 1,000 *g* for 60 s. The ion concentrations in the pellet containing the fixed cells were determined. To release the intracellular ions for measurement, the fixed and the nontreated control cells were lysed by boiling the cells in 1 N NaOH solution for 2 h. This procedure was adequate to release magnesium and potassium from fixed cells as shown by determining the concentrations of these ions both in the supernate after fixation and in the lysed pellet suspension of the fixed cells in two series of experiments. The samples were neutralized with 1 N HCl, and potassium and magnesium determinations were performed according to the methods of Kingsley and Schaffert (21), Aull and Hempling (2), and Sparrow and Johnstone (33), as adapted for Ehrlich ascites tumor cells in this laboratory by Laiho et al. (24). The samples were analyzed with a Jarrell Ash atomic absorption spectrophotometer (Fisher Scientific Co., Pittsburgh, Pa.), and it was found that OsO₄ did not interfere with the analysis.

Deionized water was used as blanks in K and Mg determinations and the NaOH treatment did not have any effect on blank values. The results of intracellular determinations were corrected by subtracting the amounts of ions in the extracellular space of the pellet, determined with [¹⁴C]dextran.

For the determination of ATP, 1-ml aliquots of cell suspension were centrifuged, the supernate was carefully removed, and the pellet was mixed with 1.75 ml of ice-cold 6.25% perchloric acid. The specimens were kept in ice and neutralized, and after centrifugation, ATP determinations were made in the supernate using the luciferase method (34) with a Farrand fluorometer (Farrand Optical Co., Inc., Valhalla, N. Y.). No inhibition of the luciferase reaction was found in GA-fixed samples. Small amounts of OsO₄ trapped by the GA-OsO₄- and OsO₄-fixed cell pellets resulted in about 5 and 27% inhibition of the initial luciferase reaction, respectively.

RESULTS

Vital Dye Permeability

Fixation with 3% GA or 1% OsO₄ did not produce any appreciable background color to the cells. Cytoplasmic and nuclear material became increasingly brown or black in the 3% GA-1% OsO₄ fixation solution, depending on the fixation time.

After only a 2-min fixation with 3% GA or 1% OsO₄ either at 0°C or 37°C the cells became stainable with methylene blue, nigrosin, or trypan blue. In cells fixed with 3% GA-1% OsO₄ the background color allowed only the intense bluish staining of methylene blue to be detected.

The staining intensity varied somewhat depending on the fixation agent, the fixation time, and the vital dye used. Methylene blue turned all fixed cells strongly blue. Trypan blue stained strongly only the GA-fixed cells, leaving OsO₄-fixed cells weakly stained. Cells fixed with either GA or OsO₄ were stained weakly and unevenly by nigrosin as compared with staining of unfixed cells. When the fixation time was longer (up to 2 days) in 1% OsO₄, staining intensity of the fixed cells decreased or they became unstainable by nigrosin or trypan blue.

Intracellular Potassium, Magnesium, and ATP

About 85–90% of intracellular potassium was released in 2 min when the cells were fixed with 5% GA, 1% OsO₄, or 3% GA-1% OsO₄ at 37°C or 0°C

or with 3% GA at 37°C (Figs. 2, 3, 6, and 7). After this initial loss the level of intracellular potassium remained practically constant during the 24-h observation period. With GA as the fixative the rate of efflux of intracellular potassium was dependent on the concentration of GA. Moreover, the fixation time and temperature affected the rate of efflux of potassium (Figs. 2 and 3).

The effect of fixation on intracellular magnesium was similar (Figs. 4–7). The percentage of magnesium (around 45–48%) retained in the intracellular compartment remained much higher than that of potassium (around 11–13%).

During the 2-min fixation period at 37°C with 3% GA-1% OsO₄ or 1% OsO₄, ATP disappeared totally from the cells. Only traces of ATP were analyzable during 3% GA fixation at 2 min and none was found later.

Volume of Normal Cells

The mean diameter of the control cells used for the present experiments varied from 15.7 to 16.5 μm³. The respective mean cell volumes calculated on the basis of the mean diameters were plotted as 100% in Tables II–IV and in Fig. 8. The standard deviations were about 10% of the respective values of the diameters.

Effects of Fixation on Cell Volume

When GA or GA-OsO₄ was used as the fixative agent the osmolality of the buffer had a negligible effect on the diameter of the cells within the range of 207–413 mosmol (Table II). The GA concentration within the range of 0.1–5.0% in isotonic buffer had little effect on the cell diameter (Table I). Some shrinkage was, however, seen when the concentration of GA was 5.0%. With OsO₄ alone as the fixing agent, the cells expanded, even with hypertonic KRP (413 mosmol) as the vehicle. This expansion was more pronounced with lower buffer osmolality. The increase of mean diameter of cells fixed with 1% OsO₄ in 413, 310, or 207 mosmol KRP for 1 h indicated respective volume increases of 29, 48, or 73% (Fig. 8).

