MICROTRABECULAR LATTICE OF THE CYTOPLASMIC GROUND SUBSTANCE

Artifact or Reality

JOHN J. WOLOSEWICK and KEITH R. PORTER

From the Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309. Dr. Wolosewick's present address is the Department of Anatomy, University of Illinois at the Medical Center, Chicago, Illinois 60612.

ABSTRACT

The cytoplasmic ground substance of cultured cells prepared for high voltage transmission electron microscopy (glutaraldehyde/osmium fixed, alcohol or acetone dehydrated, critical-point dried) consists of slender (3–6 nm Diam) strands—the microtrabeculae (55)—that form an irregular three-dimensional lattice (the microtrabecular lattice). The microtrabeculae interconnect the membranous and nonmembranous organelles and are confluent with the cortices of the cytoplast. The lattice is found in all portions of the cytoplast of all cultured cells examined. The possibility that the lattice structure is an artifact of specimen preparation has been tested by (*a*) subjecting whole cultured cells (WI-38, NRK, chick embryo fibroblasts) to various chemical (aldehydes, osmium tetroxide) and nonchemical (freezing) fixation schedules, (*b*) examination of model systems (erythrocytes, protein solutions), (*c*) substantiating the reliability of critical-point drying, and (*d*) comparing images of whole cells with conventionally prepared (plastic-embedded) cells. The lattice structure is preserved by chemical and nonchemical fixation, though alterations in ultrastructure can occur especially after prolonged exposure to osmium tetroxide. The critical-point method for drying specimens appears to be reliable as is the freeze-drying method. The discrepancies between images of plastic-embedded and sectioned cells, and images of whole, critical-point dried cells appear to be related, in part, to the electron-scattering properties of the embedding resin. The described observations indicate that the microtrabecular lattice seen in electron micrographs closely represents the nonrandom structure of the cytoplasmic ground substance of living cultured cells.

KEY WORDS microtrabecular lattice · cytoplasmic ground substance · high voltage electron microscopy · whole cultured cells · fixation and artifact

A number of recent reports call attention to the lattice-like morphology of the cytoplasmic ground substance as it appears in electron micrographs of thinly spread, cultured cells (5, 7, 10, 23, 53–58). In preparation for microscopy, such cells have ordinarily been fixed with glutaraldehyde, post-fixed with OsO_4, and dehydrated in alcohol or acetone. Finally, the specimens have been trans-

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/07/0114/26 $1.00
Volume 82 July 1979 114–139

ferred to liquid CO_2 and dried by the critical-point method (2) to avoid the damaging effects of surface tension. After these treatments, the cytoplasmic ground substance appears to consist of slender strands (3–6 nm in diameter), which we have called microtrabeculae (55), interconnected so as to form an irregular, three-dimensional, microtrabecular lattice (MTL). This lattice is confluent with the upper and lower cortices of the cytoplast and interconnects most of the better-known components of the cell such as microtubules, cisternae of the endoplasmic reticulum, polysomes, and stress fibers. We have suggested that the strands of the MTL constitute a polymerized, protein-rich phase, whereas the intertrabecular spaces contain water-rich solutions of low molecular weight metabolites (35).

The possibility that the observed lattice might be an artifact condensed during fixation or dehydration from a homogeneous, protein-rich cytosol or cytogel has occurred to most observers. This concern has not been dispelled by observations that a similar, though less distinct lattice is present in conventionally prepared thin sections of a number of cell types (our interpretations of published micrographs, e.g., references 6, 8, 9, 21, 24, 25, 39, 45, and 59). Various arguments for (16) and against (5, 55, 58) the artifactual nature of the MTL have been published, but these notwithstanding, some doubts persist as to whether the lattice is merely a condensation artifact, or, if not that, just how closely the visualized image resembles the structure of the ground substance before fixation, dehydration, and critical-point drying.

To help resolve these issues, we have (*a*) subjected whole cultured cells to various chemical and nonchemical fixation schedules, (*b*) examined model systems after similar fixations, (*c*) investigated the reliability of critical-point drying, and finally, (*d*) compared the images obtained from whole cells with conventionally prepared thin sections of cells. The resulting observations are discussed and evaluated along with arguments supporting our belief that the lattice seen in electron micrographs closely represents the nonrandom structure of the ground substance of living, cultured cells.

MATERIALS AND METHODS

Because the observations reported below are best understood in the context of the experimental conditions employed, procedural details are given in association with the respective experiments and observations. We hope this departure from convention makes this report more readable.

Cell Lines

The procedures used throughout this study for growing cells in preparation for high voltage electron microscopy were described previously (13, 55, 56). In brief, cultures were established on Formvar-carbon-coated gold grids held on coverslips. When enough cells were visible on the grids (phase-contrast microscopy), the cultures were used for experiments without other disturbance to the cells.

For most of these studies we used the well-known cell line, WI-38 (human fibroblast [18]). Cells were maintained under constant growth conditions as recommended by the American Type Culture Collection. In a few instances, however, normal rat kidney (NRK [1]) and chick embryo cells grown from primary explants of lung tissue (14-d incubation) were used.

