THE ANATOMICAL RECORD 252:254–263 (1998)

Morphology of Human Neutrophils: A Comparison of Cryofixation, Routine Gluteraldehyde Fixation, and the Effects of Dimethyl Sulfoxide

CHARLES S. GILBERT AND RICHARD T. PARMLEY*
Carolinas Medical Center, Department of Pediatric Research,
Charlotte, North Carolina 28203

ABSTRACT

Cell shape and density are critical to the evaluation of neutrophil function and/or activation. Dimethyl sulfoxide-cryofixation-freeze-substitution processing (DCF) instantly preserves cell processes and ultrastructural elements with fewer artifacts than routine chemical fixation with glutaraldehyde and postfixation osmium tetroxide (GO). This study morphometrically examined density-separated neutrophils to assess differences in DCF and GO processing procedures and studied the effect of dimethyl sulfoxide followed by GO fixation (DGO) on morphology.

Fifteen consecutive neutrophils were analyzed using computerized planimetry for differences in DCF v. GO treatments (n = 4) and DGO v. GO treatments (n = 4). Cryofixed and DGO-fixed cells were significantly rounder than GO cells which had a more irregular surface with membrane projections. The cell volume of GO cells was 27–30% smaller than in DCF or DGO processing, while the surface area was similar. The increased volume in DCF and DGO cells did not appear to be due to abnormal cell swelling, since membranes, nuclear envelope, and mitochondrial cristae were more intact than in GO cells. Preservation of mitochondria as well as endocytic caveolae with a subplasmalemmal coating was best in DCF samples, moderate in DGO, and poorest in GO. Morphometric data showed that the nuclear compartment was 22% smaller, while the cytoplasm (and its associated compartments) was 29% smaller in GO compared to DCF-processed neutrophils. This was consistent with the more dense cytoplasm in GO cells. Pretreatment of neutrophils with dimethyl sulfoxide (DMSO) resulted in volume preservation and improved the morphology of GO fixation.

In summary, DCF appears to be an excellent method for preserving neutrophil membranes and cytoplasmic organelles (particularly mitochondria), and prevents a number of artifacts caused by routine GO fixation. Morphology can also be improved by using DMSO in conjunction with GO. Anat. Rec. 252:254–263, 1998.

Key word: **neutrophils (human); cryopreservation; morphometry; electron microscopy; dimethyl sulfoxide; fixation; glutaraldehyde**

Chemical fixation, using glutaraldehyde, has been comparatively studied in several different tissue types and species. Glutaraldehyde solutions are generally considered to be hypertonic, resulting in some osmotic shock and tissue shrinkage (Hayat, 1989; Lee, 1984). Morphometric methods have been used to measure (linearly) tissue shrinkage with glutaraldehyde fixation of mouse ova and demonstrated approximately a 3% decrease in diameter and up to a 12% decrease when embedded in Epon (Konwinski et al., 1974). Hillman and Deutsch (1978)

*Correspondence to: Dr. Richard T. Parmley, Carolinas Medical Center, Department of Pediatrics, 1000 Blythe Blvd., Charlotte, NC 28203. Fax: (704) 355–8424. E-mail: rparmley@carolinas.org

Received 7 January 1998; Accepted 5 May 1998

reported a 20–30% decrease in tissue cross-sectional areas of rat brain with glutaraldehyde fixation and ethanol dehydration. Similarly, cell volume changes of 10–30% were reported in cultured rat smooth muscle tissue (Lee et al., 1982). These area and volume differences related to chemical fixation need to be understood and minimized so that comparisons between fixed tissue and living tissue may be made. Cryofixation and freeze-substitution is a technique that can be used in such comparisons, since it better preserves cell ultrastructure and antigenicity (Aldrich, 1989; Hayat, 1989).

Several studies have compared the morphology of cryopreservation methods with conventional glutaraldehyde fixation in nonhematologic tissues (Benchimol, 1994; Meissner and Schwarz, 1990), normal human peripheral blood basophils (Hastie, 1990), and nonhuman lymphocytes (Pfaller and Rovan, 1978). Improved ultrastructural preservation was reported; however, these cells were not analyzed morphometrically nor have human neutrophils been investigated with these two fixation methods. The purpose of this study was to evaluate the general differences in normal human peripheral blood neutrophils and their cellular components preserved using routine procedures of dimethyl sulfoxide (DMSO, a cryoprotectant)-cryofixation-freeze substitution (DCF) and glutaraldehyde-postglutaraldehyde osmium tetroxide (GO) and to examine the effect of DMSO on GO-fixation (DGO) morphology. A concentration of 10% DMSO has some practical significance in that this is the concentration used to freeze and store bone marrow for subsequent biologic and transplant studies (Herzig, 1981). Portions of this paper have been presented in abstract form (Gilbert and Parmley, 1993).

MATERIALS AND METHODS

Specimen Preparation

Peripheral blood was collected in heparin or EDTA from six male donors (on one or more occasions) by venipuncture after obtaining informed consent. Blood was centrifuged at 500 × g for 45 min to separate the granulocytes using a one-step density gradient (Kalmar et al., 1988). The granulocytes, designated as fraction 2 (FR2) cells, were aspirated into a siliconized Pasteur pipette. These FR2 cells were then washed twice in RPMI 1640 cell culture medium (Sigma, St. Louis, MO; 286 mOsm; centrifuged at 2,000 rpm for 5 min at room temperature) and divided into aliquots.

Abbreviations

DCF	dimethyl sulfoxide-cryofixation-freeze-substitution
DMSO	dimethyl sulfoxide
GO	glutaraldehyde-post-glutaraldehyde-osmium tetroxide
DGO	DMSO pretreatment - glutaraldehyde-post-glutaraldehyde-osmium tetroxide
Ac	cross-sectional area of the cell
Pc	cross-sectional perimeter of the cell
FFc	form factor of the cell
Svc	surface to volume ration of the cell
Vc	cell volume
Sc	cell surface area
sd	standard deviation
UALC	uranyl acetate - lead citrate
V_V	volume fraction
fl	femtoliter

Glutaraldehyde-Osmium Tetroxide Fixation (GO)

A matched simultaneous portion of FR2 cells (n = 8) was fixed in 3% glutaraldehyde in 0.1 M cacodylate, pH 7.35 (total osmolarity of fixative/buffer = 554 mOsm; buffer alone = 187 mOsm) for 1 hr at 4–6°C. An additional three experiments compared morphology from specimens fixed in 1% glutaraldehyde in cacodylate buffer (318 mOsm); and 1% glutaraldehyde in cacodylate buffer with 0.1 M sucrose added (419 mOsm; buffer alone = 307 mOsm). Osmolarity of the buffers and fixative solutions was measured using a freezing-point depression osmometer (Osmette A, model 5002; Precision Systems, Inc., Natick, MA). Glutaraldehyde-fixed cells were washed and stored in 0.1 M cacodylate buffer containing 7% sucrose, pH 7.35. Samples were postfixed in 1% OsO_4 in 0.1 M cacodylate, pH 7.35 for 1 hr at room temperature. Cells were routinely dehydrated in graded ethanols, propylene oxide, and embedded in Spurr's low viscosity resin.