The cells expanded prominently with all three fixing agents when the fixatives were made up in deionized water. With GA, GA-OsO₄, or OsO₄, the corresponding volume expansions were 37, 35, and 82% (Table IV, Fig. 8). However, these cells did not expand as drastically as nonfixed cells incubated in extremely hypotonic KRP-buffer of 31 mosmol for 2 min. The volume of the latter cells

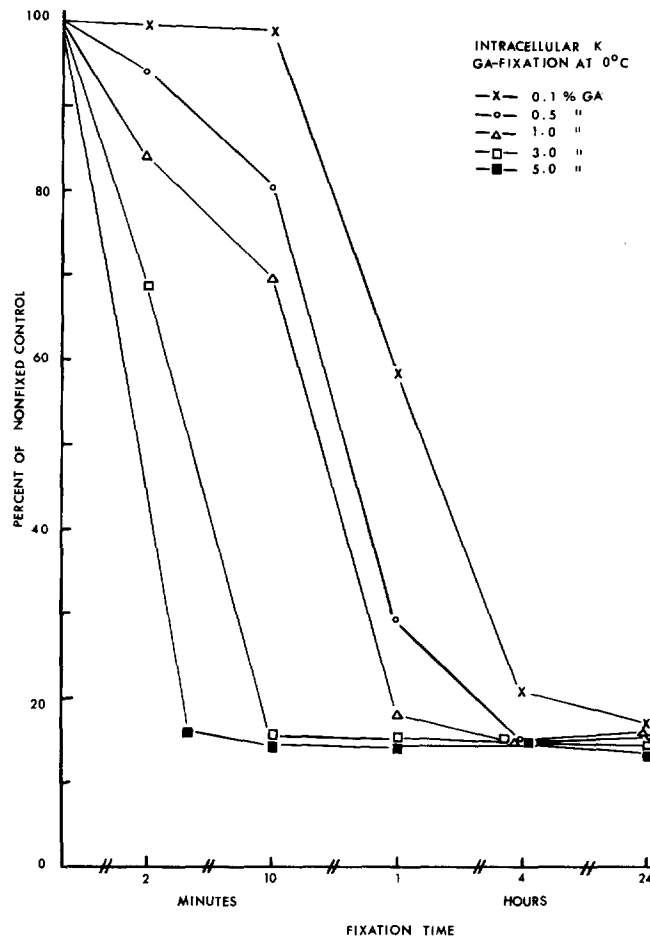


FIGURE 2

FIGURES 2-5 Effects of GA fixation on intracellular potassium and magnesium. 100% is the pre-fixation control value of normal, nonfixed cells. Each point is a mean of four determinations.

increased by approximately 330% from that of the normal cells, and furthermore, only a small proportion of the cells was disrupted in these extreme conditions (Table IV).

Effects of Postfixation Treatment On Cell Volume

Postfixation of 3% GA-fixed cells with 1% OsO₄, as is commonly done in the preparation of tissue for electron microscopy, had no effect on cell diameter (Table III).

Fixation of cells for 60 min with any of the three fixing agents buffered with KRP of widely different osmolalities (103-413 mosmol) seemed to render the cells resistant to any significant volume

alterations during postfixation treatment with the same series of KRP buffers as above. Likewise, varying the time of the postfixation treatment had virtually no effect (Table II).

If the cells were transferred to deionized water after a 60-min fixation with OsO₄ in isotonic KRP, some expansion of cell volume was noted. When GA or GA-OsO₄ was the fixing agent, the cells showed a tendency to shrink rather than to expand when moved into deionized water after fixation (Table IV).

The electronic particle size analyzer did not reflect the real size of the cells when they were fixed with either 3% GA or 1% OsO₄. If OsO₄ was used as a fixative agent the electric particle size analyzer graphs indicated significant shrinkage of

the fixed cells. When only GA was the fixative agent, some shrinkage was registered. In Fig. 9 an example is presented of the effect of 3% GA or 1% OsO₄ fixation on the cell size distribution curves when the fixative vehicle was deionized water. In this particular experiment, the nonfixed cells in isotonic KRP seemed to shrink from the volume of approximately 1,800 μm³ (graph A) to approximately 700 μm³ (graph C) when fixed with OsO₄ in deionized water, and to approximately 1,700 μm³ (graph B) when fixed with GA in deionized water. Based on microscope analysis, however, the values were 1,980 μm³ (unfixed cells), 3,260 μm³ (OsO₄-fixed) and 2,510 μm³ (GA-fixed cells), indicating a significant increase of the cell volume. This error of electric particle counter measurements on treated cells is related to change in membrane conductivity and is discussed below.

Effects of Sonication on Cell Volume and Cell Disruption

All the nonfixed cells were disrupted rapidly when they were sonicated using 9.7 g of sonic pressure (Fig. 10). Fixation for only 10 s with any of the three fixatives made the cells significantly more resistant, and prolongation of the fixation time to 60 min further increased the resistance to disruption; GA had the strongest stabilizing effect. After fixation for 10 s with GA, only about one-third of the cells was disrupted by 1-min sonication, and after 5 min of sonic stress about 22% of the cells still remained intact when viewed with a light microscope. GA-OsO₄- and OsO₄-fixed cells were disrupted more easily (Figs. 10 and 11). The diameters of the fixed, sonicated, but nondisrupted cells were of the same order as before