Viewing the Micrographs

Stereo viewing is essential in the examination of micrographs of thick preparations taken with the high voltage microscope. In fact, there is little advantage in using the microscope to examine thick specimens unless one takes stereo pairs and studies them as a fused image. The superimposition of structures in a single image of a thick specimen is unavoidable, as well as confusing, and such images are essentially uninterpretable. Most of the images presented here to illustrate our observations are accordingly reproduced as stereo pairs.

These can be observed with the aid of small binocular desk sets manufactured especially for stereo viewing (obtainable, e.g., from Abrams Instrument Corp., Lansing, Mich.). Alternatively, the viewer can learn to fuse such pairs without mechanical aids. What one does is to focus with both eyes on an object at ~30 feet. Without changing focus, one then interposes the stereo pair in the line of sight. The observer should see three images, and the middle one will be stereoscopic. Practice will perfect the technique to the point where fusing stereo pairs will become rapid and comfortable.

EXPERIMENTAL

I. Chemical Fixation

Fixation of cells and tissues with various mixtures of chemicals has long been regarded as a

source of structural artifacts. Among the many reagents tried, osmium tetroxide has received more than passing attention because of the unusually good quality of preservation achieved. This was made especially evident by Strangeways and Canti (46) in their study of the action of several fixatives on cultured cells. Their observations were confirmed in the early period of biological electron microscopy in studies involving both cultured cells and whole tissues (30, 36, 37). It was also recognized early that, although OsO_4 fixes well during initial contact with the cells, this reagent eventually solubilizes most components of the cytoplasm, membranes excluded, if allowed to act over periods as long as 12–24 h (31). In this and other respects, glutaraldehyde is more reliable, and combined with OsO_4 it appears to be ideal (41). Since other classical fixatives are known to be strongly acidic or to contain known protein coagulants (e.g., mercuric chloride), we chose for this study to experiment only with the currently popular aldehydes and OsO_4.

A. The Aldehydes: Glutaraldehyde and Paraformaldehyde

PROCEDURES: As described earlier (55, 56), cells were incubated on Formvar-coated gold grids until a suitable population was reached. The culture medium was poured off the grids, leaving only a thin layer covering the cells, and immediately replaced with 2% glutaraldehyde (E. M. grade, Polysciences, Inc., Warrington, Pa.) in 0.1 M sodium cacodylate (Sigma Chemical Co., St. Louis, Mo.) (pH 7.2–7.4, 37°C). Fixation in glutaraldehyde was continued for 15 min at 37°C, after which the primary fixative was removed, and the cells were rinsed briefly (three changes in 3 min) in buffer (0.2 M sodium cacodylate) alone before being covered with a 1% solution of OsO_4 (Polysciences, Inc.) in 0.1 M sodium cacodylate for 5 min. Preparations were then rinsed in distilled H_2O (two changes in 1 min), stained for 1–2 min in 0.5% uranyl acetate in 0.2 M s-collidine (pH 4.5), washed in 0.2 M s-collidine, and placed in a multiple-grid holder (Tousimis, Rockville, Md.) for further processing.

Dehydration in increasing concentrations of reagent grade acetone (15, 25, 50, 70, 95, and 100%; 1–2 min per change) was followed by drying from liquid CO_2 by the critical-point method according to Anderson (2). Grids were subsequently carbon-coated on both sides and stored over a desiccant (silica gel) for later observation.

The quality of preservation realized with this procedure is regarded in this laboratory as the standard against which the effects of other fixatives or experimental conditions are evaluated.