Dimethyl Sulfoxide-Cryofixation-Freeze-Substitution (DCF)

One heparinized simultaneous GO-matched portion (n = 4) was resuspended in RPMI with 10% dimethyl sulfoxide (DMSO) for 10–15 min at room temperature. A drop of this cell suspension 1–2 mm in diameter was placed on a Teflon disc attached to a specimen rod. This sample was cryofixed using the Eiko RF-2 (Eiko Engineering, Ltd., Mito, Japan) slam-freezing device. This device has a gold-plated copper block that is cooled to liquid nitrogen (LN2) temperatures. The frozen sample was stored in LN2 for up to 12 days and processed (batched) for freeze-substitution. Specimens were processed in 4% OsO_4 in acetone for 48 hr at -80°C. The temperature of these samples was incrementally raised to room temperature: 2 hr to -40°C, 1 hr to -30° C, 1 hr in a -20°C freezer, 1 hr in a refrigerator at 4°C, and 1 hr at room temperature. Cells were washed in three changes of anhydrous acetone for 15 min each and three changes of propylene oxide for 20 min each. The cells were placed in 1:1 propylene oxide/Spurr's resin for 2 hr, a 2:1 mixture overnight, and full Spurr's resin for 4 hr followed by embedment in Spurr's resin. Since the cells did not adhere together, they were embedded as a homogeneous cell suspension as were the other samples processed in this study.

Dimethyl Sulfoxide-Glutaraldehyde-Osmium Tetroxide Fixation (DGO)

A simultaneous GO-matched portion of FR2 cells in EDTA (n = 4) were resuspended (as above) in RPMI with 10% DMSO for 10–15 min, centrifuged, and fixed in 3% glutaraldehyde and processed as above in GO fixation.

Electron Microscopy

Thin sections (60–90 nm) of morphologic preparations were cut using a diamond knife on an Ultrotome NOVA (LKB, Bromma, Sweden) or a Reichert Ultracut S (Leica, Inc., Deerfield, IL) ultramicrotome. Sections were collected on 200 mesh copper grids. The grids were routinely counterstained with methanolic uranyl acetate (UA) and aqueous lead citrate (LC) as described previously (Gilbert et al., 1993). Grids were evaluated in a Zeiss EM109 electron microscope (Carl Zeiss, Inc., Thornwood, NY) at

50 kV or a Philips CM-10 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ) at 80 kV.

Morphometric Analysis

The profiles of 15 consecutive evaluable neutrophils were scored for each DCF and DGO preparation along with their simultaneous GO controls. Cells were evaluated if they had two or more nuclear lobes, identifiable neutrophil granules, and were otherwise evaluable. The cells were photographed at an original magnification of 7,000× and enlarged 3.2 times (Zeiss EM109) or at 6610× and enlarged 2.55–2.75 times (Philips CM10) using standard darkroom techniques.

The plasmalemma was outlined using a Numonics 2200 electromagnetic tablet connected to an IBM-compatible computer, SigmaScan v3.9 software (Jandel Scientific, San Rafael, CA), and a magnifying lamp. The cross-sectional areas (A) and perimeters (P) of the cell, nuclei and mitochondria were recorded for each cell. The nucleus was outlined at both the inner (IM) and outer (OM) membranes of the nuclear envelope. Mitochondria were measured at the outer membrane. Cell area and perimeter were recorded for DGO-processed cells and their simultaneous GO control.

The cytoplasmic area was calculated from the difference between the total cell area and the OM nuclear area. Volume fraction (V_V) was determined by the area fraction (A_A) based on the standard morphometric relation, $V_V = A_A$, and reported as percent (Aherne and Dunnill, 1982). A cell form factor [$FF_C = (4\pi A)/P^2$] with a value of 1.0 being a perfect circle (Fernandez-Segura et al., 1995) and the cell's surface/volume density [$S_{VC} = (4/\pi)(P/A)$] (Weibel and Bolender, 1973) were calculated. Cell volume [$V_C = (4/3)\pi r^3$] was calculated assuming the cell to be a sphere with radius based on profile area [$r = (3A/2\pi)$] (Sokol et al., 1987). This assumption is considered appropriate even when cells deviate from sphericity (Weibel and Bolender, 1973). Cell surface area [$S_C = (S_{VC})(V_C)$] was calculated as described previously (Sokol et al., 1987; Weibel and Bolender, 1973). Organelle volumes were determined by multiplying the V_C by the V_V for the specific organelle (Sokol et al., 1987; Weibel and Bolender, 1973).

Plates were made of a calibration grid and the calculated magnification was then used in a correction factor (Expected magnification/Actual magnification) for the data. Measured areas were multiplied by the square of the correction factor. Linear measures were multiplied by the first-order correction factor.

Statistical Analysis

Data analysis was done using Number Cruncher Statistical Software v5.0 (NCSS, Dr. J. L. Hintze, Kaysville, UT) on an IBM-compatible computer. Graphing was done in Grapher for Windows v1.0 (Golden Software, Golden, CO). Data were initially screened using ANOVA and nested ANOVA models for analysis. Nested analysis looked at the treatment and donor variables. This design examined paired simultaneous donor samples varying only in treatment. These data were clustered in two ways: by treatment pooling individual cells (n = 60) and treatment by donor/experiment (n = 4). These differing views show no difference in mean value; only the variance was different with the treatment cluster appearing to show the population variance of human neutrophils while the donor cluster showed the variance of donor means.

TABLE 1. Morphological Comparison of Fixation[a]

	3% GO[b]	Pre-DMSO 3% GO (DGO)	DCF (RPMI)
Plasma membrane	2	2–3	4
Coated pits	1	3–4	4
Cytoplasmic matrix	2	2–4	4
Golgi membranes	1–3	2–3	4
Mitochondria	1–3	2–3	4
Microtubules	1	1–4	4
Nuclear pores	1–2	2–3	4
Perinuclear space	1–3	1–4	4
Cell shape (ref: circle)	2–3	4	4

[a]Cell organelles/characteristics were graded according to the following scheme: 1, structures were disrupted, obscured, rarely seen or absent; 2, structures were partially visible; boundaries are noncontinuous; 3, structures were visible; boundaries are continuous and visible but not well-defined; 4, structures were prominent and well-defined.
[b]For abbreviations, see list.

Two treatments were compared in each experiment analyzed using two-tailed *t*-tests, Mann-Whitney, normality, and homogeneity of variance testing. Normality of the data was checked using histogram plots and the measures of skewness and kurtosis. When adherence to the parametric assumptions were weak, a Mann-Whitney test (nonparametric analog of the unpaired *t*-test) was used. However, parametric and nonparametric tests both offered similar results, with the *t*-test most often yielding a smaller *P*-value.

RESULTS

The ultrastructural cytochemistry and morphology of neutrophils in this study was similar to that described previously for glutaraldehyde-fixed specimens (Bainton et al. 1971; Zucker-Franklin, 1968). Generally, the reports of GO-processed neutrophils describe numerous cytoplasmic granules, poorly developed Golgi body, rare and small mitochondria, and rare microtubules. As outlined below, pretreatment with DMSO offered improved morphology when followed by GO fixation, while DCF processing offered the best morphology even though some cells showed visible ice crystal formation. A morphologic comparison of GO, DGO, and DCF fixed neutrophils is presented in Table 1.

