ULTRASTRUCTURE OF HUMAN LEUKOCYTES AFTER SIMULTANEOUS FIXATION WITH GLUTARALDEHYDE AND OSMIUM TETROXIDE AND "POSTFIXATION" IN URANYL ACETATE

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ABSTRACT

Human leukocytes in suspension or in monolayer cultures have been processed for electron microscopy by fixation in a freshly made cold mixture of glutaraldehyde and osmium tetroxide and by "postfixation" in uranyl acetate. Simultaneous exposure to glutaraldehyde and osmium tetroxide eliminates many of the shortcomings seen when either of these agents is used alone as the initial fixative. Specimens are processed to the stage of dehydration as single cell suspensions or as very small clumps to assure rapid penetration of fixatives and efficient washing. The technique is rapid and reproducible. Electron micrographs presented in this report illustrate the ultrastructural features of human white cells prepared by this method.

Neutrophilic polymorphonuclear leukocytes are commonly not well preserved by standard methods of fixation for electron microscopy. Cold osmium tetroxide produces in these cells variable destruction in their cytoplasmic matrix and other damage probably attributable to oxidative effects or to inadequate fixation of proteins. Neutrophils fixed with glutaraldehyde followed by osmium tetroxide frequently show mottled granules, occasional myelin figures, and poorly defined membranes, findings which may reflect some degree of autolysis or lipid extraction during the aldehyde exposure.

We report here a method for obtaining improved fixation of neutrophils and of other white blood cells. Essential features of the method are the following: (*a*) the cells are fixed and processed to the stage of dehydration as single cells in suspension or as very small clumps; (*b*) initial fixation is accomplished with a freshly made mixture of glutaraldehyde and osmium tetroxide, similar to that employed by Trump and Bulger (1); (*c*) the cells are postfixed[1] by suspension in uranyl acetate solution modified from Kellenberger (2). This "mixed fixative," as we call it, has been applied, with good results, to several other types of cells in suspension or in monolayer cultures, some of which were illustrated in separate reports (3, 4).

MATERIALS AND METHOD

Collection of Leukocytes

Venous blood from healthy adults was collected with minimal trauma in a plastic syringe and immediately mixed with heparin (Connaught Medical Research Lab., Toronto, Canada) to give a final concentration of 0.1 mg/ml. The heparinized blood was mixed with an equal volume of similarly heparinized 2% clinical dextran (Abbott Laboratories, North Chicago, Ill.) in physiologic saline. The tube was slanted at 45° and kept at room temperature for 45 min to allow red cell sedimentation. The opalescent cell- and platelet-rich supernatant plasma layer was then collected and centrifuged at room temperature

[1] Postfixation in uranyl acetate may be incorrect terminology since uranyl ions are probably acting primarily as a stain rather than as a fixative. The term will nevertheless be used since it signifies clearly the technical procedure and avoids confusion with the separate later step of staining of the sections with uranyl acetate.

for 5 min at 200 *g* to deposit the white cells while leaving most of the platelets in suspension. Sedimented leukocytes were fixed directly or were washed once in heparinized saline and suspended in 10–20% autologous serum-Hanks' solution for short term (1–2 hr) culture in plastic Petri dishes or glass T flasks.

The Fixation, Staining, and Washing Solutions

Stock solutions of 2.5% glutaraldehyde (Fischer Scientific Co., Fairlawn, New Jersey) in 0.1 M cacodylate pH 7.4 and of 1% osmium tetroxide in 0.1 M cacodylate pH 7.4 were maintained separately at 4°C. These were brought to 0°C in an ice bath and mixed, one part glutaraldehyde plus two parts osmium tetroxide, within an hour of use. Under these conditions such a mixture remained clear and colorless or faintly tan during the fixation.

Ice cold physiologic saline was used for washing.

Uranyl acetate (0.25% in 0.1 M acetate buffer at pH 6.3) was employed for postfixation of the cells in suspension.

The Fixation and Processing Procedure

The sedimented leukocytes at room temperature from 1 ml (approximately 5×10^6 cells or 5-mm^3 packed cells) of the dextran supernatant cell-rich plasma were suspended in 2 ml of the cold, mixed fixative. After approximately 2 min, the suspension was transferred to a 3 ml conical centrifuge tube and spun in a tabletop clinical centrifuge (International Equipment Co. Needham Heights, Mass.) at 300 *g* for 1 min. The supernatant fluid was decanted and aspirated with a Pasteur pipette. The pellet was chilled briefly on ice, was resuspended in another 2 ml of mixed fixative, and was allowed to stand on ice for 10–30 min. Centrifugation (as above) was followed by two washes in cold saline; each involved gentle suspension of the cells with a Pasteur pipette and then prompt sedimentation in the centrifuge. The pellet was next suspended in uranyl acetate solution and allowed to stand at 0°C for 15–30 min. Two saline washes (as above) followed. The cell pellet was warmed in a 50°C water bath for 5 min and then suspended in a few drops of 2% Noble agar which had been boiled or autoclaved and allowed to cool to 50°C in a water bath. Care had to be taken to transfer the agar with a warm Pasteur pipette to avoid solidification. The cell suspension in fluid agar was centrifuged at 750 *g* for 2 min in a carrier half-filled with hot tap water. (If solidification of the agar had been avoided, the cells formed a firm pellet at the bottom of the tube; otherwise, they were distributed in a fuzzy zone. In the latter event the tube could be heated in a steam bath until the agar had melted, and then it could be recentrifuged. Such steaming of fixed cells had little or no discernible effect on their ultrastructure.) The tube was cooled in ice to solidify the agar. After addition of 70% alcohol and further standing on ice for an hour or more, the agar filling the conical tip of the tube could then be displaced by careful pipetting, and the black cell pellet was trimmed to small blocks for dehydration and embedding by standard methods (5).

Cells cultured in vitro on glass or plastic surfaces were fixed by draining off the fluid culture medium and flooding the cell layer with a freshly made, cold mixture of osmium tetroxide and glutaraldehyde. The cells were then gently scraped from the surface with a plastic policeman. This cell suspension was transferred to a 3 ml conical tube and centrifuged at 300 *g* for 1 min. The supernatant was thoroughly removed, and the pellet was chilled on ice, suspended in a fresh aliquot of mixed fixative, and kept on ice for 15–30 min. Washing, postfixation in uranyl acetate, and subsequent processing were as those described above for blood leukocytes.

Thin sections were cut with diamond knives on a Porter-Blum microtome and picked up on 200-mesh grids coated with thin layers of Formvar and carbon. Sections were stained with uranyl acetate and lead citrate (6) and were examined and photographed in a Siemens Elmiskop I at 80 kv with a 50 μ objective aperture.

RESULTS

In our early attempts to employ a mixture of glutaraldehyde and osmium tetroxide as a fixative, we observed, as have others (1), that the two fixatives interacted to produce a brownish purple product; this reaction was more rapid and extensive at higher temperatures and apparently was accelerated by the presence of organic material in the specimen. Although in many instances well-preserved cells were obtained after exposure to these discolored fixative mixtures, the results were variable. We found that cold stock solutions of glutaraldehyde and osmium tetroxide, both in cacodylate buffer at pH 7.4, could be mixed and kept at 0° C for 1 hr with little or no discoloration and no precipitation, and that this mixture gave rapid, reproducible fixation of various cell types.