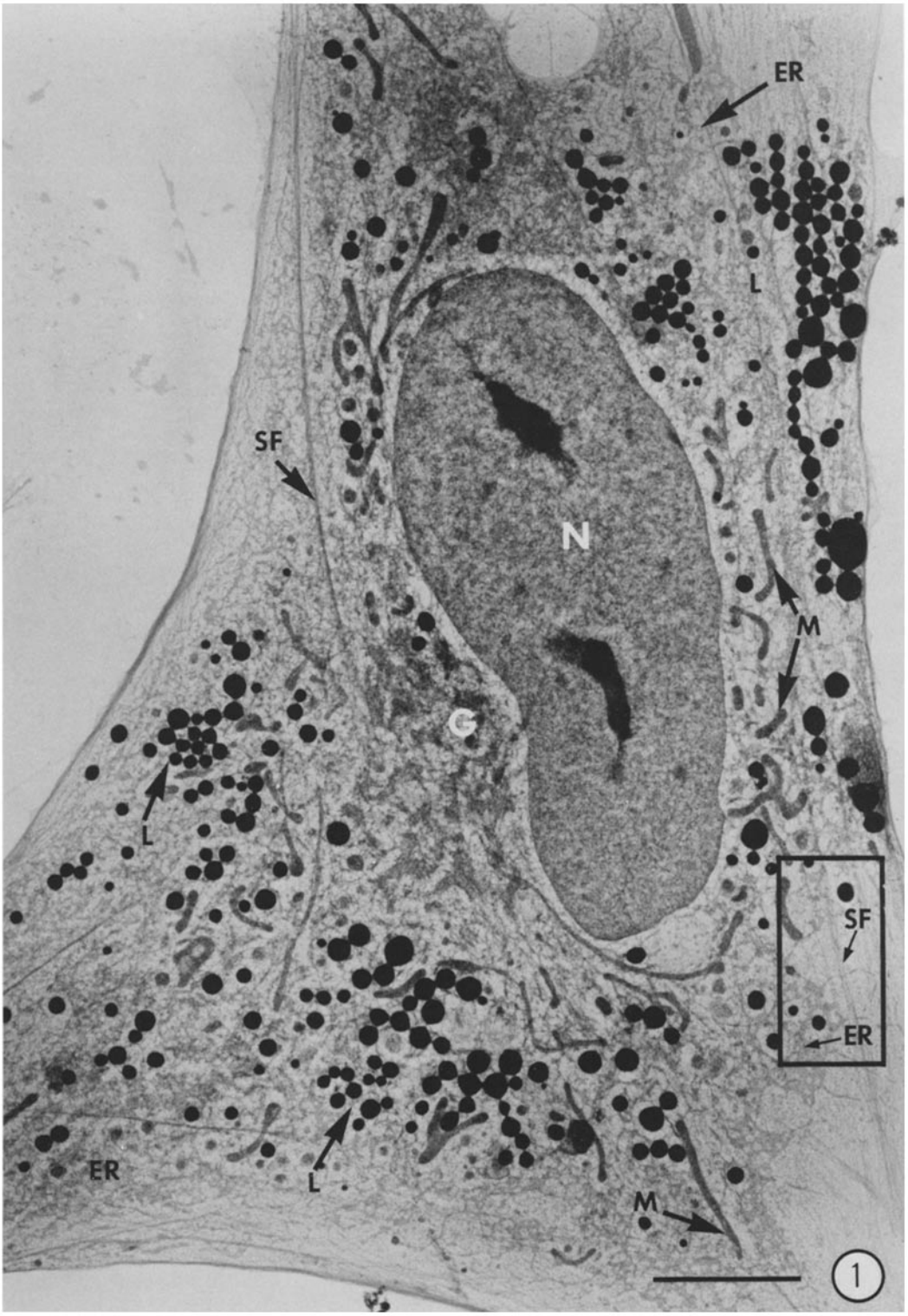
In five cases, 2% paraformaldehyde was used as the primary fixative instead of glutaraldehyde; the buffers and fixation times were as just described. In other experiments, fixation was continued for 3 h. Finally, some preparations were fixed in either glutaraldehyde or paraformaldehyde and processed without any additional stain (i.e., osmium or uranyl acetate).

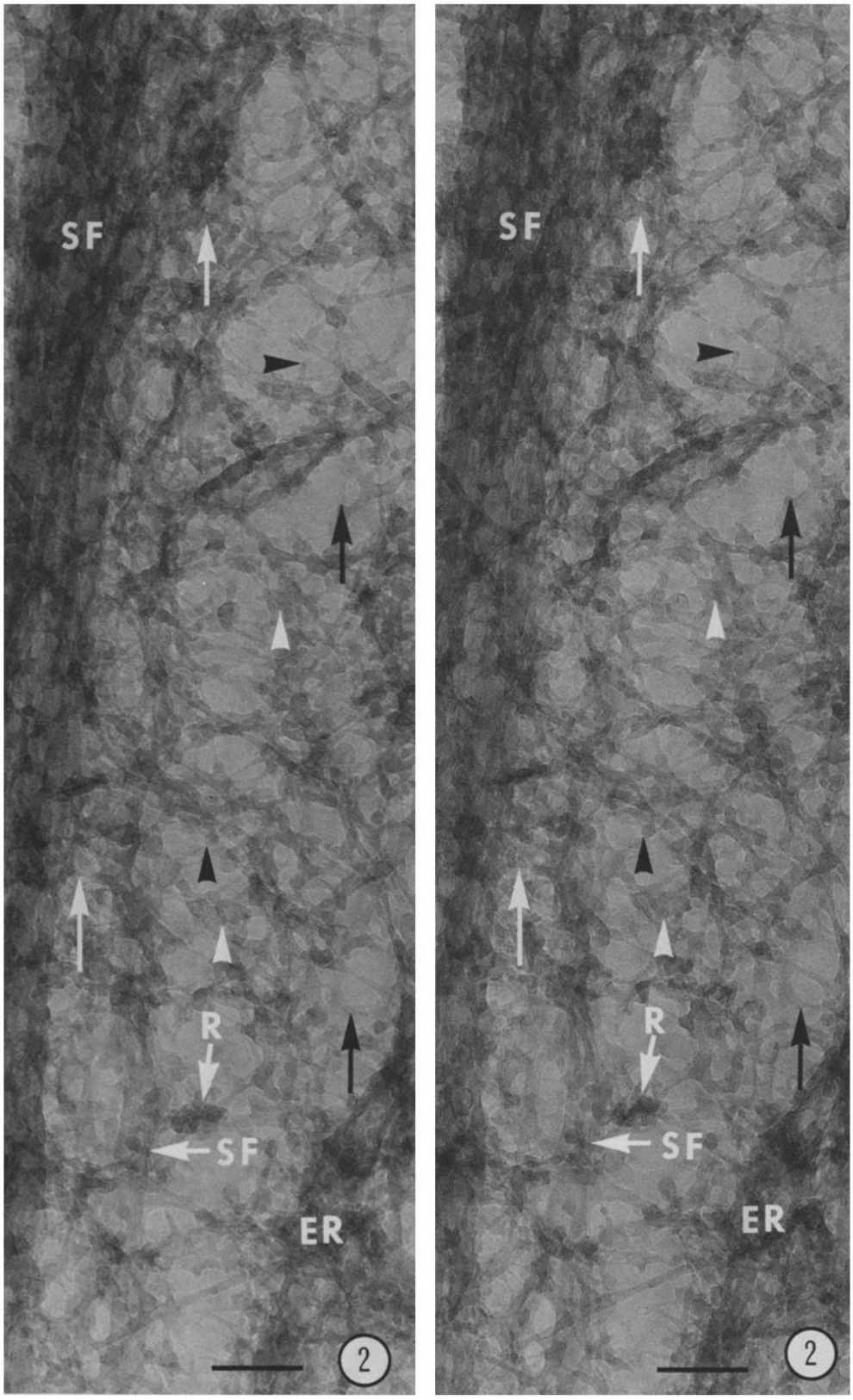
OBSERVATIONS: Low magnification micrographs of cultured chick cells grown in monolayer consistently show the fine structural features depicted in Fig. 1. Chick cells are smaller, thinner, and less dense than human WI-38 cells; measurements from thin sections and stereo pairs show that thinly spread chick cells are never thicker than 1.0 μm. These combined characteristics yield informative images of all parts of the cell, and the absence of any embedding resin in these preparations greatly enhances contrast and resolution.