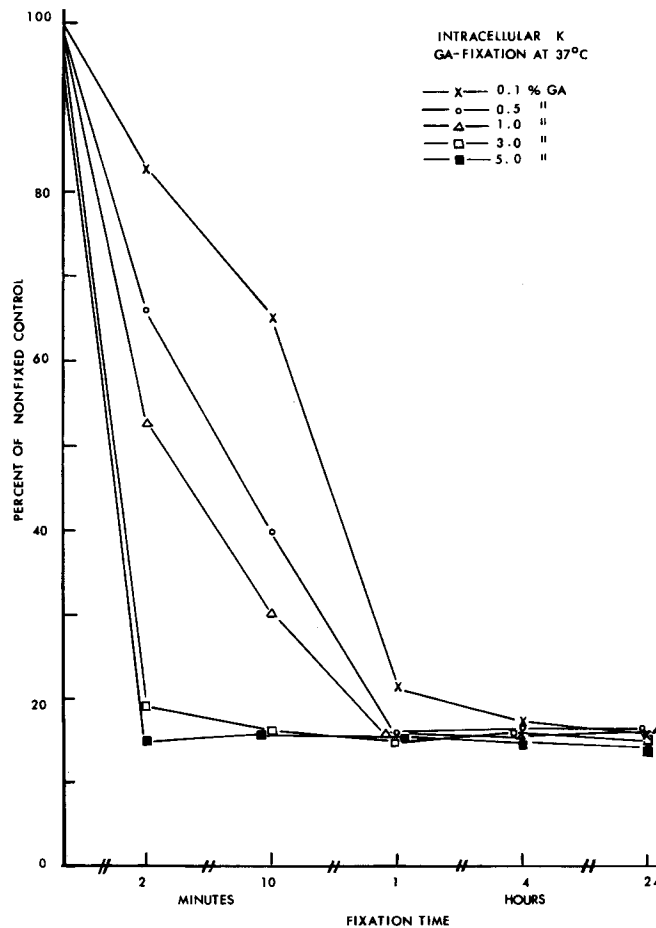


FIGURE 3

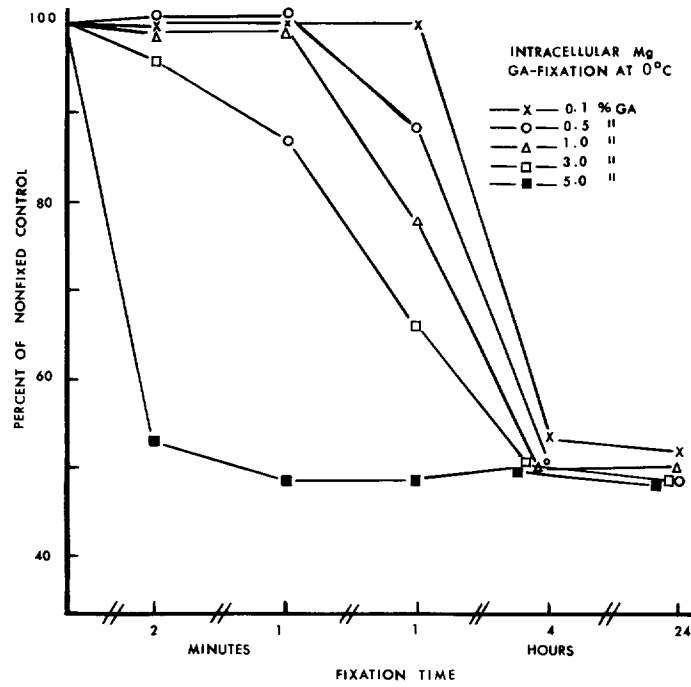


FIGURE 4

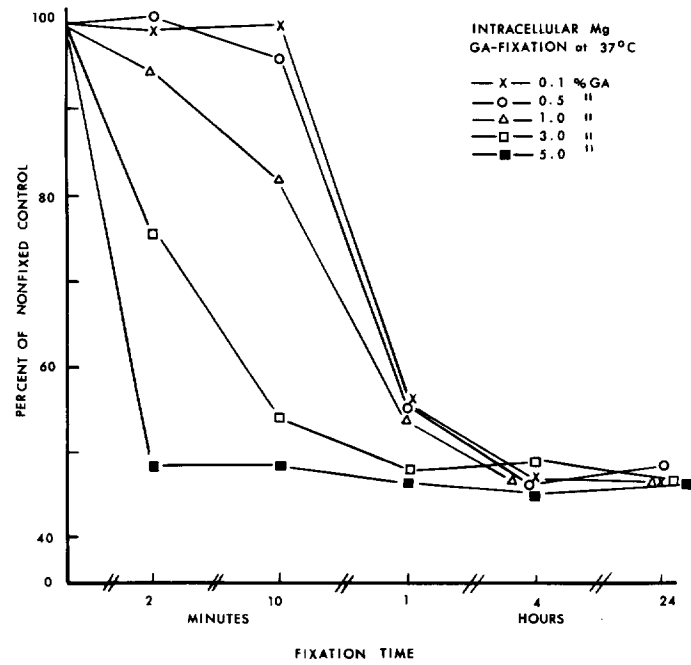


FIGURE 5

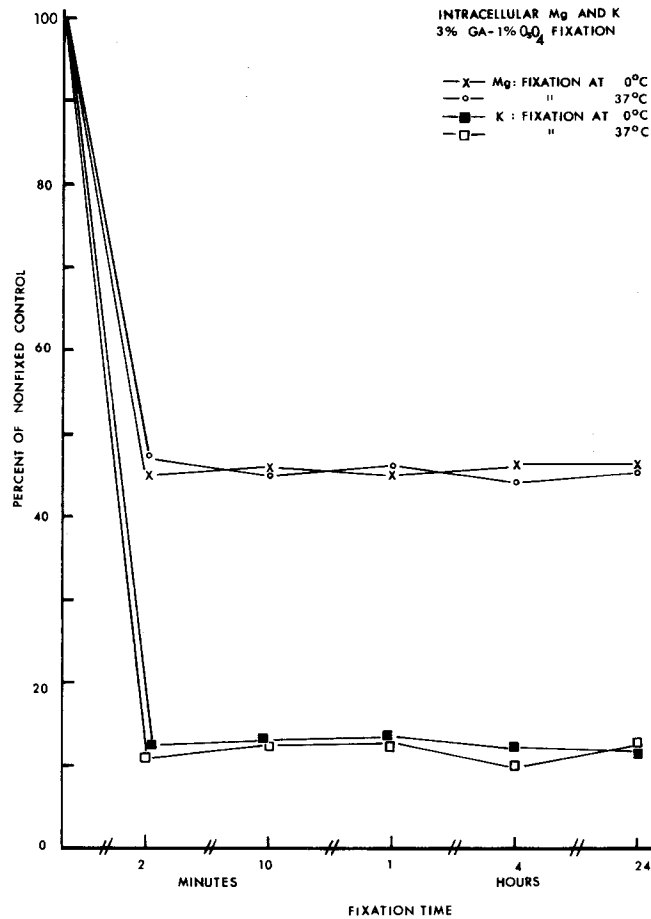


FIGURE 6

FIGURES 6 and 7 Effects of GA-OsO₄ and OsO₄ fixations on intracellular potassium and magnesium. 100% is the prefixation control value of the normal and nonfixed cells. Each point is a mean of two determinations.

sonication, or with longer sonication times, diameters became slightly smaller.