In order to avoid possible change in the shape of the cells or damage to them by chilling or washing prior to fixation, the cell pellet or culture, at room or incubator temperature, was suspended in cold fixative; after the suspension had stood for approximately 2 min, the cells were collected as a pellet, the supernatant was discarded, and the pellet was chilled thoroughly and then suspended in a fresh aliquot of the mixed fixative and held at 0° C. Studies were made on the relative concen-

trations of osmium tetroxide and glutaraldehyde in the mixture and on the optimal duration of fixation. These concentrations and times were found not to be critical. Similar fixation was obtained with mixtures ranging from 0.5 to 2.5% of each of the components, and no differences were apparent after fixation times ranging from 10 min to 1 hr. Care was required not to use too large a quantity of cells for the volume of mixed fixative. Overloading with cells led to discoloration of the solution during fixation and to variability in results. It was essential to suspend the cells in premixed fixative solution; cells that were exposed first for only 1 min to one of the reagents and then to the other reagent by its addition to the solution exhibited features of sequential rather than simultaneous fixation.

Early experience with the mixed fixative established that its preservative qualities were good, but that membranes were poorly defined. Application of a modified Kellenberger technique for staining of the fixed cells by suspension in uranyl acetate solution resulted in striking improvement in membrane definition. Postfixation in uranyl acetate for periods ranging from 10 to 30 min gave essentially identical results. The usefulness of uranyl acetate postfixation was related, in some unknown manner, to the initial treatment of the cells with mixed fixative; exposure to uranyl acetate of cells sequentially fixed with glutaraldehyde and osmium tetroxide did not significantly improve the poor membrane definition.

This mixed fixation procedure thus far has been applied with success to human white blood cells, to peritoneal cells of mice, and to tissue culture monolayers of mouse macrophages and L cells. Figs. 1–11 illustrate general and detailed features of the ultrastructure of human white blood cells processed by this method.

A low power view of a blood monocyte is shown in Fig. 1. The monocyte nucleus is commonly horeshoe-shaped, with extensive, light-staining, central nucleoplasm and dense, granular, peripheral chromatin. Nucleoli are rarely seen. Perinuclear filament bundles (Figs. 1 and 3) are often present in monocytes. In transverse section (not shown here) these bundles are clearly composed of filaments, not tubules. The Golgi apparatus is well developed and often multicentric. Small vesicles (50–200 mμ in diameter) are scattered throughout the cytoplasm but are especially numerous in the Golgi region. These vesicles can be classified into three general types: (*a*) a few showing surface spikes typical of coated vesicles; (*b*) some larger vesicles with an electron-lucent content, probably of pinocytic origin; and (*c*) many vesicles with a smooth surface and a matrix density similar to that of cytoplasmic ground substance, most likely representing elements derived from the Golgi apparatus or from smooth endoplasmic reticulum (ER). Monocyte mitochondria (Figs. 1 and 2), commonly located in the peripheral cytoplasm, are round or elongated with a dense matrix and well-defined cristae. Variable numbers of small (100–200 mμ), oval or rod-shaped, dense bodies are seen in the perinuclear region of most monocytes. Usually only a few short strips of slightly dilated, partly rough and partly smooth ER are present in the peripheral cytoplasm. The cytoplasmic ground substance is moderately dense and has a microgranular appearance. The plasma and nuclear membranes (Figs. 1 and 2) are well defined and generally smooth. In some instances a faint layer of amorphous material appears to be attached to the outer surface of the cell limiting membrane. At high magnification the cell surface membrane shows a typical trilaminar unit structure (Fig. 2).

Human blood lymphocytes processed by the mixed fixation technique are illustrated at low magnification in Fig. 4. Lymphocyte nuclei are oval with indentations or kidney-shaped and show a chromatin distribution of approximately equal amounts of centrally located light-staining material and of peripheral dense-staining granular elements. Some sections of lymphocytes show prominent nucleoli (Fig. 5) which usually are composed of three zones: an outer zone consisting of loosely packed granules identical in appearance to aggregated nuclear chromatin; a central core of tightly packed smaller granules (nucleolar granular area); and electron-opaque, nongranular material (fibrillar zone) coursing around and through the central zone. Also demonstrated in Fig. 5 is the good preservation of nuclear membranes and pores. The cytoplasm of lymphocytes is relatively sparse. Centrioles are frequently seen and appear to be well fixed. Several oval or rod-shaped mitochondria are typically grouped in a rosette pattern about the centrosomal region in the lymphocyte. The Golgi apparatus is poorly developed or not seen, and only small numbers of cytoplasmic vesicles are present. A common noteworthy feature, however, is the occurrence of large (300–400 mμ in diameter) multivesicular bodies (Figs. 4 and 6) distributed among the mito-