The total osmolarity of the fixative solution appeared to have a significant effect on the neutrophils studied here. The 1% glutaraldehyde in 0.1 M cacodylate buffer with a total osmolarity of 318 mOsm offered some improvement in cell density and shape over 3% glutaraldehyde (total: 554 mOsm; 0.1 M cacodylate: 187 mOsm). When the buffer was made isotonic (0.1 M cacodylate with 0.1 M sucrose: 307 mOsm) in 1% glutaraldehyde (419 mOsm), the cell density was equivalent, while the cell shape was less round and inferior to the GO-fixed cells.

The neutrophils appeared rounder and larger with DCF (Fig. 1a) and DGO (see Fig. 1c) than in GO samples (Fig. 1b). Monocytes fixed with DGO appeared less rounded than neutrophils (Fig. 1c). Morphometric evaluations of cell volume indicated that the volume of neutrophils was greater with DCF or DGO samples when compared to GO samples. The cell volume was about 27–30% smaller in GO-processed cells. In contrast, the cell surface area of all matched cells (paired experiments) was similar regardless of fixation. The differences between paired samples of

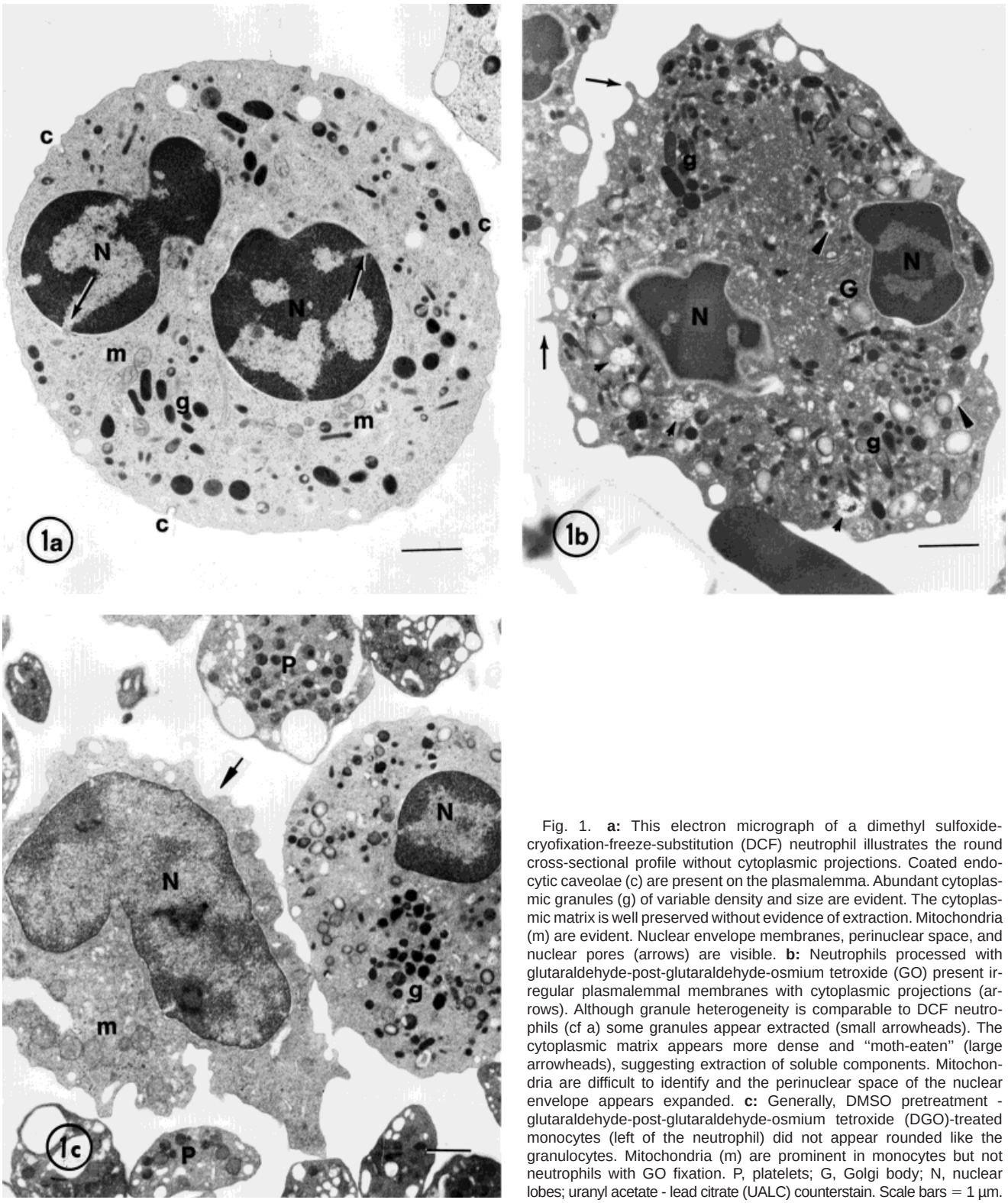


Fig. 1. **a:** This electron micrograph of a dimethyl sulfoxide-cryofixation-freeze-substitution (DCF) neutrophil illustrates the round cross-sectional profile without cytoplasmic projections. Coated endocytic caveolae (c) are present on the plasmalemma. Abundant cytoplasmic granules (g) of variable density and size are evident. The cytoplasmic matrix is well preserved without evidence of extraction. Mitochondria (m) are evident. Nuclear envelope membranes, perinuclear space, and nuclear pores (arrows) are visible. **b:** Neutrophils processed with glutaraldehyde-post-glutaraldehyde-osmium tetroxide (GO) present irregular plasmalemmal membranes with cytoplasmic projections (arrows). Although granule heterogeneity is comparable to DCF neutrophils (cf a) some granules appear extracted (small arrowheads). The cytoplasmic matrix appears more dense and "moth-eaten" (large arrowheads), suggesting extraction of soluble components. Mitochondria are difficult to identify and the perinuclear space of the nuclear envelope appears expanded. **c:** Generally, DMSO pretreatment - glutaraldehyde-post-glutaraldehyde-osmium tetroxide (DGO)-treated monocytes (left of the neutrophil) did not appear rounded like the granulocytes. Mitochondria (m) are prominent in monocytes but not neutrophils with GO fixation. P, platelets; G, Golgi body; N, nuclear lobes; uranyl acetate - lead citrate (UALC) counterstain. Scale bars = 1 µm.

DGO-processed cells appeared similar to DCF-processed paired cells and cell volume did not differ significantly between them while being significantly different from standard GO-processed cells (Fig. 2).