Effects of Freezing and Thawing on the Cell Volume

All nonfixed cells were disrupted when they were frozen with liquid nitrogen and thawed five times in a water bath. Fixation as short as 10 s with GA, OsO₄, or GA-OsO₄ followed by freezing and thawing caused neither disruption of cells nor any essential change in the cell volume (Table V).

Effects of Centrifugation Force on the Cell Volume of Fixed Cells

When the cells were fixed with 3% GA or 3% GA-1% OsO₄ for 1 h and thereafter exposed to 10,000 or 100,000 g centrifugation force for 10 min, no essential changes were found in cell volume (Table VI). OsO₄-fixed cells were clearly shrunken at 10,000 g but no additional shrinkage occurred at 100,000 g.

DISCUSSION

Ehrlich ascites tumor cells were chosen as a model in this study for several reasons. They have been

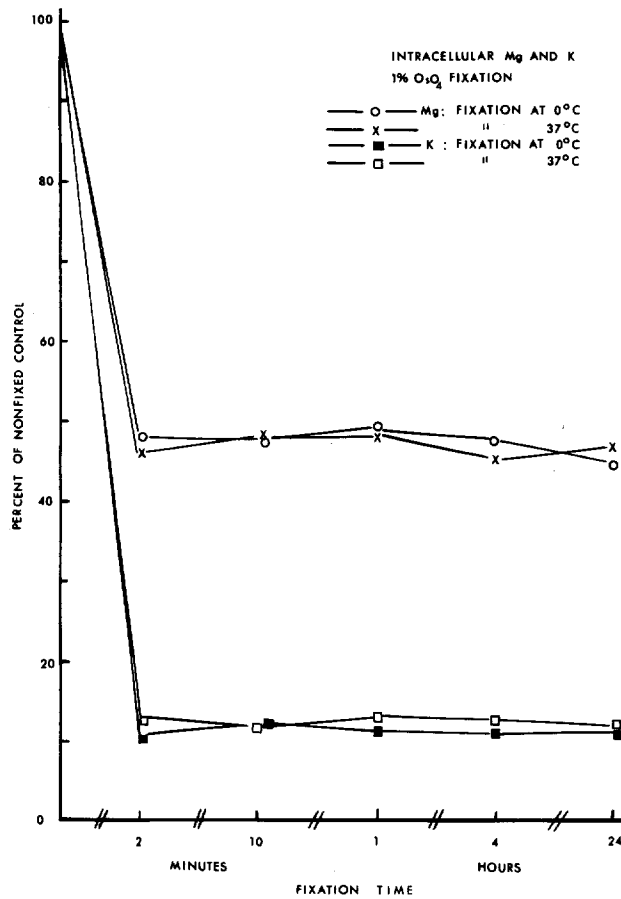


FIGURE 7

widely used in experimental cell biology and pathobiology, and thus the behavior of the cells is known under many experimental conditions. These cells contain organelles typical for most cells, they are practically spherical (19), and they can be directly, fairly homogeneously, and simultaneously exposed to injurious agents and fixation solutions. Therefore, the pathophysiological effects of the fixatives on the cells may be analyzed more precisely than is possible with tissues, where diffusion factors, intercellular space, etc., make analysis of intracellular composition much more difficult. The results presented in the present study cannot, of course, be applied directly to tissues for the same reasons. In this study the effects of only a few fixatives on cell size and cell membrane changes during and after fixation have been analyzed.

Effects of Fixatives on the Cell Membrane

The present study indicates that GA, GA-OsO₄, and OsO₄ in the concentrations commonly used in fixation for electron microscopy markedly increased the permeability of Ehrlich ascites tumor cells to several agents.

Vital dyes were able to pass the cell membrane almost immediately after exposure of the cells to fixative. Several earlier observations on Ehrlich ascites tumor cells from this laboratory (24, 38) indicate that the permeability of the cell membrane to vital dyes is closely associated with irreversible changes in the cells and is a good indicator of cell death resulting from various injurious mechanisms. In view of these observations the present results indicate that Ehrlich ascites tumor cells were lethally injured virtually immediately during

fixation when 3% GA and/or 1% OsO₄ were used as the fixation agents.

Intensity of staining with vital dyes has been shown to be dependent on the fixative used (12). This is doubtless the reason that the staining intensity of Ehrlich cells, especially after OsO₄ fixation, was heterogeneous. Gradual reduction or disappearance of stainability of the cells after fixation for 20 min with OsO₄ may be one

indicator of completeness of fixation since reaction of the fixative with components of the nucleus such as histones apparently renders the nucleus unstainable to vital dyes.

After suspending Ehrlich ascites tumor cells in 3% GA and/or 1% OsO₄ fixatives, cellular ATP disappeared within a few minutes. This result and the minimal intracellular potassium values obtained during the first minutes of fixation indicate that the active transport mechanism for monovalent cations and the passive permeability properties of the cell membrane for these ions are rapidly destroyed. Direct potassium and sodium determinations on the 1% OsO₄-fixed left ventricle of the rat heart (22) and on 3.3% GA-fixed human red blood cells (40) are in agreement with the present results.