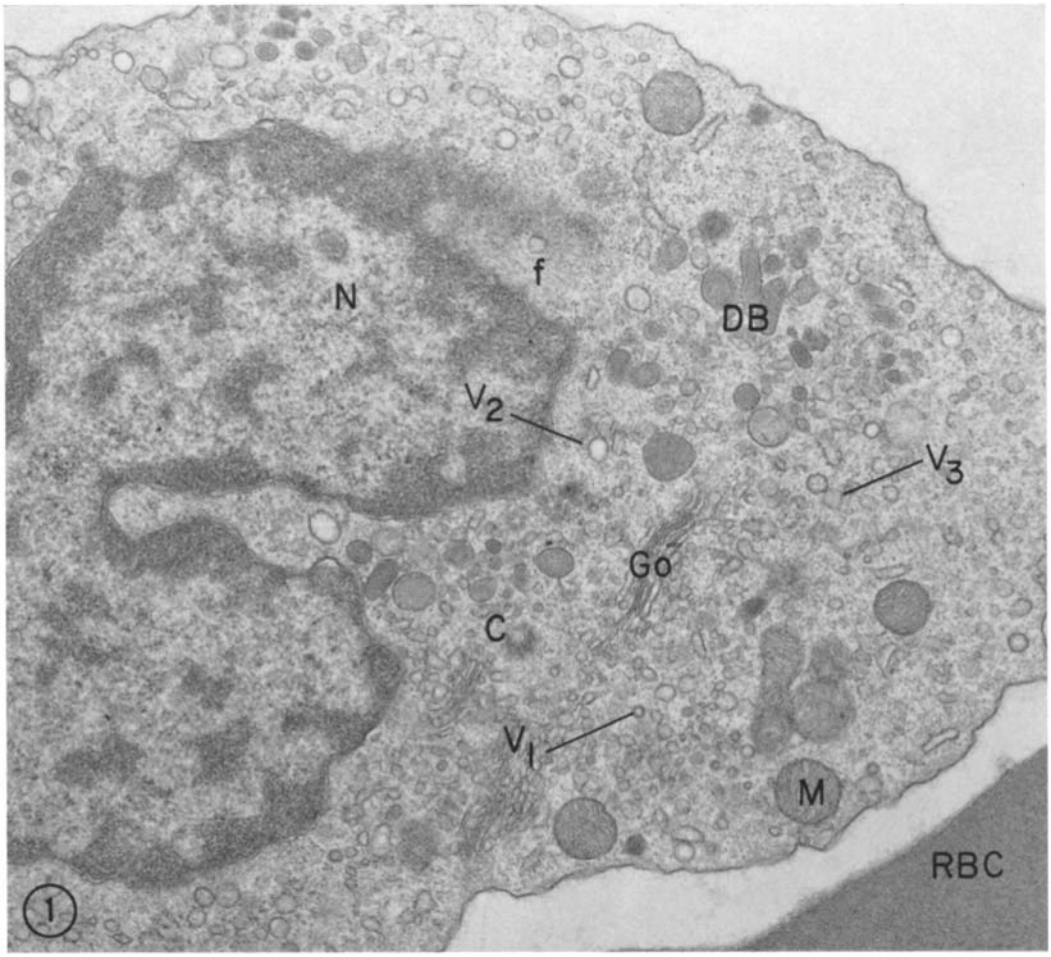

FIGURE 1 A human blood monocyte at low magnification. The nucleus (*N*) is horseshoe-shaped. Nucleoplasm consists predominantly of light-staining areas with a peripheral rim of aggregated dense granules. No nucleolus is seen. The cytoplasm is abundant and presents a variety of organelles. The centriole (*C*) is located in the nuclear hof area and is surrounded by a well-developed Golgi apparatus (*Go*) with multicentric stacks of cisternae and associated vesicles. Collections of small round or rod-shaped dense bodies (*DB*) are also present. An array of filaments (*f*) is seen adjacent to the nucleus in one area; individual filamentous elements are poorly visualized at this low magnification (see Fig. 4). Perhaps the most striking feature of monocyte cytoplasm is the presence of large numbers of small vesicles, most numerous in the centrosomal region but also present in peripheral zones. These vesicular elements appear to be of three general types: a small number of vesicles (V_1) showing surface spikes typical of so-called coated vesicles; a moderate number of slightly larger vesicles (V_2) with an electron-lucent content; and a large number of vesicles (V_3) varying in size and containing material with a density similar to or slightly greater than that of the cytoplasmic matrix. Several mitochondria (*M*) are seen in the peripheral cytoplasm. A few short strips of partly rough and partly smooth endoplasmic reticulum are present. The cytoplasmic matrix is of moderate density and has an amorphous or microgranular appearance. The cell membrane is sharply defined; in some areas amorphous material appears to coat the surface of the cell. Part of a red blood cell (*RBC*) is seen in the lower right corner. × 22,000.

chondria. Dense bodies may be present, but they are small in size and few in number. The surface membranes of lymphocytes processed in this manner are well defined; in some instances amorphous material is seen as a thin layer apparently attached to the cell surface. The ground substance of lymphocyte cytoplasm is composed of ribosomes and finely granular elements showing little or no

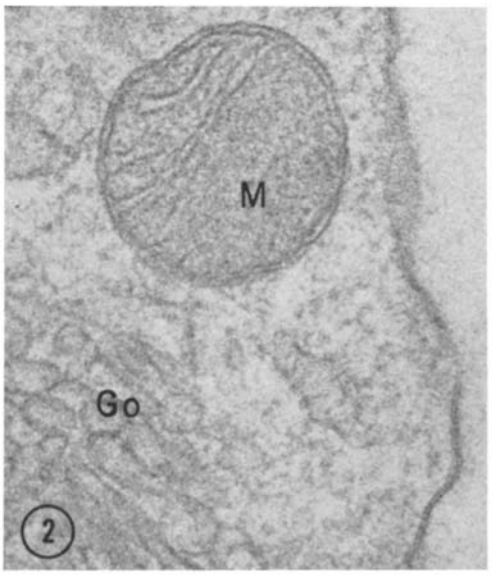

FIGURE 2 A portion of a monocyte at higher magnification. A mitochondrion (*M*) shows a moderately dense matrix and well-defined membranes; origin of cristae from the inner limiting membrane can be seen at several places. The outer margin of the Golgi complex (*Go*) is seen at the lower left. At lower right a segment of limiting cell membrane has been well sectioned and stained, and the typical trilaminar unit structure is evident. × 96,000.

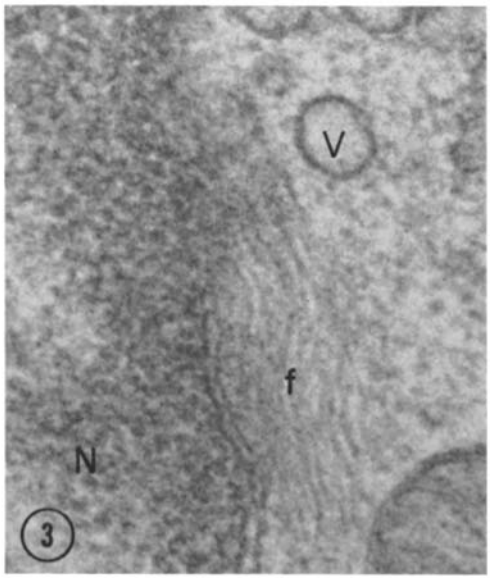

FIGURE 3 This figure shows the perinuclear filaments (*f*) in another monocyte. Individual filaments vary from 30 to 70 A in thickness and follow a curved course. They seem to originate from, or to be anchored to, the nucleus (*N*) in an area where nuclear membrane is not visible. A cytoplasmic vesicle (*V*) has a clear content. × 96,000.

pattern of distribution. One or two short strips of smooth ER may be present. Resting lymphoid cells show no filaments or microtubules in their cytoplasm, but cells fixed while crawling on glass or plastic show microtubular structures approximately 150 A in diameter (Fig. 6), especially prominent at the base of pseudopods or uropods in the constricted zone between the cytoplasmic extension and the cell body.

An example of a human blood neutrophil processed by the mixed fixation method is shown in Fig. 7. Nuclear lobes show a preponderance of dense, granular chromatin located peripherally. Nucleoli are not seen. Thin strands connecting the nuclear lobes are composed of a core of aggregated chromatin surrounded by nuclear membranes. Centrioles are well preserved (Figs. 7 and 8). A few vesicles of varying structure may be present in the centrosomal region. The cytoplasm of the neutrophil leukocyte is in general characterized by the scarcity or the absence of the formed elements usually seen in other cells. There is little or no Golgi complex; pinosomes or multivesicular bodies are usually not seen; mitochondria are rare; and ER is entirely absent or is limited to a few scattered elements. The predominant cytoplasmic structures are dense bodies or granules which vary in size (100–400 mμ), shape, and internal density. Some of this variation, especially in size, no doubt is due to sectioning through different planes of the individual granules, but the heterogeneity is too great to be accounted for entirely by this technical aspect. No separation into distinct types of granules is suggested by their morphology. At higher magnification a typical neutrophil granule (Fig. 9) shows a moderately dense structureless matrix and a well-defined, limiting unit membrane. The cytoplasmic ground substance of the neutrophil is finely granular and moderately electron opaque. Resting neutrophils show no cytoplasmic microtubules, but cells fixed while engaged in locomotion on glass or plastic show abundant microtubules or filaments in the pseudopod in the region of its origin from the cell body. The limiting membrane of the neutrophil fixed by this method sometimes shows a thin amorphous outer coating.