In DCF cells, the plasma membrane appeared distinctly trilaminar (Fig. 3a) and averaged 150 angstroms. This contrasted with GO specimens in which membranes were difficult to clearly identify (Fig. 3b) and adjusting osmolar-

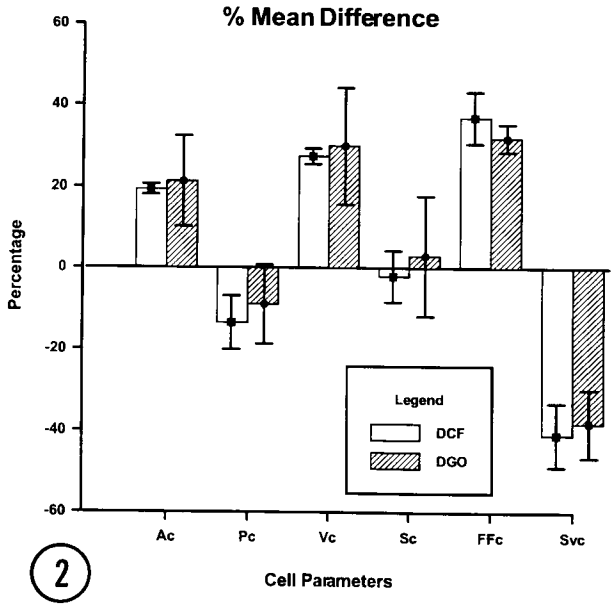


Fig. 2. This graph shows, in order, the cell area (Ac), cell perimeter (Pc), cell volume (Vc), cell surface area (Sc), cell form factor (FFc), and cell surface to volume density (Svc) parameter comparisons of the two dimethyl sulfoxide (DMSO) treatments. Differences were determined by subtracting the paired simultaneous GO measurements from the DCF or DGO measurements. No significant differences in area, volume, or shape were found between DCF and DGO processing. While cell shape and volume changes were significant between DCF or DGO and their respective GO treatment, the surface areas were not different. Error bars are ± 1 standard deviation (sd).

ity of the fixative solution did not remedy the problem. Membrane morphology was improved with the DMSO treatment (Fig. 3c) and averaged 100 angstroms, but was not as well preserved as with DCF. Coated endocytic caveolae were more evident in DCF cells (Figs. 1a, 3a). They ranged from 0 to 5 per cell profile with an average of 1 to 2 per cell. They had an overall incidence of 1 per 20 linear microns of cell surface (n = 60 cells). These endocytic caveolae contained a particulate subplasmalemmal coating ranging from 175- to 262-angstroms (averaging 200 angstroms)-thick (Fig. 3a). Although rare indentations consistent with caveolae were observed in GO specimens (2 in 60 cells), none of these contained an identifiable subplasmalemmal coating (Fig. 3b) to clearly identify them as the coated caveolae and vesicles observed in DCF specimens. Compared to GO cells, DGO-processing of cells slightly increased the number of visible coated pits. Coating of the caveolae and vesicles was more prominent and averaged 120 angstroms.

Mitochondrial membranes were best preserved in DCF cells (Fig. 4a). These mitochondria had distinct cristae. These structures were not as easily or readily observed in the GO samples (Fig. 4b) regardless of the osmolarity of the glutaraldehyde. The mitochondrial matrix in DCF cells appeared less dense, in contrast to the GO cells in which the small dense appearance of the mitochondria prevented clear identification and separation of these structures from cytoplasmic granules. The number, size, and cytoplasmic volume fraction of mitochondria was greater in cryofixed samples (Fig. 4a, Tables 2 and 3). For DCF, approximately 4– 5 mitochondria were observed per cell profile with an overall incidence of 16 per 100 μm^2 of cytoplasm. In GO cells, the mitochondria averaged 1 – 2 per cell profile with about 3 per 100 μm^2 of cytoplasm. Prior treatment with DMSO only minimally improved mitochondrial morphology after GO processing.

A variety of other cytoplasmic structures appeared better defined with more contrast in DCF samples. The Golgi body and granule membranes were more prominent in DCF samples (Fig. 5a). Coated vesicles were readily identified in the Golgi region of DCF cells but not in GO cells (Fig. 5) in all osmolarities tested. Although DGO treatment did improve the morphologic appearance (Fig. 5c), the optimal preservation was obtained with DCF treatment. Some granules in GO specimens often appeared more floculent or extracted, and contained pseudomembranes or lamellae in greater frequency than that observed for cryofixation (cf Figs. 4b, 5b). The cytoplasmic matrix of GO samples appeared more dense and somewhat moth-eaten (cf Figs. 1b, 5b). Glycogen particles appeared more extracted in GO samples. The ninefold triplet microtubule structure of the centriole in cross-section was easily identified in cryofixed samples (Fig. 6). Similarly, microtubules associated with centrioles and the cytoplasm were more easily identified with DCF (Figs. 5a, 6). Microfilaments were also more visible centrally as well as in the peripheral cytoplasm with DCF. Microtubules were visible with DGO but not with GO processing alone.

Nuclear cross-sectional areas were significantly different between DCF and GO (Table 2). (This was not identified in the preliminary report [Gilbert and Parmley, 1993] due to failure to apply a magnification correction factor.) However, nuclear profile perimeters were generally similar in both treatment samples (Table 2). This yielded a nuclear volume for GO samples that was 22% smaller than in DCF samples when measured at the outer nuclear envelope membrane (Table 3). The nuclear membranes of the DCF samples were distinctly more evident and less distorted when compared to GO samples (Figs. 1a,b, 7). In DCF samples, distinct inner and outer membranes were observed in the nuclear envelope (Fig. 7a). These two membranes were trilaminar and averaged 98 angstroms in width. Occasional areas of wide separation or swelling of the perinuclear space were evident in several GO samples. Although analysis showed an increase in volume (7%) of the perinuclear space in GO samples (Table 3), this was not felt to be conclusive. Nuclear pores were observed with similar frequency in DCF and GO treatments. In DCF specimens, the euchromatin appeared more continuous with the cytoplasm at the nuclear pore junction and less dense than in corresponding GO cells (Figs. 1a, 7a). In contrast, the separation of the euchromatin from the cytoplasm was greater in GO samples and the euchromatin was more dense (Figs. 1b, 7b). This separation also appeared with DGO treatment.

DISCUSSION

This study compared DCF- and GO-processing as well as the effect of DMSO on neutrophils in suspension and demonstrated deficiencies and artifacts with one commonly used GO method. Greater shrinkage of cells was observed with GO treatment, consistent with results described for other cell types (Hillman and Deutsch, 1978; Lee, 1984; Lee et al., 1982). This shrinkage was observed