The change in permeability to potassium of the cell membrane was dependent on the concentration and the temperature used for fixation in GA when the GA concentration was less than 3%. Because the cells lost potassium quite slowly even during fixation at 0°C it appears that the mechanism involves the passive permeability of the cell membrane for monovalent cations. It seems obvious that a certain number of GA molecules must react with the cell membrane, leading to a change in membrane conformation and in the loss of permeability properties. It seems reasonable that reaction velocity was reduced at lower temperature. Reduced activity might also indicate that the reactions between reactive groups of the cell membrane and GA are endothermic in nature (30).

The effect of GA fixation on intracellular magnesium was different from that on potassium. This could be due to different intracellular compartmentalization of the magnesium ions or to Mg binding to macromolecules. High levels of retained intracellular magnesium after OsO₄ and formaldehyde fixation have also been noted in rat heart muscle (22).

The cell membrane is almost exclusively responsible for the electrical resistance of the normal cell (4), and the operation of the electronic particle counter depends on the difference in electrical resistance between the electrolyte and the particles suspended in it. The present observations indicate that the electrical resistance of the cell membrane changed when the cells were fixed with GA or OsO₄. When OsO₄ was used as the fixative agent the membrane resistance was obviously lowered, resulting in cell size distribution curves which

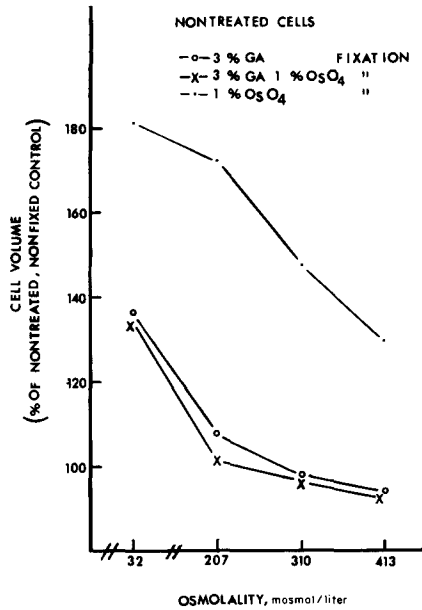


FIGURE 8 Volume changes of cells fixed with 3% GA, 1% OsO₄, or 3% Ga-1% OsO₄ made in various modifications of the KRP buffer. The fixation time was 1 h at 37°C. 100% represents the volume of nonfixed cells in isotonic KRP. Osmolality of the buffers used in fixation is presented on the axis.

TABLE I
Effects of 1-h GA Fixation on the Diameter of Normal Ehrlich Ascites Tumor Cells

| Treatment of cells | GA % | Total osmolality mosmol | Cell diameter | |
|-----------------------------|------|-------------------------|---------------|------------|
| | | | Mean μ m | SD μ m |
| Control | | 310 | 16.1 | 2.1 |
| 0.1-5.0% GA fixation in KRP | 0.1 | 319 | 16.3 | 1.9 |
| | 0.5 | 370 | 16.5 | 2.0 |
| | 1.0 | 419 | 16.4 | 2.4 |
| | 3.0 | 650 | 16.1 | 1.8 |
| | 5.0 | 865 | 15.5 | 1.9 |

TABLE II
Effects of GA and/or OsO₄ Fixation and the Buffer Osmolality of Postfixation Treatment on the Diameter of Ehrlich Ascites Tumor Cells

| Treatment of cells | Fixative | Osmolality of the buffers | | | Cell diameter | | | |
|--------------------|------------------------|---------------------------|------------------------|------------------------|-----------------------------|-----------------------------|-----------------------------|----------|
| | | Osmolality of fixative | Incubation or fixation | Postfixation treatment | Mean SD | | | |
| | | <i>mosmol/liter</i> | <i>mosmol/liter</i> | <i>mosmol/liter</i> | μm μm | μm μm | μm μm | |
| Control | — | — | 310 | — | 60 min | 16.5 2.0 24 h | 7 days | |
| Fixation | 3% GA | 737 | 413 | — | 16.2 2.5 | 16.5 2.1 | * | |
| | | 644 | 310 | — | 16.4 2.6 | 16.8 2.4 | 16.2 2.1 | |
| | | 540 | 207 | — | 16.9 2.1 | 17.4 2.0 | * | |
| | Postfixation treatment | | | 413 | 413 | 16.1 2.1 | 16.2 2.0 | * |
| | | | | 413 | 310 | 15.7 1.9 | 16.3 1.9 | * |
| | | | | 413 | 207 | 15.9 1.6 | 16.4 2.4 | * |
| | | | | 310 | 413 | 16.3 2.2 | 16.7 1.9 | * |
| | | | | 310 | 310 | 16.2 1.8 | 16.7 1.6 | 16.5 1.9 |
| | | | | 310 | 207 | 16.5 1.9 | 17.1 2.3 | * |
| | | | | 207 | 413 | 17.1 1.9 | 17.5 2.1 | * |
| | | 207 | 310 | 17.4 1.9 | 17.6 2.1 | * | | |
| | | 207 | 207 | 17.0 2.6 | 17.4 1.7 | * | | |
| Fixation | 3% GA | 778 | 413 | — | 16.1 1.5 | 16.2 2.2 | * | |
| | | 680 | 310 | — | 16.3 1.8 | 16.8 2.2 | 16.2 2.0 | |
| | | 576 | 207 | — | 16.6 1.7 | 17.1 2.5 | * | |
| | Postfixation treatment | | | 413 | 413 | 16.5 1.8 | 16.6 2.1 | * |
| | | | | 413 | 310 | 16.7 2.3 | 17.0 1.9 | * |
| | | | | 413 | 207 | 16.7 2.2 | 16.4 1.8 | * |
| | | | | 310 | 413 | 16.6 2.5 | 16.3 1.9 | * |
| | | | | 310 | 310 | 16.9 1.8 | 16.7 2.1 | 17.3 1.8 |
| | | | | 310 | 207 | 17.0 1.9 | 16.7 1.6 | * |
| | | | | 207 | 413 | 17.2 2.4 | 17.1 1.8 | * |
| | | 207 | 310 | 16.8 2.5 | 17.2 2.1 | * | | |
| | | 207 | 207 | 17.5 1.9 | 17.1 1.8 | * | | |
| Fixation | 1% OsO ₄ | 454 | 413 | — | 18.0 1.9 | 18.1 2.1 | * | |
| | | 347 | 310 | — | 18.8 2.4 | 18.6 2.5 | 18.5 2.1 | |
| | | 246 | 207 | — | 19.8 2.4 | 19.5 2.0 | * | |
| | Postfixation treatment | | | 413 | 413 | 18.1 2.2 | 18.5 1.8 | * |
| | | | | 413 | 310 | 17.8 1.7 | 18.0 1.9 | * |
| | | | | 413 | 207 | 18.0 2.8 | 18.5 2.1 | * |
| | | | | 310 | 413 | 18.3 2.3 | 19.2 2.5 | * |
| | | | | 310 | 310 | 18.7 2.4 | 19.0 2.3 | 18.8 2.2 |
| | | | | 310 | 207 | 18.7 2.2 | 19.3 2.4 | * |
| | | | | 207 | 413 | 19.2 2.7 | 19.2 1.9 | * |
| | | 207 | 310 | 19.3 2.8 | 19.2 1.9 | * | | |
| | | 207 | 207 | 20.0 2.2 | 19.6 2.3 | * | | |