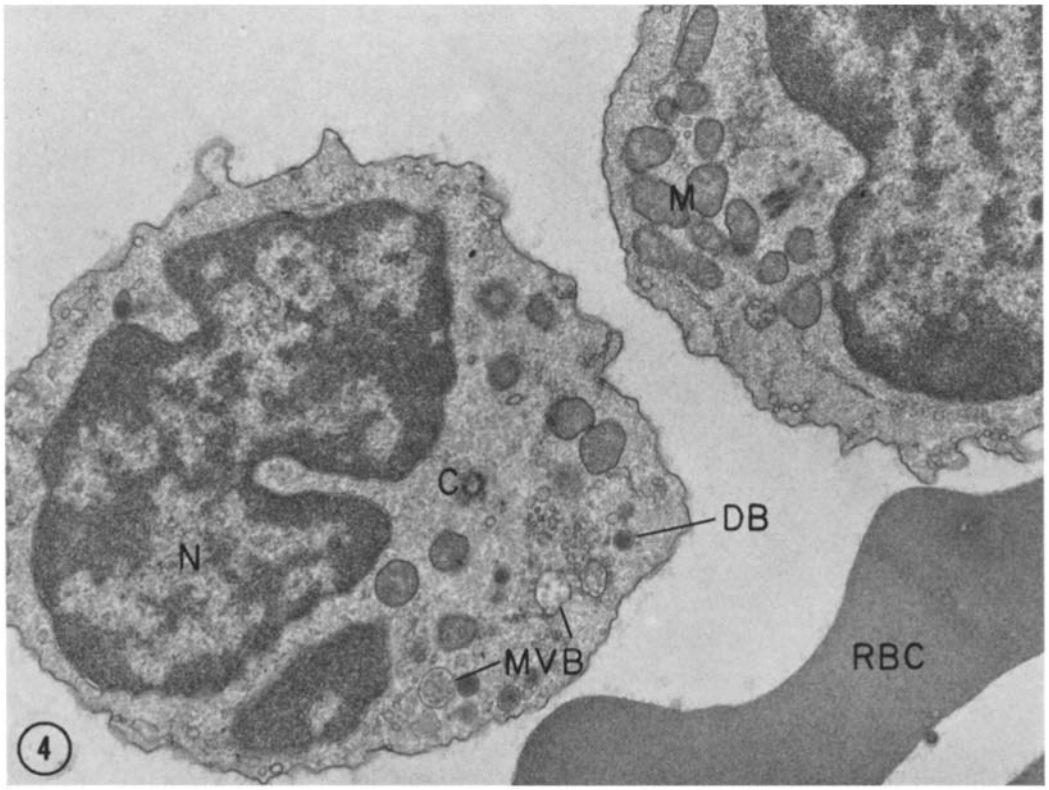

FIGURE 4 Human blood lymphocytes at low magnification. The nucleus (*N*) is oval with indentations. Nucleoplasm consists of approximately equal areas of peripheral aggregated dense material and of scattered central light-staining regions. Although not present in these cells, nucleoli are often present in lymphocytes (see Fig. 5). Cytoplasm of the lymphocyte is not extensive; the cytoplasmic matrix is moderately dense and of a granular appearance. Centrioles (*C*) are prominent, but Golgi apparatus is poorly developed or not seen; and dense bodies (*DB*) are few in number and small in size. Only a few cytoplasmic vesicles are seen, but one or more large multivesicular bodies (*MVB*) are commonly present. Lymphocyte mitochondria (*M*) are large in size and in number and tend to be arranged in a rosette pattern about the centrosomal region. A few isolated strips of predominately smooth endoplasmic reticulum may be seen. The cell membrane is well stained and in places appears to have a thin outer coating of amorphous material. A red blood cell (*RBC*) is at lower right. × 15,600.

A portion of a human eosinophil processed by the mixed fixation technique is presented in Fig. 10. The eosinophil nucleus usually consists of two lobes and shows abundant aggregated chromatin located peripherally. Eosinophil granules are large (approximately 800 mμ in diameter), and most of them have a sharply defined limiting membrane, a matrix of amorphous material of variable electron opacity, and one or more electron-opaque crystalloid inclusions. The lamellar pattern seen in granule crystals in rat eosinophils after glutaraldehyde fixation (7) has thus far not been noted in human cells processed by mixed fixation. Mitochondria and ER are sparse in eosinophils, but small vesicles of various types are numerous, especially in the central region of the cell. Vesicles seem to be distinctly more common in eosinophils than in neutrophils (compare Figs. 7 and 10).

Specimens of human blood examined thus far have contained too few basophils to allow evaluation of the preservation of these cells by the mixed fixation procedure.

None of the leukocytes discussed thus far has a well-developed rough ER. We include therefore an illustration (Fig. 11) showing a portion of the

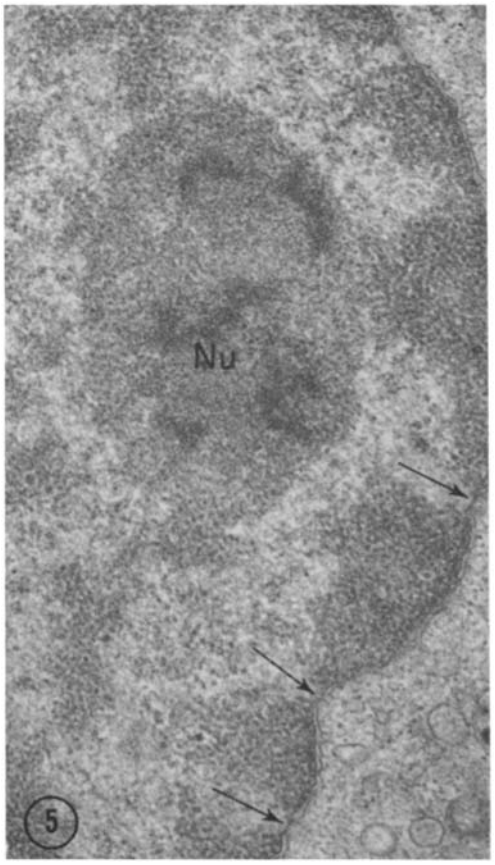

FIGURE 5 This figure shows a nucleolus (*Nu*) in another lymphocyte. The nucleolus appears to be composed of three parts: an outer zone consisting of loosely packed granules identical in appearance to aggregated nuclear chromatin; a central zone containing smaller, more tightly packed granules (nucleolar granular zone); and clumps or strands of dense-staining material (nucleolar fibrillar zone) located in and about the central granular area. Aggregates of granular chromatin are located at the periphery of the nucleus. The light-staining nucleoplasm shows scattered granules and some amorphous material. The nuclear membranes are well defined. Nuclear pores (arrows) occur where light-staining areas of nucleoplasm extend to the nuclear membranes; nuclear membranes appear to fuse to form a "diaphragm" at these pore sites. × 35,000.