The organelles and structures evident in low magnification images are readily identified. It is common in these thinly spread cultured cells for fibers, mitochondria, and lipid granules to be more or less radially disposed with respect to the cell center, suggesting a nonrandom organization of the cytoplast (Fig. 1).

At higher magnification, i.e., in micrographs

FIGURE 1 Low magnification image of an unsectioned fibroblast from a primary explant of lung from a 14-d chick embryo. The high energy (1 MeV) electrons have penetrated all portions of the cell including the nucleus, as evidenced by the clarity and contrast of the image. Organelles readily identified in this cell include the nucleus (*N*), mitochondria (*M*), endoplasmic reticulum (*ER*), centrosphere region and Golgi (*G*), stress fibers (*SF*) composed of microfilaments, and lipid inclusions (*L*). Such low magnification images illustrate the organization of the cytoplast in these thinly spread cells. The distribution of mitochondria, stress fibers, and rows of lipid inclusions tends to be radial. Larger organelles are excluded from regions above and below the nucleus, probably by the thickness of the nucleus. The matrix occupying the space between the major organelles supports the fine lace-like divisions of the ER and other organelles and has a fine structure depicted in Figs. 2 and 3. The outlined area (lower right) if enlarged would be similar in its content and structure to that shown in Fig. 2. Bar, 5 μm. × 4,500.


ER
L
SF
N
M
G
L
SF
ER
ER
L
M
1


SF
R
SF
ER
2
SF
R
SF
ER
2

depicting less than a few cubic micrometers of the cell, the previously described lattice of microtrabeculae (55) emerges as a three-dimensional continuum apparently supporting other cytoplasmic components (Figs. 2 and 3). With appropriate tilt angles, the lattice is easy to observe in stereo.

Individual microtrabeculae vary enormously in length and thickness. Indeed, it is most difficult to measure accurately structures that are so nonuniform throughout the cytoplast. Microtrabeculae may measure more than 10 nm thick at their ends, i.e., where they fuse with other trabeculae, microtubules, and microfilaments, etc. but only 2–3 nm thick at their midpoints. The variation is especially great when lattice elements near the cell margin are compared with those near the cell center. Where microtrabeculae approach the continuous layer of cortical matrix material and become confluent with it, they flatten out and align with their broadest aspects parallel to the surface of the cortex.

Microtrabeculae also vary enormously in length, some reaching 100–200 nm; others are much shorter. There is a corresponding variation in the size of the interstices outlined by the trabeculae. We estimate that ~80% of the cytoplasmic volume comprises intertrabecular space, but this varies with cell type.

Stress fibers are prominent in chick fibroblasts and WI-38 cells and seem to be integral parts of the lattice in which parallel and often elongated trabeculae have become laterally associated in compact bundles. This structure is made particularly striking by the fact that, in regions adjacent to the stress fibers, the trabeculae appear randomly oriented in space. At least two sizes of stress fibers are evident in Fig. 2. One consists of only a few trabeculae in parallel array; the other comprises many. Fibers seem to develop in regions closer to the upper and lower surfaces (cortices) of the cytoplast (the latter, as used here, refers to all of the cell, exclusive of nucleus and plasma membrane).