* Not determined.

indicated a prominent "reduction" in the size of the cells. However, the opposite results were obtained with direct light-microscope mensuration. A similar phenomenon in human erythro-

cytes after OsO₄ fixation was presented by Carstensen et al. (10), but they did not give any comparative light-microscope data. The membrane-bound OsO₄ is in itself conductive, thus,

together with membrane injury it may reduce the membrane's resistance to electric current. When 3% GA was used as a fixation agent, only small changes between the results obtained with an electric particle counter and light-microscope methods were noted when the osmolality of the vehicle was isotonic or slightly hyper- or hypotonic. The difference increased significantly when the cells were fixed with 3% GA in very hypotonic media (32 mosmol). It is possible that the effect of osmotic stress on the membrane is more important than the effect of the fixation agent itself during GA fixation. The cells lost about 85–90% of intracellular potassium almost immediately during GA fixation in isotonic buffer. It seems, therefore, that the membrane resistance and the potassium

permeability during and after GA fixation are different properties of the cell membrane, making it difficult to draw conclusions concerning the concentrations of intracellular ions on the basis of electrophysiological observations on GA- and OsO₄-fixed tissues (11, 13).

Osmotic Effect During Fixation

When the cells were fixed in hypo- or hypertonic media they were exposed to (a) the osmotic stress caused by the vehicle of the fixation agent; (b) the osmotic stress of the fixative itself; and (c) the fixation effect (chemical reaction) between the fixation agent and reactive groups in the cell.

Earlier studies indicate that the osmolality of the fixative agent, i.e., aldehyde or OsO₄, does not

TABLE III
Effects of OsO₄ Postfixation on the Cell Diameter of GA-Fixed Ehrlich Ascites Tumor Cells

| Treatment of cells | No fixation | | Osmolality of the buffer in fixation or postfixation | Postfixation in 1% OsO ₄ for 1 h | | | |
|--------------------|---------------|---------|--|---|---------|---------------|---------|
| | Cell diameter | | | Fixation in 3% GA for 1 h | | Cell diameter | |
| | Mean | SD | | Mean | SD | Mean | SD |
| | μm | μm | <i>mosmol</i> | μm | μm | μm | μm |
| Control | 16.5 | 1.6 | 310 | 16.4 | 1.8 | 16.3 | 1.5 |

TABLE IV
Effects of GA and/or OsO₄ Fixation and the Postfixation Treatment in Deionized Water on the Diameter of Ehrlich Ascites Tumor Cells

| Treatment of cells | Fixation or postfixation treatment | Cell diameter | | | |
|--|--|---------------|---------|------|------|
| | | Mean | SD | Mean | SD |
| Control | — | μm | μm | | |
| 31 mosmol KRP buffer for 2 min | — | 16.4 | 1.9 | | |
| | | 26.7 | 3.1 | | |
| | | | 1 h | | 24 h |
| Fixation of cells in: | 3% GA in 31 mosmol KRP | 18.2 | 2.3 | 18.5 | 2.1 |
| | 3% GA + 1% OsO ₄ in 31 mosmol KRP | 18.1 | 1.9 | 17.9 | 2.1 |
| | 1% OsO ₄ in 31 mosmol KRP | 20.0 | 2.3 | 19.5 | 2.4 |
| Control | — | 16.3 | 1.9 | | |
| Fixation of cells in isotonic KRP buffer for 1 h | 3% GA | 15.9 | 1.7 | | |
| | ↓ water | 15.8 | 1.8 | | |
| Postfixation treatment in water for 24 h | 3% GA + 1% OsO ₄ | 16.2 | 2.1 | | |
| | ↓ water | 15.6 | 1.7 | | |
| | ↓ 1% OsO ₄ | 18.3 | 2.1 | | |
| | ↓ water | 19.6 | 2.4 | | |

seem to have much effect on the volume of tissues during fixation (6, 7, 16, 27). When the total fixative osmolality, osmolality of the GA or GA-OsO₄, osmolality of the buffer, and registered volumes in various GA and GA-OsO₄ fixations (Table II) are compared, the same conclusion also seems to apply to Ehrlich ascites tumor cells. The behavior of the cells during fixation with OsO₄ alone as the fixing agent is different and will be discussed later.