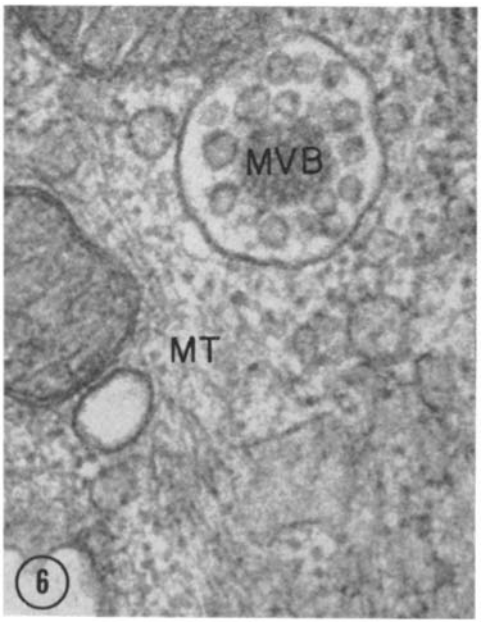

FIGURE 6 This figure shows at high magnification a portion of a lymphoid cell which had been fixed while engaged in locomotion. Mitochondria are seen at upper left, and a well-developed multivesicular body (*MVB*) is present. The cytoplasm shows numerous microtubules (*MT*) or filaments. Microtubules are not seen in resting lymphocytes. × 80,000.

cytoplasm of a plasma cell which was present in one of the leukocyte suspensions. Membranes and ribosomes of the rough-surfaced ER are sharply delineated and appear to be well preserved. A helical arrangement of the ribosomes is suggested in some areas. Also illustrated are the differences in structure and dimensions between ER membranes and cell surface membranes.

DISCUSSION

White cells constitute only a small fraction of blood; therefore, some method of concentration must be used to permit their study. Buffy coat preparations are difficult to handle, and they contain many more platelets than leukocytes. Much recent work on blood leukocyte morphology and function has therefore been done on cells harvested by the dextran sedimentation method. The addition to heparinized blood of high molecular weight dextran causes the red cells to form rouleaux and to settle rapidly when it stands at room temperature; the cell- and platelet-rich supernatant plasma is then collected and centrifuged at low speed to sediment the white cells but not the platelets. This provides a relatively pure and a representative leukocyte pellet. Recent studies in many laboratories indicate that the cells so obtained have normal morphology and functions (8, 9). Spontaneous settling of red cells occurs in

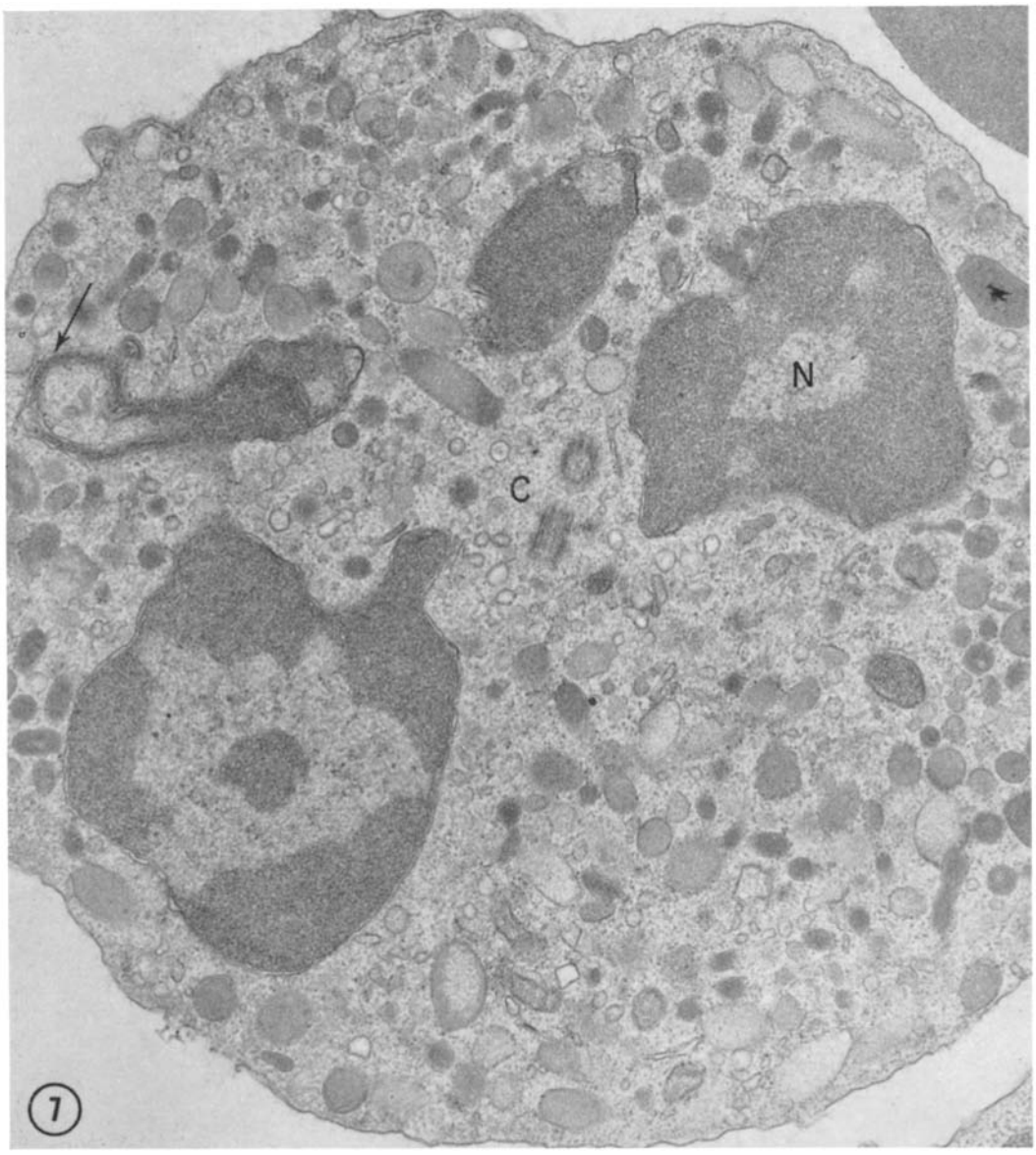

FIGURE 7 Low power view of a human neutrophil leukocyte. Nuclear lobes appear as multiple nuclei (*N*) in the thin section. Nucleoplasm consists predominately of dense, aggregated granular material located peripherally; nucleoli are not seen. In one place (arrow) a thin strand connecting nuclear lobes has been suitably sectioned to show a dense core of heterochromatin surrounded by nuclear membranes. Neutrophil cytoplasm is extensive. The cytoplasmic matrix is moderately dense and appears to be composed of dispersed microgranular and amorphous material. Centrioles (*C*) are well preserved. The cytoplasm contains very large numbers of dense granules ranging in size from 100 to 400 mμ and varying widely in staining properties. These granules are in many instances clearly membrane bounded and have a structureless content (see Fig. 9). On the whole, the small granules appear darker than do the larger ones, but many exceptions to this rule can be found. Other organelles in neutrophil cytoplasm are notable for their scarcity or their absence. Golgi apparatus is poorly developed or not seen; mitochondria are few in number and small in size; vesicles are present in small numbers predominately in the central region; and endoplasmic reticulum is absent or consists of only a few short strips. The neutrophil surface membrane is sharply defined, and in places (top part of cell in Fig. 7) it shows an amorphous outer coating. × 24,000.