Elongated vesicles of the endoplasmic reticulum, which in these cells form a lace-like configuration, are contained in the lattice at a level more or less midway between the upper and lower cortices (Figs. 2 and 3), and have only a few ribosomes on their surfaces. Other "free" ribosomes and polysomes are contained in the trabeculae and are especially prominent at points where trabeculae fuse, that is, at cross-points in the lattice (Fig. 4).

Microtubules are not detected in some images (Fig. 2), probably because none is present in the small volume of cytoplasm represented. Where evident, they have essentially the same density as the trabeculae and are not much larger in diameter than some of the latter. However, because they tend to be straight and extend over relatively long distances (several micrometers), they can ordinarily be identified (see black arrow, Fig. 3). The microtubules are coated with matrix material, and trabeculae extend from or into this coating. Thus, the microtubules, too, are integral to the lattice and essentially suspended in it.

In experiments using paraformaldehyde as a fixative, the morphology of the cytoplasmic ground substance had the same general features as after glutaraldehyde. The differences, if any, are so insignificant that they cannot be convincingly depicted or described.

Similarly, experiments to test the effects of prolonged fixation reveal that in cells fixed with glutaraldehyde for 3 h the MTL has the same mor-

FIGURE 2 Stereo micrograph of a region similar to that outlined in Fig. 1, but at higher magnification. Two parallel stress fibers (*SF*) are shown, one broad and thick, close to the upper surface, the other much thinner and nearer the lower cortex. Microtrabeculae are continuous with individual filaments of the stress fiber as well as with filaments not part of the stress fiber. The microtrabeculae which make up the space-filling lattice are distinctly more random in distribution and orientation farther from the stress fiber. For the most part they vary in "diameter" from 3–6 nm, though some are much broader, especially when they fuse with other microtrabeculae. The intertrabecular space, which is continuous throughout the cytoplast, is represented in the image by microtrabecula-free areas 80 to over 100 nm across. Many trabeculae (white arrows) appear more globular and at junctional regions are frequently occupied by polysomes (*R*). Where the microtrabeculae fuse with the cortices, they adopt more lamellar forms (black arrows). A few microtrabeculae (black arrowheads) have free ends; these may represent local areas of deformation/reformation of the protein-rich phase of the lattice. Alternatively, these free-ending strands may develop during specimen preparation or examination. *ER*, endoplasmic reticulum. 8° total tilt. Bar, 0.1 μm. × 140,000.