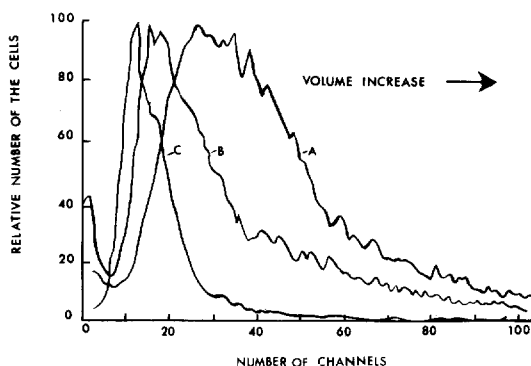


FIGURE 9 Cell size distribution graphs of nontreated, nonfixed cells in isotonic KRP (graph A), and nontreated cells fixed in 3% GA (B) or in 1% OsO₄ (C) in deionized water for 10 min at 37°C. Cell size distributions were determined in isotonic (310 mosmol) KRP. One channel corresponds to about 60 μm in the case of the nonfixed and OsO₄-fixed cells, and the corresponding value of GA-fixed cells (graph B) is approximately two times larger. The graphs are copies of original data made by Coulter Counter R model ZBI particle size analyzer and model C-1000 channelizer.

Cell size during fixation appears, then, to be determined chiefly by a net force related to the osmotic stress resulting from the balance between the buffer and the cell interior, the strength of the bonds resulting from reactions between the fixation agent and cellular components, the loss of semipermeable properties of the cell membrane, and the number of osmotically active particles which might be formed during fixation in the cell interior.

Observations on nonfixed Ehrlich cells in hypotonic media indicate that the volume of these cells has an almost linear correlation with the reciprocal of the incubation medium and that increases in volume of up to four to five times that of normal cells are possible without disruption (Penttila and Trump, unpublished observations). The cell membrane is thought to be freely permeable to water, and so even quite small changes in the osmolality of the fixative buffer would be expected to produce readily observable volume changes if the semipermeable properties of the cell membrane were intact. The great difference between the intra- and extracellular osmotic pressures that exist when cells are suspended in very hypotonic media causes some initial swelling; soon thereafter the cell membrane loses its permeability characteristics, at least with respect to small molecules. Finally, the reaction between the cell proteins and fixatives renders the cell rigid and resistant to various kinds of stresses.

When OsO₄ alone was used as a fixative, its effect was to make the cell membrane extremely

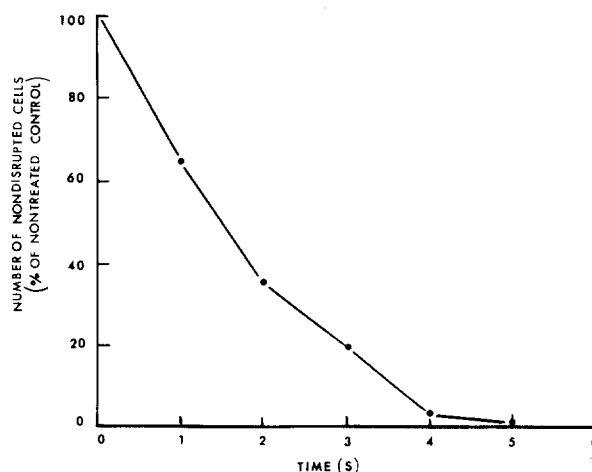


FIGURE 10 Effects of sonicating on the nonfixed cells. The graph presents counts of light-microscopically intact cells in percent after sonicating. 100% is the presonation cell count. Each point is the mean of five determinations. The time of processing is on the axis.

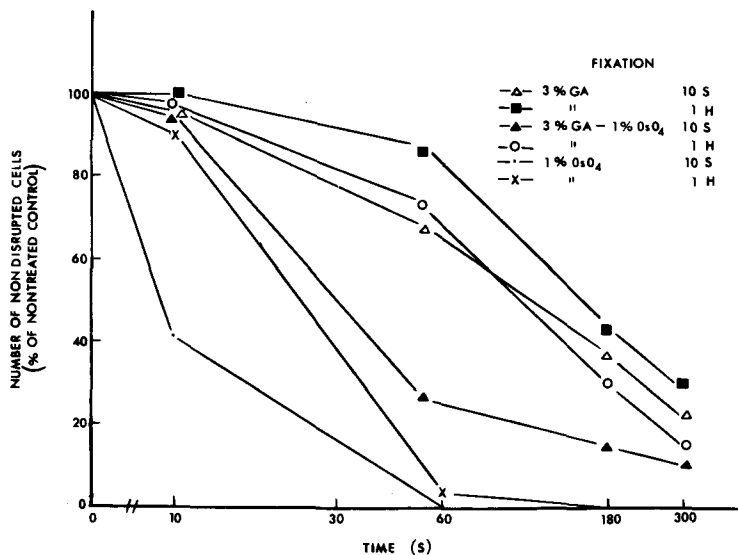


FIGURE 11 Effects of sonication on the fixed cells. Fixation agent and time of fixation is marked for each curve. See Fig. 10 for other explanations.