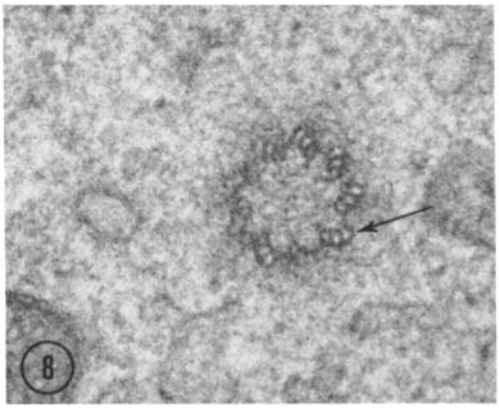

FIGURE 8 This figure shows one element of a neutrophil centriole at high magnification. In some places (arrow) the triplet of tiny rings is readily visible. The centriole in this transverse section is seen to consist of nine of these triplets. × 96,000.

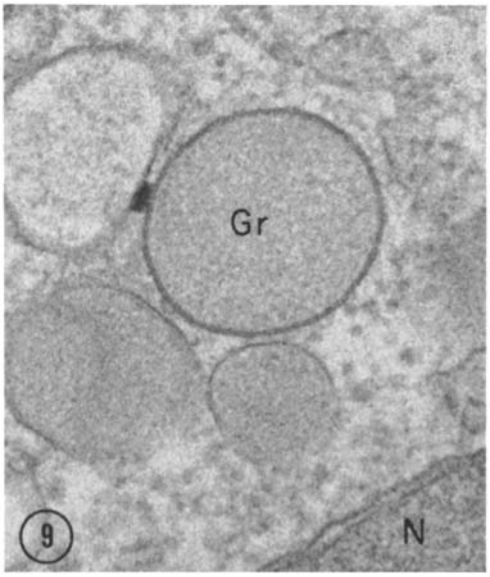

FIGURE 9 This figure illustrates a neutrophil granule (*Gr*) at high power. The trilaminar unit structure of the granule membrane is clearly resolved. The granule content is moderately densely stained and structureless. A portion of the nucleus (*N*) is at lower right. × 96,000.

some blood specimens without the addition of dextran; we could find no detectable differences between the ultrastructure of leukocytes harvested from the supernatant plasma of such specimens and that of leukocytes collected after the addition of dextran. The white cell pellet obtained from dextran-sedimented blood showed, after fixation and processing for electron microscopy, a distribution of the various types of cells essentially the same as that in the original blood sample.

The blood leukocytes were fixed, washed, and postfixed as single cells or as very small clumps in suspension, not as pellets or cell blocks. Problems of penetration of fixative, especially problems related to the different rates of penetration of the aldehyde and the osmium tetroxide components, were thus minimized or eliminated since the radius of the individual cells was only a few microns. Efficient, rapid washing was also facilitated by dealing with suspensions of single cells. Although centrifugation was required to collect the cells between each step, a very brief spin at slow speed sufficed to sediment them completely, and no evidence was obtained of any cell damage attributable to the repeated centrifugation. The cells, once fixed by this method, appeared in fact to be remarkably resistant to damage. As mentioned parenthetically above, even heating the cells to 100°C in a steam bath caused little or no change in their ultrastructure. The cells in the fixed and washed pellet did not adhere to one another firmly enough to allow their subsequent processing as a clump. They were therefore embedded in agar to facilitate handling during the dehydration and Epon-infiltration steps.

A distinct advantage of the mixed fixation method is its reproducibility. When employing glutaraldehyde as the initial fixative, we experienced, as had others, the bothersome problem of occasional poorly preserved specimens, and we attributed this problem to polymerization or other change in the glutaraldehyde stock solution. With mixed fixation, however, we used the same bottle of concentrated glutaraldehyde for over 6 months and the same lot of diluted 2.5% glutaraldehyde in buffer for up to a month with no indication of change in the quality of fixation.

As noted previously by Trump and Bulger (1), simultaneous exposure to glutaraldehyde and osmium tetroxide seems to give pictures intermediate between those seen with either of these agents alone as the initial fixative, in terms of distribution of nuclear chromatin, mitochondrial swelling, and preservation of cytoplasmic ground substance. It is admittedly difficult to relate cellular preservation as seen under the electron microscope, to the structure and organization obtained in the living cell. Such comparison be-

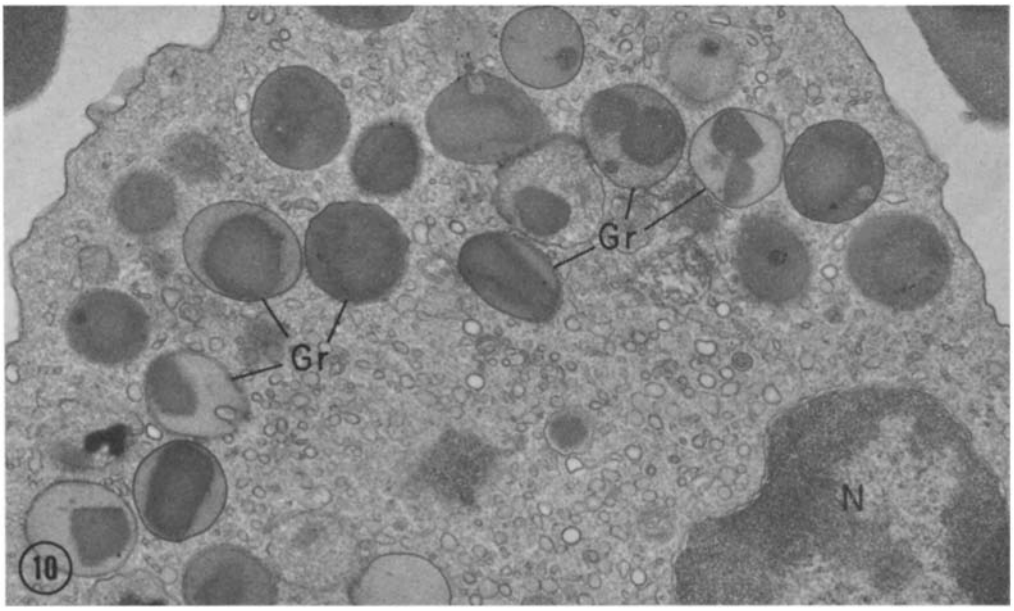

FIGURE 10 A portion of a human blood eosinophil. The nucleus (*N*), part of which is at lower right, usually shows two lobes with abundant aggregated chromatin located peripherally. Numerous small vesicles of varying size, shape, and content are seen in the cytoplasm, especially in the perinuclear area. Eosinophil granules (*Gr*) range from 500 to 800 mμ in diameter. Many of the granules contain very electron-opaque inclusions, some of which have sharp corners and straight edges suggesting a crystalline nature. The granule matrix around these inclusions is amorphous and of varying electron opacity. The granules have a distinct limiting membrane. Mitochondria and endoplasmic reticulum are sparse or absent. × 20,000.

tween living and fixed material is, however, possible at least to some degree with leukocytes. Living white cells have been studied extensively by phase-contrast light microscopy in recent years in our laboratory and elsewhere; therefore, considerable information is available on such features as nuclear chromatin patterns, granule distribution, and pinocytic activity. These features of white cells appear very similar in the living specimens and in material processed by mixed fixation, indicating that this procedure is relatively free of artifact production.