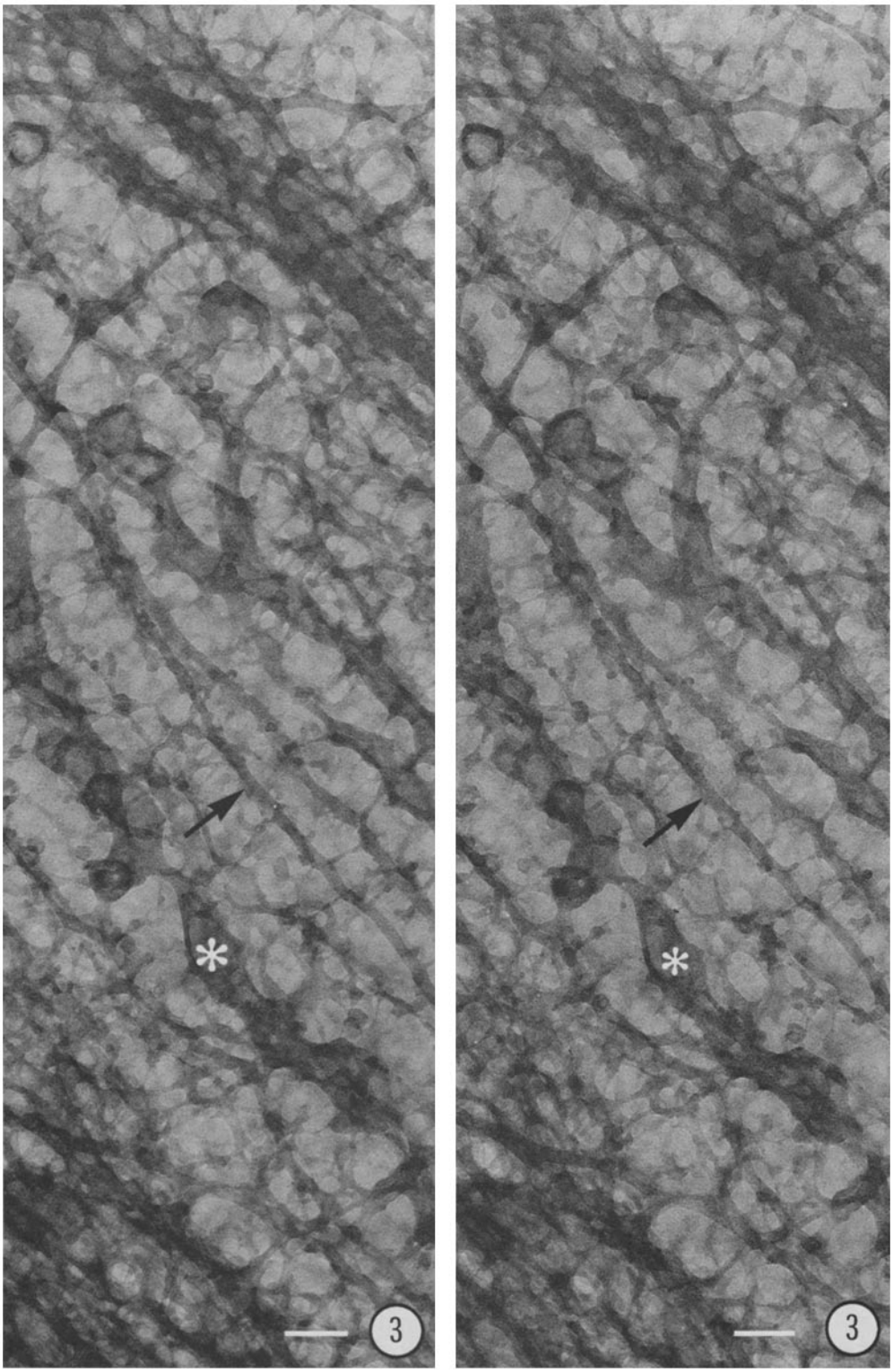


FIGURE 3 Stereo micrograph of a portion of a WI-38 cell (passage 31) dried by the critical-point method. The microtrabecular lattice in this cell resembles the lattice in chick fibroblast (Fig. 2). Microtrabeculae are prominent here and exhibit the wide variation in both length and breadth observed in cultured chick embryo cells. Here, the fusion of microtrabeculae with the surfaces of microtubules (arrow), the cisternae of the smooth endoplasmic reticulum (asterisk), and the microfilaments is easily seen in three dimensions. As in all the cells studied, microtrabeculae are broader where they fuse with organelles, or other

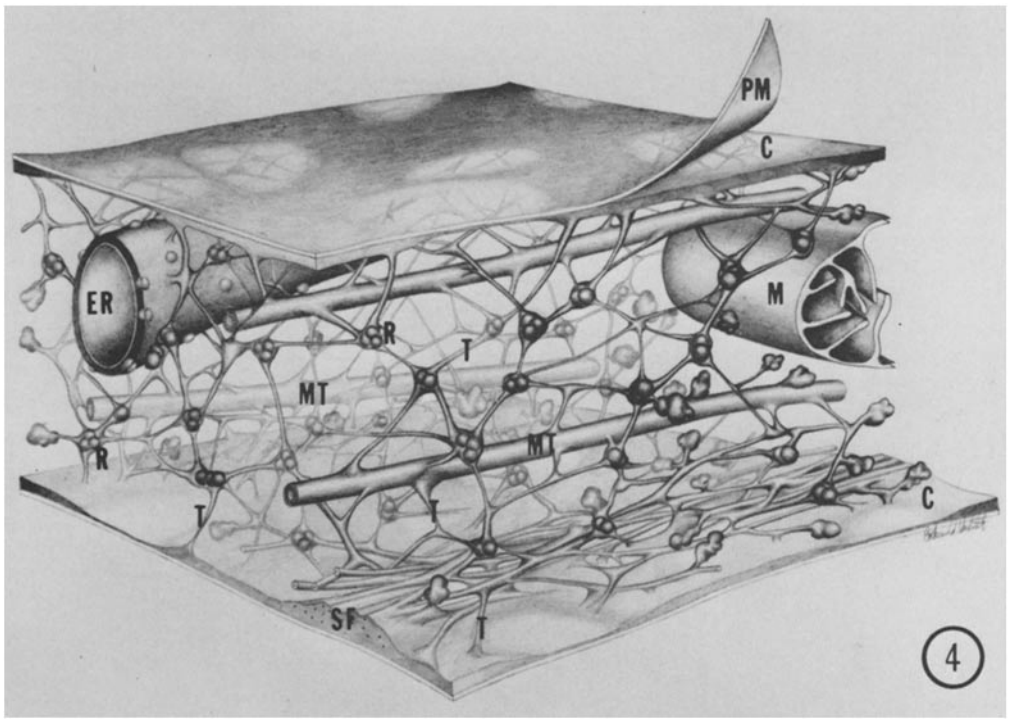


FIGURE 4 A model of the microtrabecular lattice illustrating the relationship of the structured elements to other organelles. This model is derived from hundreds of stereo images of cultured cells prepared by the techniques described in this report. The lattice of slender microtrabeculae is a constant feature of the ground substance in all cells studied. In this model, material of the lattice is depicted as continuous with the upper and lower cortices (*C*), the surfaces of the endoplasmic reticulum (*ER*), with microtubules (*MT*), with the filaments in stress fibers (*SF*), and other trabeculae (*T*). "Free" polysomes (*R*) are depicted at the junctional regions of the trabeculae. We suggest that this lattice supports or contains the organelles of the cell with the possible exception of mitochondria (*M*). *PM*, plasma membrane. × 150,000 (approx.).

phology as in cells fixed for only 15 min.