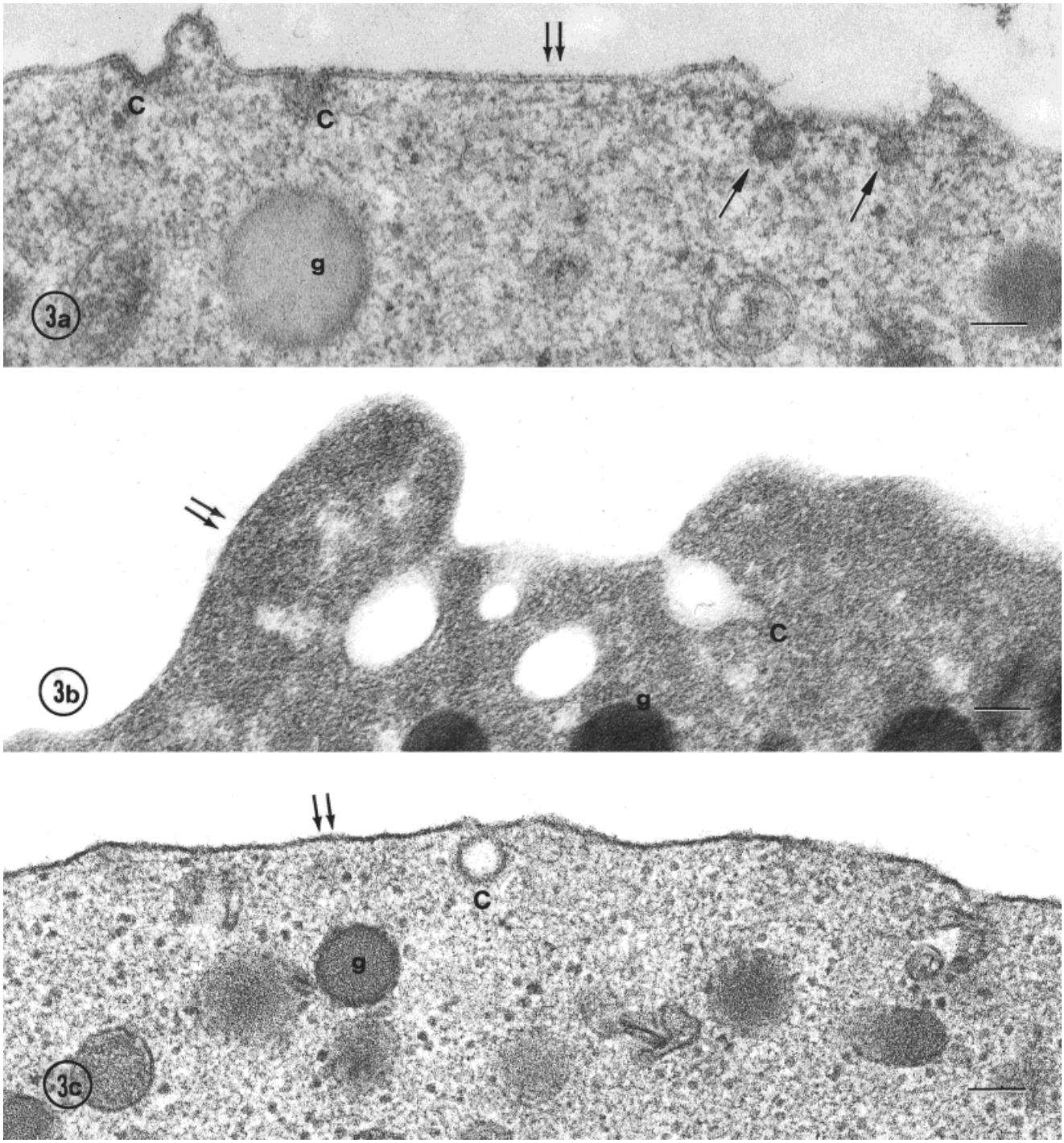


Fig. 3. **a:** At high magnification, the trilaminar structure of the plasmalemma in DCF neutrophils is very distinct (double arrow). The subplasmalemmal coating of an endocytic caveolus (C) is evident. A series of caveolae at the plasmalemma show a sequence from coated pit (C) to vesicle (arrows). Granule (g) membranes are intact. **b:** Distinct membrane bilayers and the coated structure of the caveolus are not readily seen in GO neutrophils. **c:** In cells treated by DGO, the membrane is more distinct than that with GO alone (cf b) but still does not clearly show the cell membrane bilayer as seen in the DCF-treated cell (cf Fig. 2a). UALC counterstain. Scale bar = 0.1 µm.

in cell volume and the size measurements of the cytoplasm, nucleus, and mitochondria (Table 3). In addition, the morphology of the membranes and matrix of both the cytoplasm and various organelles suggested improved preservation with DCF. This is similar to the improved preservation of basophil membranes with cryofixation (Hastie, 1990). The effects of DCF processing appeared to be multifactorial, since DMSO exposure in DGO process-

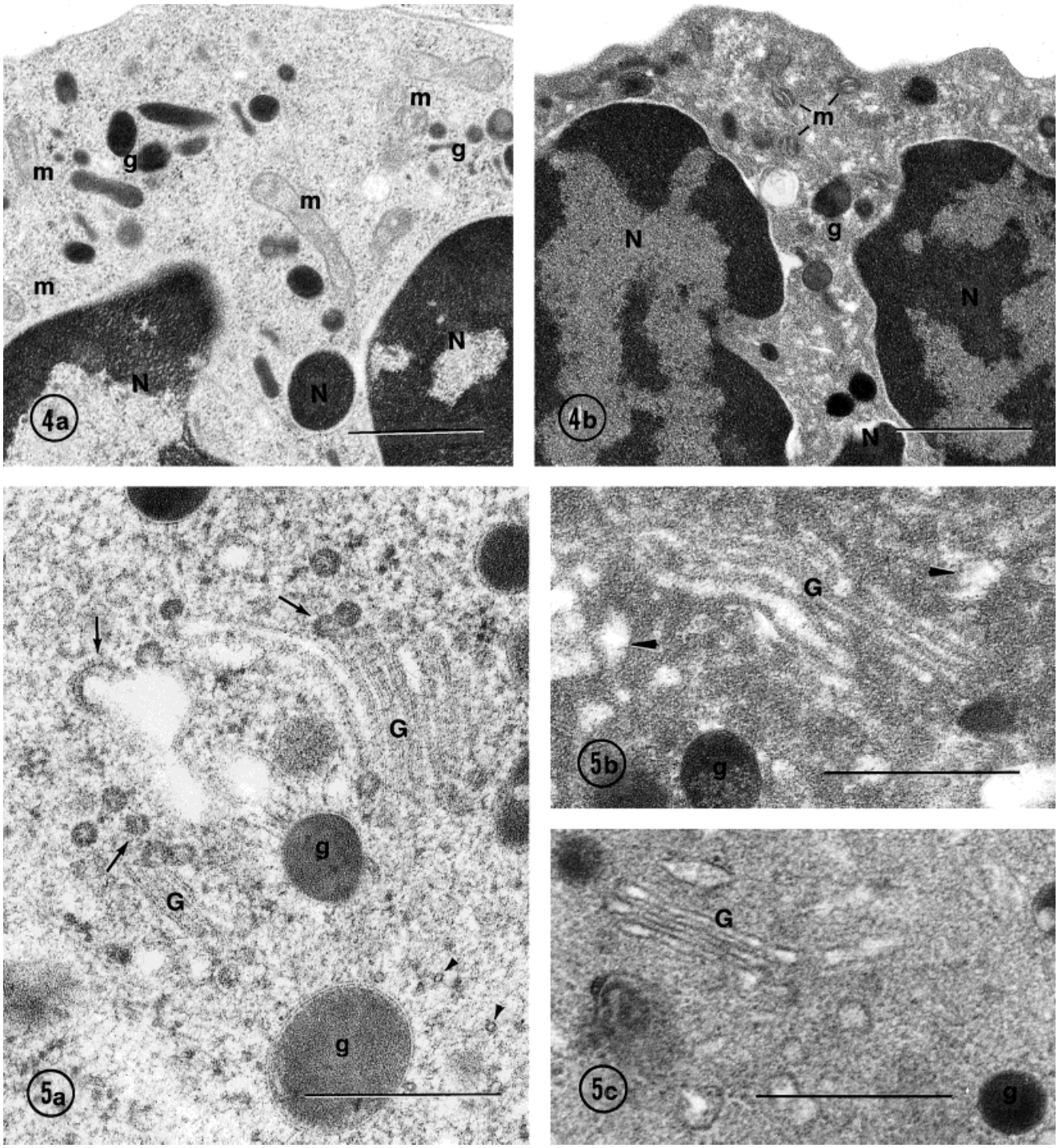


Fig. 4. **a:** The DCF neutrophil contains easily identifiable mitochondria (m, cf Fig. 1a), which are distinctly larger than those found in (**b**) GO neutrophils (cf Fig. 1b, Table 2). While the mitochondrial matrix appears less dense than the cristae in DCF, preparations this finding is reversed for the GO samples shown here. N, nuclear lobes; g, granules; UALC counterstain. Scale bars = 1 µm.