Many of the fine structural features of leukocytes processed by mixed fixation are essentially the same as those observed with other types of fixation in these or other types of cells and thus need not be discussed further (10–14). Included among these well-known features are nucleoplasm, nucleoli, nuclear membranes and pores, centrioles, mitochondria, Golgi complex, ER, and cell surface unit membrane.

Filaments in the perinuclear cytoplasm are seen quite often in human blood monocytes after mixed fixation. Similar bundles of filaments have been observed previously in blood cells, chicken macrophages, and leukemia cells (15–21). The origin and possible functions of these filaments are unknown. Many of our pictures suggest that these filaments are attached to the nuclear membrane, and Sutton and Weiss (21) noted in some cells a similar relationship between filaments and the outer mitochondrial membrane.

Preservation and definition of small vesicular elements are particularly striking in cells fixed simultaneously with glutaraldehyde and osmium tetroxide and post-fixed with uranyl acetate. Fine features of these vesicles—surface spikes, membrane thickness, content—allow classification into types and permit assessment of the role of vesicles in physiologic processes such as pinocytosis (3) or in pathologic events such as autophagic vacuole formation after drug intoxication (4). The number of vesicles is very large in monocytes, in keeping with the well-developed Golgi

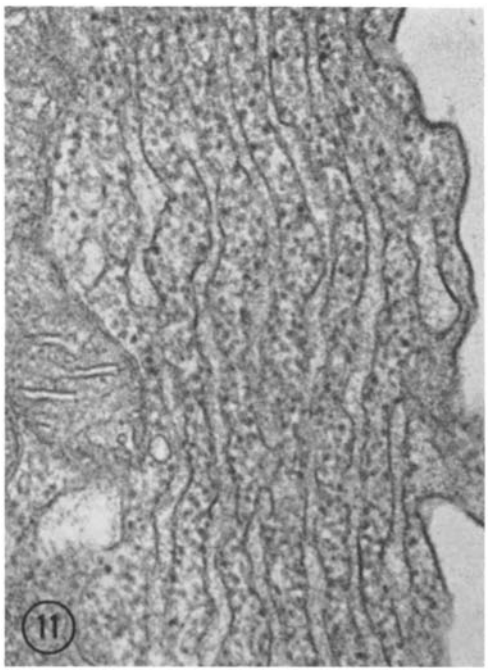

FIGURE 11 This figure shows an area of cytoplasm and surface membrane of a human plasma cell encountered in one of the specimens. It illustrates fixation of rough endoplasmic reticulum obtained with the new method described in this report. Membranes of the endoplasmic reticulum are sharply defined; note the difference in structure and in dimension between these reticulum membranes and the unit surface membrane at right. Ribosomes are abundant and appear to be aligned in a helical pattern in several areas. Part of a mitochondrion is seen at left. × 60,000.

apparatus and high level of pinocytic activity of these cells, and is small in lymphocytes and neutrophils, cells which normally do not exhibit active pinocytosis and show little or no Golgi complex. Of some interest is the occurrence of large numbers of vesicles in eosinophil cytoplasm, suggesting that eosinophils possibly have physiologic activities which might provide leads in studying their function which remains unknown (reviewed in reference 22).

Multivesicular bodies (MVB), which have been reported in lymphocytes by other researchers (11, 23), are especially prominent in lymphocytes after they had been processed by mixed fixation. The lymphocyte MVB are large and well developed, averaging 400 mμ in diameter and often containing up to a dozen small (40 mμ) vesicles (see Fig. 6). MVB in general are thought to be formed following endocytosis and/or autophagy (reviewed in references 24, 25). Although normal lymphocytes do not engage in phagocytosis or active pinocytosis under various conditions in vitro, they may do so when stimulated in vivo, and their MVB might thus represent a type of residual body in a cell poorly endowed with digestive enzymes. Autophagy could also give rise to MVB in lymphocytes, but no evidence is now available to suggest that this process occurs normally in these cells.

The limited observations made thus far on lymphocytes and neutrophils fixed while crawling indicate that microtubules or filaments occur in pseudopods or uropods, especially in the region of attachment of these extensions to the cell body. Additional observations are needed to establish more clearly the structure of these linear elements. Microtubules apparently play a role in movement of various types of cells (26), but the mechanism of ameboid locomotion remains unsettled despite extensive study (27). With adequate methods of fixation now available, it should be profitable to investigate ultrastructural features of locomotion in leukocytes.

Many previous investigators have noted heterogeneity of the cytoplasmic granule population in electron microscopic studies on rabbit heterophils (28–32). There has been no general agreement, however, on the number of distinct granule classes. As shown here, human blood neutrophil granules do not seem to fall into types, but rather they display essentially a continuous spectrum of variation in size and in internal electron opacity. Further studies are required on histochemical reactivity at the electron microscope level, and on enzyme analysis of granule populations separated on the basis of granule density or size, before any conclusions can be drawn on the question of different granule types in these cells.

The surface coating seen on some of the cells illustrated here may be a true component of the cell membrane which is not preserved by previous methods of handling, or it may represent serum proteins or other constituents of the surrounding medium which are adsorbed onto the cell surface at the time of fixation. Amorphous surface material is commonly present on amebae (33).

The difficulty of distinguishing between large lymphocytes and small monocytes has bedeviled cytologists for over a century, and the difficulty has by no means been eliminated by the extension of observation to the ultrastructural level. With

the present method of fixation there remain rare cells which defy classification, but, on the whole, distinctions between monocytes and lymphocytes are clear. Among the features, some old and well known and others new, helpful in classifying these mononuclear cells are the following. The monocyte is a larger cell and has more abundant cytoplasm than does the lymphocyte. Monocyte nuclei tend to be horseshoe-shaped or even multiple in thin sections, whereas the lymphocyte nucleus is typically oval with indentations. Monocyte nuclei show a higher proportion of light-staining areas than do those of lymphocytes. Perinuclear filaments are often present in monocytes, but they have not been observed in resting lymphocytes. Lymphocyte mitochondria are elongated structures usually gathered in a rosette pattern about the centrosomal area, whereas monocyte mitochondria tend to be oval or round and are scattered in the peripheral cytoplasm. Monocytes have as a rule many more cytoplasmic vesicles and small dense bodies than do lymphocytes, but multivesicular bodies are more common in circulating lymphocytes than in monocytes. Monocytes usually exhibit a prominent Golgi apparatus, whereas lymphocytes have a poorly developed Golgi apparatus. When the distinguishing between lymphocytes and monocytes is of particular importance, we have found it useful to incubate the cells with colloidal gold or with heat-killed, washed *Staphylococcus albus*; monocytes display active phagocytosis and pinocytosis, and gold or bacteria are thus seen in digestive vacuoles or eventually in dense bodies. Lymphocytes exhibit neither pinocytic nor phagocytic activity demonstrable by these methods under various in vitro conditions studied thus far in our laboratory.