Among the cells used in this study, chick cells yielded images showing greater openness in the lattice than either the WI-38 or NRK cells. More recently, the range of cell types brought to the high voltage microscope has been broadened considerably, as has our awareness of a great variability in the compactness of the lattice, related in all probability to the degree of hydration of the cells examined. In general, the lattice is more finely divided in differentiated cells than in the cells from explants of embryonic tissue. These observations will be reported in detail elsewhere.

The schematic representation of the lattice shown in Fig. 4 is the image of the cytoplasmic ground substance that is common to all of the cultured cells fixed by the procedures outlined above. It is this image that is to be evaluated for its relation to what actually exists in the living cell.

B. Osmium Tetroxide

The qualities of OsO_4 as a fixative have been repeatedly extolled since it was introduced to histology over a century ago by Schultze (43). For electron microscopy it combines in one reagent both fixative and stain, and for this reason, among others, it found early favor among electron microscopists (30, 36). Since the chemical reactions between OsO_4 and the components of the cell are very different from those of the aldehydes (17, 37), we were particularly interested to see whether, when used alone, osmium would preserve the lattice described above.

PROCEDURE (1): OsO_4 IN SOLUTION: Cells were handled identically with those for glutaraldehyde fixation except that a solution of osmium tetroxide (1% in 0.2 M sodium cacodylate, pH 7.2–7.4) replaced the glutaraldehyde. Fixation was continued for 15 min, after which the preparations were rinsed in distilled water,

dehydrated through an acetone series, and dried by the critical-point method.

OBSERVATIONS: When used as the primary fixative, osmium tetroxide preserves the microtrabeculae and their arrangement in an irregular three-dimensional lattice in all cells studied (Fig. 5). As expected, cellular membranes, especially those of the endoplasmic reticulum and mitochondria, fix and stain satisfactorily with osmium tetroxide, as do also lipid inclusions. Microfilament bundles (stress fibers) appear consistently in these preparations, although individual 6- to 7-nm (actin) filaments often are difficult to resolve. Though not normally preserved by OsO_4, microtubules are seen occasionally in the cortical regions of osmium-fixed cells. On the other hand, trabeculae comprising the lattice are less distinct than in the aldehyde-fixed cells. Closer examination also reveals many discontinuities in the lattice. Many microtrabeculae terminate as free ends (arrowheads, Fig. 5), as if there had been some decay of the original structure and perhaps a flowing together of the material of the matrix to form a slightly different structure from that evident in aldehyde-fixed cells. This phenomenon may be similar to the partial disassembly of bundles of actin microfilaments reported by others as taking place during fixation in solutions of OsO_4 (33).

PROCEDURE (2): OsO_4 VAPORS: In another series of experiments, cultured cells (WI-38 and NRK) were fixed with vapors of OsO_4. This was done by quickly draining all but a thin film of culture medium from the grids and then inverting them over a 1% solution (in H_2O) of OsO_4 in a Maximow well slide for 1, 10, 60, or 120 min. All preparations were subsequently processed as described above.

OBSERVATIONS: The image of the cytoplasmic ground substance after a 1-min exposure to OsO_4 vapors (Fig. 6) essentially duplicates the appearance after fixation by immersion in solutions of OsO_4. If there is any significant difference in fixation, it is in the appearance of the cytoplasmic cortex underlying the plasma membrane, and in the smaller number of microtrabeculae. The cortex shows a reticular pattern of thick and thin areas which may have developed as a consequence of the relative slowness with which the OsO_4 vapor reaches the cytoplasmic ground substance. Possibly the material of some of the microtrabeculae mingled with that of the cortex before fixation was complete. Fixation with osmium is evidently not an instantaneous achievement, as with glutaraldehyde, but seems rather to be prolonged with the products always changing.

This could, on the other hand, be the early stage of a decay in the quality of the initial fixation that becomes much more evident after 120 min of exposure to osmium vapors (Fig. 7). Here, the residual trabeculae are few and most of the osmium-fixed material, representing the residue of the lattice, is associated with either the upper or lower cortex in the form of irregularly fenestrated sheets. One has the impression that, after initial "fixation" the structural integrity fails and the lattice is to some extent deformed and solubilized. Eventual stabilization of the residue may result from dehydration in acetone.

II. Fixation by Freezing

These experiments were designed to test the possibility that chemical fixation or other conventional procedures induce meaningless artifacts. We therefore included in this study freeze-drying from both the nonfixed and fixed states, and freeze-substitution using OsO_4 as the fixative. In all the experiments, cells on coated grids were frozen without the aid of cryoprotectants.