Fig. 5. **a:** The DCF-processed cell shows a well-preserved Golgi body (G). Note the several coated vesicles (arrows) that are in various stages of fusion with or budding from the Golgi cisternae and resemble the coated pits seen at the plasmalemma (cf Fig. 3a). Some microtubules are seen in cross-section (arrowheads). **b:** A GO-processed cell shows a denser ''moth-eaten'' cytoplasm (arrowheads) surrounding a Golgi zone that appears less defined. **c:** Cells pretreated with DMSO yielded better Golgi membrane preservation than GO but were still inferior to DCF. g, granules. UALC counterstain. Scale bars = 0.5 µm.

TABLE 2. Comparisons of Mitochondria and Nuclei by Fixation Treatment[a]

	Mitochondria area (μm^2)	Nuclear area (μm^2)	Nuclear perimeter (μm)
DCF			
(n = 60 cells)[b]	0.063 ± 0.032[c]	9.4 ± 2.9	18.2 ± 4.4
(n = 4 donors)	0.063 ± 0.005	9.4 ± 0.7	18.3 ± 0.8
GO			
(n = 60 cells)[b]	0.032 ± 0.028[d]	8.1 ± 2.2	19.5 ± 5.0
(n = 4 donors)	0.033 ± 0.008	8.1 ± 0.4	20.5 ± 2.7
t-test			
(n = 60 cells)[b]	<0.001	0.009	0.137
(n = 4 donors)	<0.001	0.021	0.142

[a]All values reported are mean ± standard deviation (μ ± sd).
[b]See the morphometric analysis portion of the Materials and Methods section.
[c]Based on 270 individual mitochondria.
[d]Based on 80 individual mitochondria.

ing offered some advantages over GO processing alone but did not preserve the membrane or cytoskeletal structures, mitochondria, or Golgi region as well as did DCF processing.

The DCF and DGO cells were rounder with a much smoother plasmalemmal surface similar to previous light and scanning electron microscope studies of unactivated neutrophils in suspension (Fernandez-Segura et al., 1995). In contrast, activated cells with DMSO/fMLP (fmlp = N-formylmethionyl-leveyl-phenylalanine) exposure had very irregular surfaces (Fernandez-Segura et al., 1995) as did resting monocytes (cf Fig. 1c) in the present study indicating that DMSO alone did not induce rounding. Similarly, GO neutrophils exhibited an irregular surface with many projections possibly accounting for the increase in perimeter noted in Figure 2.

Membrane addition due to degranulation could not be ruled out as the reason for the increase in perimeter, although gross differences in granule number were not observed. The cell surface area was not significantly different between DCF and GO treatments, arguing against significant degranulation even though the average perimeter difference (3.3 μm) was statistically greater in GO-treated cells.

This similarity in cell surface area with a concomitant decrease in the cell volume of GO cells was consistent with the isometric model of cell shrinkage described by Gittes and Bolender (1987). Volume regulation requires an intact cytoskeleton in human neutrophils and initial volume changes occur within the first few minutes of exposure to an osmotic insult (Downey et al., 1995). Furthermore, neutrophilic shape changes have been shown to occur within seconds (Hoffstein et al., 1982) and in association with microtubule disassembly (Oliver and Berlin, 1982). Since glutaraldehyde fixation may take from seconds to minutes (Buckley, 1973) and because microtubules were not readily seen in GO cells (Table 1), it suggested that volume changes occur predominantly at the beginning of GO fixation when the cell regulatory mechanisms can still respond to environmental insults. This volume change was overcome by pretreatment with DMSO in this study, a known membrane permeabilizer (Grace and Llinás, 1985), which may either enhance the initial glutaraldehyde penetration and subsequent fixation or stabilize the cytoskeleton (Katsuda et al., 1988; Lampugnani et al., 1987) or both. Alternatively, the DMSO may have resulted in volume and shape changes that preceded fixation; however, since neutrophils are predominantly spherical in suspension or peripheral circulation unless activated or leaving the bloodstream (McCarthy et al., 1990; Oliver and Berlin, 1982; Schmid-Schönbein et al., 1980), the change in volume was more consistent with deleterious shrinkage and not the DMSO treatment.

The plasmalemma's trilaminar appearance as well as granule and Golgi membranes was preserved with DCF. Similarly, neutrophil organelle membranes were always more prominent in the DCF samples. In contrast, the GO method resulted in less optimal preservation of the membrane's lipid bilayer. Although pretreatment with DMSO did appreciably improve membrane morphology, the bilayer was still not as distinct as with DCF (cf Fig. 3). This was consistent with previously observed retention of membrane lipids by the method of freeze-substitution (Humbel and Schwarz, 1989), and their extraction during chemical fixation (Hayat, 1989).

The DCF process provided a method for demonstration of previously unappreciated coated pits in neutrophils. Previous work with basophils showed that coated pits were better preserved with cryofixation (Hastie, 1990). Work with rabbit neutrophils showing that exocytosis is inhibited by hyperosmolarity (Kazilek et al., 1988) raises the possibility that the hyperosmolar glutaraldehyde decreased the number of coated pits observed in the present study. The failure of the GO or DGO process to more optimally preserve this membrane structure in neutrophils resulted in an underestimation of the occurrence of coated pits in glutaraldehyde-fixed samples.