TABLE V
Effects of Freezing and Thawing on the Diameter of Fixed Ehrlich Ascites Tumor Cells

| Treatment of cells | Fixation time | Cell diameter | |
|---|---------------|---------------|---------------|
| | | Mean | SD |
| | | μm | μm |
| Nonfixed control cells, no freezing and thawing | — | 16.3 | 2.0 |
| 3% GA in KRP | 10 s | 16.1 | 2.1 |
| | 60 min | 16.7 | 2.3 |
| 1% OsO ₄ in KRP | 10 s | 18.5 | 2.6 |
| | 60 min | 19.1 | 2.5 |
| 3% GA-1% OsO ₄ in KRP | 10 s | 16.3 | 1.9 |
| | 60 min | 16.5 | 2.3 |

TABLE VI
Effects of Centrifugation Force on the Diameter of Fixed Ehrlich Ascites Tumor Cells

| Treatment of cells | Centrifugation 10 min | Cell diameter | |
|------------------------------------|-----------------------|---------------|---------------|
| | | Mean | SD |
| | | μm | μm |
| Control, no fixation | | 16.1 | 2.1 |
| 3% GA fixation | | 15.8 | 2.2 |
| | 10,000 | 15.9 | 2.0 |
| | 1000,000 | 15.8 | 2.2 |
| 3% GA-1% OsO ₄ fixation | | 16.0 | 1.9 |
| | 10,000 | 15.8 | 1.8 |
| | 1000,000 | 15.7 | 1.7 |
| 1% OsO ₄ fixation | | 17.6 | 2.6 |
| | 10,000 | 16.6 | 2.5 |
| | 1000,000 | 16.6 | 2.1 |

leaky to potassium and presumably sodium. This leads to so-called colloid osmotic lysis because of the Donnan effect. Since OsO₄ reacts rather slowly with protein it is possible that the intracellular protein is not significantly altered and that cell expansion occurs as total cation increases, water following, as in the case of many well-studied membrane-damaging agents such as complement lysis or treatment with nonpenetrating mercurials such as *p*-chloromercuribenzenesulfonic acid (35). When, however, GA was used as the fixative agent, the membrane also became leaky, although cell expansion was much less dramatic. This could be due either to a cross-linking effect of GA on

intracellular proteins which effectively could reduce the number of negatively charged particles, or to the fact that the cell was strengthened through extensive protein cross-linking and thus was able to withstand the hydrostatic pressure gradient, as is the case with normal plant cells. The present experiments do not permit a distinction to be made between these two possibilities.

Osmotic Properties of the Cells After Fixation

Ion leakage and the loss of ATP and of stainability of the cells with vital dyes indicate that the

cell membrane is leaky to monovalent cations and vital dyes and that no active transport will occur. On the basis of this and the stability of the fixed cells against mechanical and thermal stress, it was expected that treatment of the cells after fixation in hyper- or hypotonic solutions would not cause significant cellular volume changes. Such insensitivity of the cells to osmotic stress after fixation was confirmed. This indicates that through reactions with cellular components, GA and/or OsO₄ stabilize the colloid-osmotic properties of the cell interior, rendering it insensitive to osmotic stress. Carstensen et al. (9) showed, by using electrical methods, that human red blood cells became osmotically inactive after GA fixation. However, there are some conflicting reports. Millonig (28) reported that GA-fixed sea urchin eggs were osmotically sensitive. Bone and Denton (6), who used the reflecting scales of the herring, found that the wavelength of light reflected by the array of guanine platelets within the cells changed in solutions of different osmolalities after GA fixation. They concluded that these changes represent osmotic volume changes and are an indication of preserved osmotic activity of the GA-fixed cells. Furthermore, Bone and Ryan (7) showed that GA-fixed crab axons remained osmotically active as evidenced by morphometric measurements. We do not know the explanation for this discrepancy; however, in both of these models the cells may have fewer intracellular protein structures per unit volume, thus precluding stabilization by protein cross-linking. In the fish scale model the cells are occupied by guanine crystals, and in the axon system the hyaloplasm is watery with few organelles.

Practical Implications

Although the fixation of individual cells differs from that of intact tissues, some general practical implications regarding fixation probably can be derived from the present results. Immediate loss of semipermeable properties of the cell membrane and stabilization of cell structure indicate a very rapid effect of a fixative agent on individual cells. The 1-h fixation time commonly used for electron microscope studies on Ehrlich cells (14, 24) could probably be reduced.

GA has been shown to be an effective fixative in electron microscopy in as low a concentration as 0.25% when perfusion techniques have been used (27). The Ehrlich ascites tumor cells retained their

volume when fixed with 0.1% GA, even though the ion leakages were delayed. It seems probable that the 3% concentration of GA, which is commonly used for individual cells (14, 24), could be reduced.

In our studies, the osmolality of the fixative agent, i.e., aldehyde or OsO₄, in the range tested seemed to be of negligible importance. The fixing effect of GA and/or GA-OsO₄ seems to be rapid and strong in that significant variations in the osmolality (up to 100 mosmol) of the buffer did not seem to have any greater effect; the use of very hypotonic buffers, however, should probably be avoided. Even though the results on individual cells are not necessarily directly applicable to tissues, the same basic facts may apply to tissues. This is suggested by the fact that good preservation of cellular structures in tissue pieces is obtained by different workers using fixative compositions (6, 7, 16, 18, 24) which vary within the above mentioned range.

As discussed above, the cells expanded considerably during OsO₄ fixation even in hypertonic buffers. It might be beneficial to add rather large molecules to the vehicle of the OsO₄ fixatives which could exert an osmotic effect counteracting the cellular expansion. Addition of sucrose to OsO₄ fixatives, as suggested by some workers (3, 16), might also be a choice for Ehrlich ascites tumor cells. Because of its tendency to increase cell volume, primary OsO₄ fixation is considered unsuitable for morphometric studies.

GA seems to have a considerably better stabilizing effect than OsO₄, and even GA-OsO₄-fixed cells were not as stable against mechanical stress (sonication) as purely GA-fixed cells. Obviously, the rapid chemical reaction between GA and OsO₄ (17) potentially reduces also the fixation power of both fixatives which, however, in the case of isolated cells, seems to be of minor importance.

The authors are grateful to Dr. Ruth E. Bulger and Dr. Elizabeth M. McDowell for their suggestions and criticisms of the manuscript.

This research was supported by grants AM-10698 and 5FO5 TW01985-02 from the National Institutes of Health.

Received for publication 4 February 1974, and in revised form 1 May 1974.

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