From the over-all view point, the mixed fixation procedure leads to distinctly better and more reproducible preservation of white blood cells than is possible with osmium tetroxide alone, with glutaraldehyde followed by osmium tetroxide, or with glutaraldehyde followed by osmium tetroxide and uranyl acetate (compare illustrations in references 10–13 and those presented here). Especially noteworthy features of cells processed by the mixed fixation method are the sharp membrane definition and the good preservation of granules and vesicles. Other methods of fixation may, of course, be preferable for special purposes; for example, glycogen deposits in neutrophil cytoplasm appear to be poorly defined after mixed fixation, which perhaps reflects extraction or modification in their staining properties. Our experience with the mixed fixative has been limited to leukocytes and to mouse macrophages and L cells in culture. Trump and Bulger (1) subjected blocks of rat and flounder renal tissue to simultaneous fixation with glutaraldehyde and osmium tetroxide and obtained generally good results. Whether our procedure will also serve for satisfactory fixation of various other cell types in suspension or in tissue blocks remains to be investigated. Suffice it to conclude, for the present, that the method described here is convenient and reliable, and that it enables studies, previously difficult or impossible, on normal and on pathologic features of leukocytes and macrophages.

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REFERENCES

1. TRUMP, B., and R. BULGER. 1966. New ultrastructural characteristics of cells fixed in a glutaraldehyde-osmium tetroxide mixture. *Lab. Invest.* **15**:368.
2. KELLENBERGER, E., A. RYTER, and J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasms. *J. Biophys. Biochem. Cytol.* **4**:671.
3. HIRSCH, J. G., M. E. FEDORKO, and Z. A. COHN. 1968. Vesicle fusion and formation at the surface of pinocytic vacuoles in macrophages. *J. Cell Biol.* **38**:629.
4. FEDORKO, M. E., J. G. HIRSCH, and Z. A. COHN. 1968. Autophagic vacuoles produced in vitro. *J. Cell Biol.* **38**:377.
5. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
6. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
7. MILLER, F., E. DE HARVEN, and G. E. PALADE. 1966. The structure of eosinophil leukocyte granules in rodents and in man. *J. Cell Biol.* **31**:349.
8. HIRSCH, J. G., and A. B. CHURCH. 1960. Studies of phagocytosis of group A streptococci by polymorphonuclear leucocytes *in vitro*. *J. Exptl. Med.* **111**:309.
9. RABINOWITZ, Y. 1964. Separation of lympho-

cytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood.* **23**:811.

10. LOW, F. N., and J. A. FREEMAN. 1958. Electron Microscopic Atlas of Normal and Leukemic Human Blood. McGraw-Hill Book, Company, New York.
11. BESSIS, M., and J. P. THIÉRY. 1961. Electron microscopy of white blood cells and their stem cells. *Intern. Rev. Cytol.* **12**:199.
12. FAWCETT, D. W. 1966. An Atlas of Fine Structure: The Cell, its Organelles, and Inclusions. W. B. Saunders Co., Philadelphia.
13. ANDERSON, D. R. 1966. Ultrastructure of normal and leukemic leukocytes in human peripheral blood. *J. Ultrastruct. Res. Suppl.* **9**:1.
14. WATANABE, I., S. DONAHUE, and N. HOGGATT. 1967. Method for electron microscopic studies of circulating human leukocytes and observations on their fine structure. *J. Ultrastruct. Res.* **20**:366.
15. BESSIS, M., and J. BRETON-BORIUS. 1957. Examen au microscope electronique des cellules des leucémies myéloides. *Bull. Microscop. Appl.* **5**:9.
16. FREEMAN, J. A., and M. S. SAMUELS. 1958. The ultrastructure of a "fibrillar formation" of leukemic human blood. *Blood.* **13**:725.
17. ACKERMAN, G. A., J. A. GRASSO, and R. A. KNOUFF. 1960. Morphological and histochemical studies of the leukemic cells from a patient with atypical myeloblastic leukemia with special reference to intracytoplasmic mucopolysaccharide vacuoles and fibrillar formation. *Blood.* **16**: 1253.
18. DE PETRIS, S., G. KARLSBAD, and B. PERNIS. 1962. Filamentous structures in the cytoplasm of normal mononuclear phagocytes. *J. Ultrastruct. Res.* **7**:39.
19. TANAKA, Y. 1964. Fibrillar structures in the cells of blood forming organs. *J. Natl. Cancer Inst.* **33**:467.
20. DE THÉ, G. 1964. Cytoplasmic microtubules in different animal cells. *J. Cell Biol.* **23**:265.
21. SUTTON, J. S., and L. WEISS. 1966. Transformation of monocytes in tissue culture into macrophages, epithelioid cells and multinucleated giant cells. *J. Cell Biol.* **28**:303.
22. HIRSCH, J. G. 1965. Neutrophil and eosinophil leucocytes. *In* The Inflammatory Process. Academic Press Inc., New York. 245.
23. ZUCKER-FRANKLIN, D. 1963. The ultrastructure of cells in human thoracic duct lymph. *J. Ultrastruct. Res.* **9**:325.
24. NOVIKOFF, A. B., E. ESSNER, and N. QUINTANA. 1964. Golgi apparatus and lysosomes. *Federation Proc.* **23**:1010.
25. DE DUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Ann. Rev. Physiol.* **28**:435.
26. PORTER, K. R. 1966. Cytoplasmic microtubules and their functions. *In* Ciba Foundation Symposium on Principles of Biomolecular Organization. G. E. W. Wolstenholme and M. O'Connor, editors. Little, Brown and Company, Boston. 308.
27. ALLEN, R. D. 1961. Ameboid movement. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press Inc., New York. **2**:135.
28. FLOREY, H. W., and L. H. GRANT. 1961. Leucocyte migration from small blood vessels stimulated with ultraviolet light: An electron-microscope study. *J. Pathol. Bacteriol.* **82**:13.
29. LOCKWOOD, W. R., and F. ALLISON. 1963. Electromicrographic studies of phagocytic cells. I Morphological changes of the cytoplasm and granules of rabbit granulocytes associated with ingestion of rough pneumococcus. *Brit. J. Exptl. Pathol.* **44**:593.
30. ZUCKER-FRANKLIN, D., and J. G. HIRSCH. 1964. Electron microscope studies on the degranulation of rabbit peritoneal leukocytes during phagocytosis. *J. Exptl. Med.* **120**:569.
31. BAINTON, D. F., and M. FARQUHAR. 1966. Origin of granules in polymorphonuclear leucocytes. *J. Cell Biol.* **28**:277.
32. WETZEL, B. K., R. G. HORN, and S. S. SPICER. 1967. Fine structural studies on the development of heterophil, eosinophil and basophil granulocytes in rabbits. *Lab. Invest.* **16**:349.
33. MERCER, E. H. 1959. An electron microscopic study of *Amoeba proteus*. *Proc. Roy. Soc. Biol.* **150**: 216.