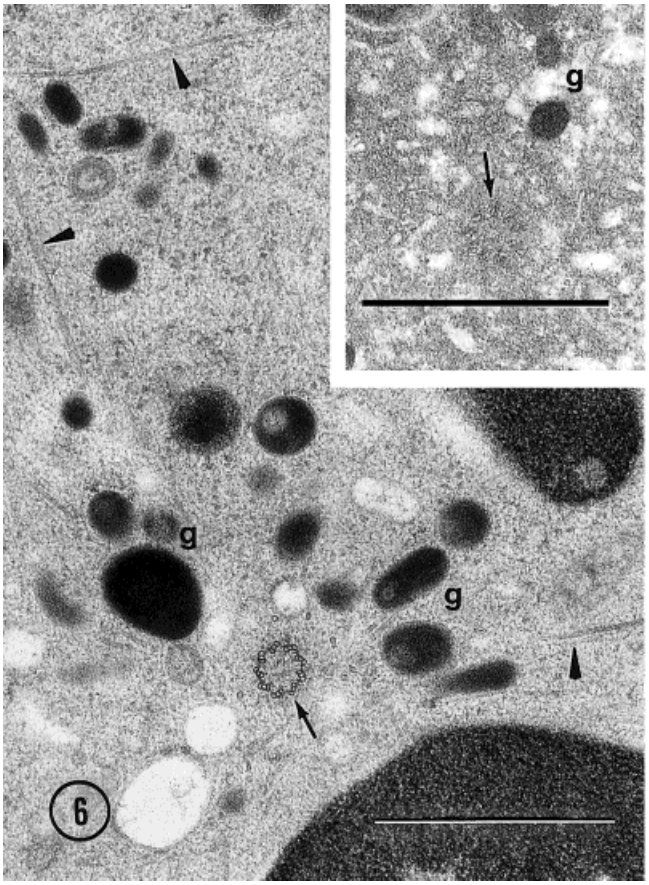
Previous studies of mitochondria in GO-fixed preparations indicated that these structures were rare in contrast to other white blood cells (Bainton et al., 1971; Zucker-Franklin, 1968). A similar result was obtained in this study with GO; however, both DGO and DCF resulted in larger, more intact and more frequent appearing mitochondria, indicating a more significant role for this organelle in mature neutrophils. Mitochondria have been shown to be easily damaged (Meissner and Schwarz, 1990) and the present study of neutrophils demonstrated that GO disproportionately shrinks mitochondria compared to the shrinkage of the cell as a whole (Table 3). The large size of mitochondria in DCF cells was not attributed to artifactual swelling since they did not appear distended or ruptured and membranes were intact. Ultrastructural changes in mitochondria have been linked to different physiological states (Hackenbrock, 1966), which may be more susceptible to chemical-induced shrinkage. The fact that GO-induced shrinkage was selective for neutrophil mitochondria compared to monocyte mitochondria further supports the role for physiologic factors in this result.

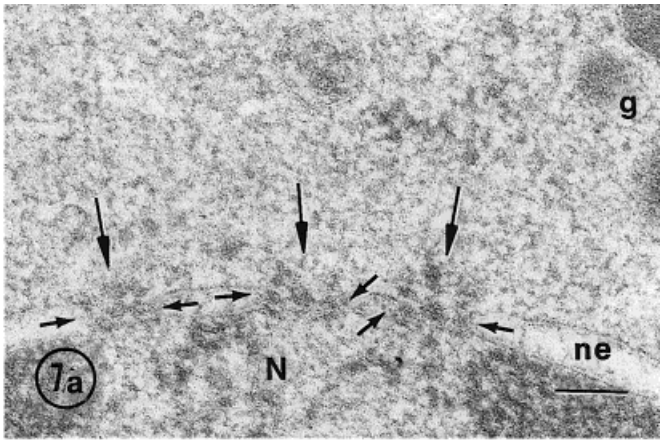
Cytoplasm and euchromatin were more dense in the GO cells than DGO and DCF. This density increase is presumably due to a mechanism that was enhanced in GO fixation and mitigated by pretreatment with DMSO. Glycogen in the GO cells appeared to be extracted as evidenced by the moth-eaten appearance of the cytoplasmic matrix in some cells, consistent with carbohydrate extraction by aldehyde fixation as previously shown (Radmehr and Butler, 1978). This appeared to be prevented by DMSO, indicating that DMSO pretreatment may be a useful adjunct for evaluating carbohydrate content and cytochemistry. The less

TABLE 3. Comparison of Cell Compartment Volumes by Fixation Treatment

	V_V-DCF (%)	V_V-GO (%)	V-DCF (fl)	V-GO (fl)	% volume difference GO v. DCF
Cell	100.00	100.00	325	236	27
Cytoplasm[a]	75.29	73.48	245	173	29
Nucleus (OM)[a,b]	24.71	26.52	80	63	22
Nucleus (IM)[a,b]	23.38	24.56	76	58	24
Perinuclear space[a]	1.33	1.96	4.3	4.6	−7.0
Mitochondria[c]	0.99	0.19	2.43	0.33	86

[a]The cellular volume fraction (V_V) for these components is reported in this table.
[b]Nuclear perimeters were measured at the outer membrane (OM) and the inner membrane (IM).
[c]The cytoplasmic volume fraction for mitochondria is reported in this table.





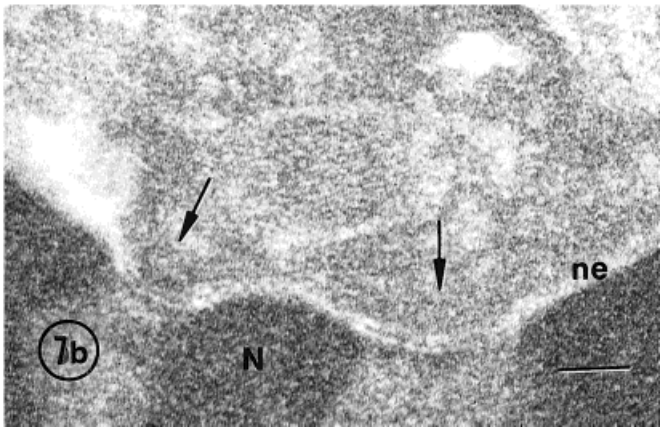


Fig. 6. The cross-sectional profile of a centriole (arrow) in this DCF neutrophil displays well-preserved typical nine sets of triplet microtubules characteristic of the centriole and microtubules (arrowheads) in longitudinal section. In GO neutrophils (**inset**), a less distinct centriole structure is found in a denser cytoplasmic matrix (arrow). The ninefold triplet microtubule structure is not as clear in the GO neutrophil. N, nuclear lobes; g, granules; UALC counterstain. Scale bars = 1 µm.

Fig. 7. **a:** Similar to the cell membrane (cf Fig. 3), the nuclear envelope (ne) in this DCF cell displays a trilaminar structure. The inner and outer membranes of the nuclear envelope are continuous with each other (small arrows) at the nuclear pore (large arrows). The nuclear pore proteins appear visible. Also, the dispersed chromatin appears somewhat continuous with the cytoplasm. **b:** In GO neutrophils, the structure of the nuclear envelope and the nuclear pore is not clear and the euchromatin appears discontinuous with the cytoplasm. g, granules; UALC counterstain. Scale bars = 0.1 µm.

dense cytoplasm in DCF and DGO did not appear to be caused by an increase in cytoplasmic volume due to abnormal cell swelling, since membranes, nuclear envelope, and mitochondrial cristae were more intact than in GO cells. Centrioles and their associated cytoskeletal structures appear better preserved in DCF, possibly due to less extraction of surrounding water-soluble components allowing for better visualization of these structures. However, DGO also preserved these structures, consistent with previous studies demonstrating promotion and stabilization of microtubules (Katsuda et al., 1988) and microfilaments (Lampugnani et al., 1987) with DMSO. Thus, the

DMSO component of the DCF process appeared to significantly contribute to cell matrix preservation, demonstrating that the ultrastructural preservation observed with DCF seems to be multifactorial.

Previous studies have correlated cell/organelle size and shape changes with various levels of activation in the neutrophil. Activated cells tended to be larger and had a more irregular shape (Fernandez-Segura et al., 1995; Hoffstein et al., 1982; McCarthy et al., 1990; Oliver and Berlin, 1982). Small mitochondrial size in neutrophils has been associated with inactivity (Hackenbrock, 1988), whereas increased coated pits and caveolae have been associated with increased endocytic and/or metabolic activity (Pearse, 1987). This study demonstrates that these morphologic features in neutrophils can be dramatically affected by fixation as well as DMSO exposure and that these parameters need to be considered in interpreting experimental results. In view of this analysis, the method of DGO fixation may offer morphological advantages with neutrophil and other cellular suspensions, saving the more complex DCF method for cases where the preservation of fine detail is critical.

LITERATURE CITED

Aherne WA, Dunnill MS. Morphometry. London:Edward Arnold (Publishers) Ltd., 1982, p 42.

Aldrich, HC. Practical aspects of freeze-substitution. EMSA Bull. 1989;19:53–59.

Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. Origin and content of azurophil and specific granules. J. Exp. Med. 1971;134:907–934.

Benchimol M. Fine structure of Tritrichomonas foetus as seen using cryotechniques. Microsc. Res. Techn. 1994;29:37–46.

Buckley IK. Studies in fixation for electron microscopy using cultured cells. Lab. Invest. 1973;29:398–410.

Downey GP, Grinstein S, Sue A-QA, Czaban B, Chan CK. Volume regulation in leukocytes: Requirement for an intact cytoskeleton. J. Cell Physiol. 1995;163:96–104.

Fernandez-Segura E, Garcia JM, Santos JL, Campos A. Shape, F-actin, and surface morphology changes during chemotactic peptide-induced polarity in human neutrophils. Anat. Rec. 1995;241:519–528.

Gilbert CS, Parmley RT. Loss of membrane structure and cytoplasmic volume in glutaraldehyde-fixed neutrophils compared to cryofixed-freeze-substituted neutrophils. In: Proc. 51st Ann. Mtg. MSA, 1993; 280–281.(Abstract)

Gilbert CS, Parmley RT, Rice WG, Kinkade, Jr JM. Heterogeneity of peroxidase-positive granules in normal human and Chédiak-Higashi neutrophils. J. Histochem. Cytochem. 1993;41:837–849.

Gittes F, Bolender RP. Counting cell nuclei with random sections: The effect of shape and size. Micron Microscopica Acta1987;18:59–70.

Grace AA , Llinás R. Morphological artifacts induced in intracellularly stained neurons by dehydration: Circumvention using rapid dimethyl sulfoxide clearing. Neuroscience 1985; 16:461–475.

Hackenbrock CR. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J. Cell Biol. 1966;30:269–297.

Hastie R. Methods in laboratory investigation: ultrastructure of human basophil leukocytes studied after spray freezing and freeze-substitution. Lab. Invest. 1990; 62:119–130.

Hayat MA. Principles and Techniques of Electron Microscopy: Biological Applications. Boca Raton:CRC Press, Inc., 1989: pp 25-31.

Herzig GP. Autologous marrow transplantation in cancer therapy. Prog. Hematol. 1981; 12:1–23.

Hillman H, Deutsch K. Area changes in slices of rat brain during preparation for histology or electron microscopy. J. Microsc. 1978;114: 77–84.

Hoffstein ST, Friedman RS, Weissmann G. Degranulation, membrane addition, and shape change during chemotactic factor-induced aggregation of human neutrophils. J. Cell Biol. 1982;95:234–241.

Humbel BM, Schwarz H. Freeze-substitution for immunochemistry. In: Verkleij AJ, Leunissen JLM, eds. Immuno-gold Labeling in Cell Biology. Boca Raton: CRC Press, Inc., 1989: 115–134.

Kalmar JR, Arnold RR, Warbington ML, Gardner MK. Superior leukocyte separation with a discontinuous one-step Ficoll-Hypaque gradient for the isolation of human neutrophils. J. Immunol. Meth. 1988;110:275–281.

Katsuda S, Okada Y, Nakanishi I, Tanaka J. Inhibitory effect of dimethyl sulfoxide on the proliferation of cultured arterial smooth muscle cells: Relationship to the cytoplasmic microtubules. Exp. Molec. Pathol. 1988;48:48–58.

Kazilek CJ, Merkle CJ, Chandler DE. Hyperosmotic inhibition of calcium signals and exocytosis in rabbit neutrophils. Am. J. Physiol. 1988;254:C709–C718.

Konwinski M, Abramczuk J, Baranska W, Szymkowiak W. Size changes of mouse ova during preparation for morphometric studies in the electron microscope. Histochemistry 1974; 42:315–322.

Lampugnani MG, Pedenovi M, Niewiarowski A, Casali B, Donati MB, Corbascio GC, Marchisio PC. Effects of dimethyl sulfoxide (DMSO) on microfilament organization, cellular adhesion, and growth of cultured mouse B16 melanoma cells. Exp. Cell Res. 1987;172:385–396.

Lee RMKW. A critical appraisal of the effects of fixation, dehydration and embedding on cell volume. In: Revel J-P, Barnard T, Haggis GH, eds. Science of Biological Specimen Preparation. Chicago, IL: SEM Inc. AMF O'Hare, 1984: 61–70.

Lee, RMKW, McKenzie R, Kobayashi K, Garfield RE, Forrest JB, Daniel EE. Effects of glutaraldehyde fixative osmolarities on smooth muscle cell volume, and osmotic reactivity of the cells after fixation. J. Microsc. 1982;125:77–88.

McCarthy DA, Bernhagen J, Liu YC, Perry JD. A rapid preparation technique for leucocytes. J. Microsc. 1990;158:63–72.

Meissner DH, Schwarz H. Improved cryoprotection and freeze-substitution of embryonic quail retina: A TEM study on ultrastructural preservation. J. Electron Microsc. Techn. 1990;14:348–356.

Oliver JM, Berlin RD. Mechanisms that regulate the structural and functional architecture of cell surfaces. Int. Rev. Cytol. 1982;74:55–94.

Pearse BMF. Clathrin and coated vesicles. EMBO J. 1987;6:2507–2512.

Pfaller W, Rovan E. Preparation of resin embedded unicellular organisms without the use of fixatives and dehydration media. J. Microsc. 1978;114:339–351.

Radmehr B, Butler WF. Leaching of glycosaminoglycan from cow uterus and vagina into fixative solution. Histochem. J. 1978;10:465–468.

Schmid-Schönbein GW, Shih YY, Chien S. Morphometry of human leukocytes. Blood 1980;56:866–875.

Sokol RJ, James NT, Wales J, Hudson G. Morphometry of eosinophils in human blood. Acta Anat. 1987;129:211–213.

Weibel ER, Bolender RP. Stereological techniques for electron microscopic morphometry. In: Hayat MA, ed. Principles and Techniques of Electron Microscopy: Biological Applications. Vol. 3. New York: Van Nostrand Reinhold Co., 1973: 237–296.

Zucker-Franklin D. Electron microscopic studies of human granulocytes: Structural variations related to function. Semin. Hematol. 1968;5:109–133.