A. Freeze-Drying Procedures without Chemical Fixation

PROCEDURE: Grids with cells were removed from their cover slips with fine forceps, dipped once for 1 s in prewarmed (37°C) isotonic ammonium carbonate (pH 7.2, 0.75%, ~320 mosmol), or 27°C distilled water, flicked free of liquid, and immediately plunged into liquid nitrogen or into Freon-12 quenched at liquid nitrogen temperatures. The entire procedure was completed in ~2 s. The grids were then placed into a solid steel grid holder submerged in liquid nitrogen, and transferred quickly to the thermocouple stage of a modified Denton freeze-drying apparatus (Denton Vacuum Inc., Cherry Hill, N. J.) operating at 5×10^{-5} Torr and with a cold trap (copper bar) in the chamber maintained at −180°C. The cells were dried *in vacuo* for 8–12 h at −80°C, after which they were allowed to warm gradually to room temperature. On several occasions the stage and the dry specimens were warmed slowly to +60°C to volatilize residual ammonium salts. Thereafter, the specimens were coated on both sides with a layer of evaporated carbon and stored as described above.

OBSERVATIONS: Fig. 8 illustrates the appearance of one of the frozen-dried specimens. The surprising contrast imparted to the images of the microtrabeculae and inclusions is apparently the

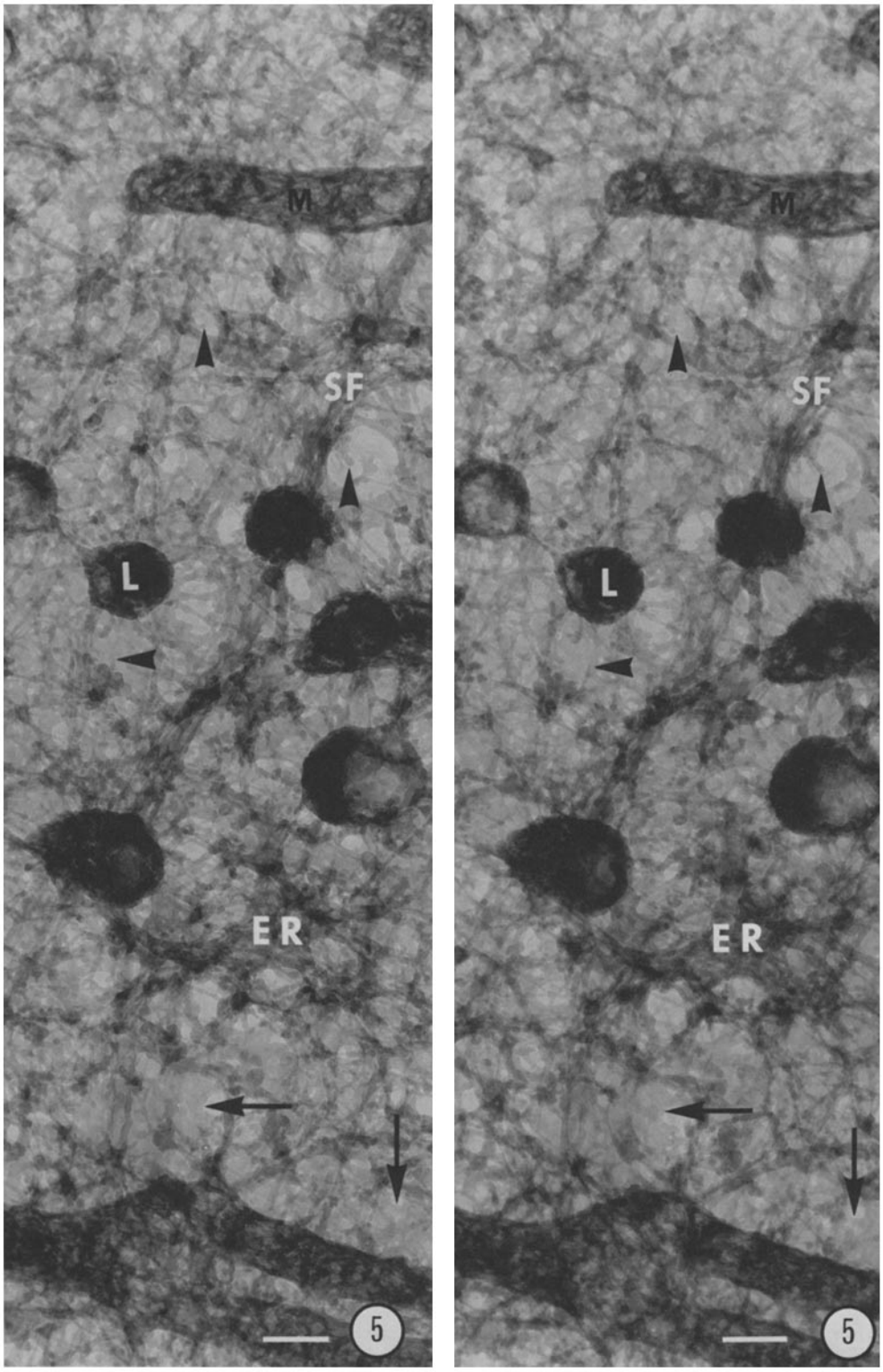
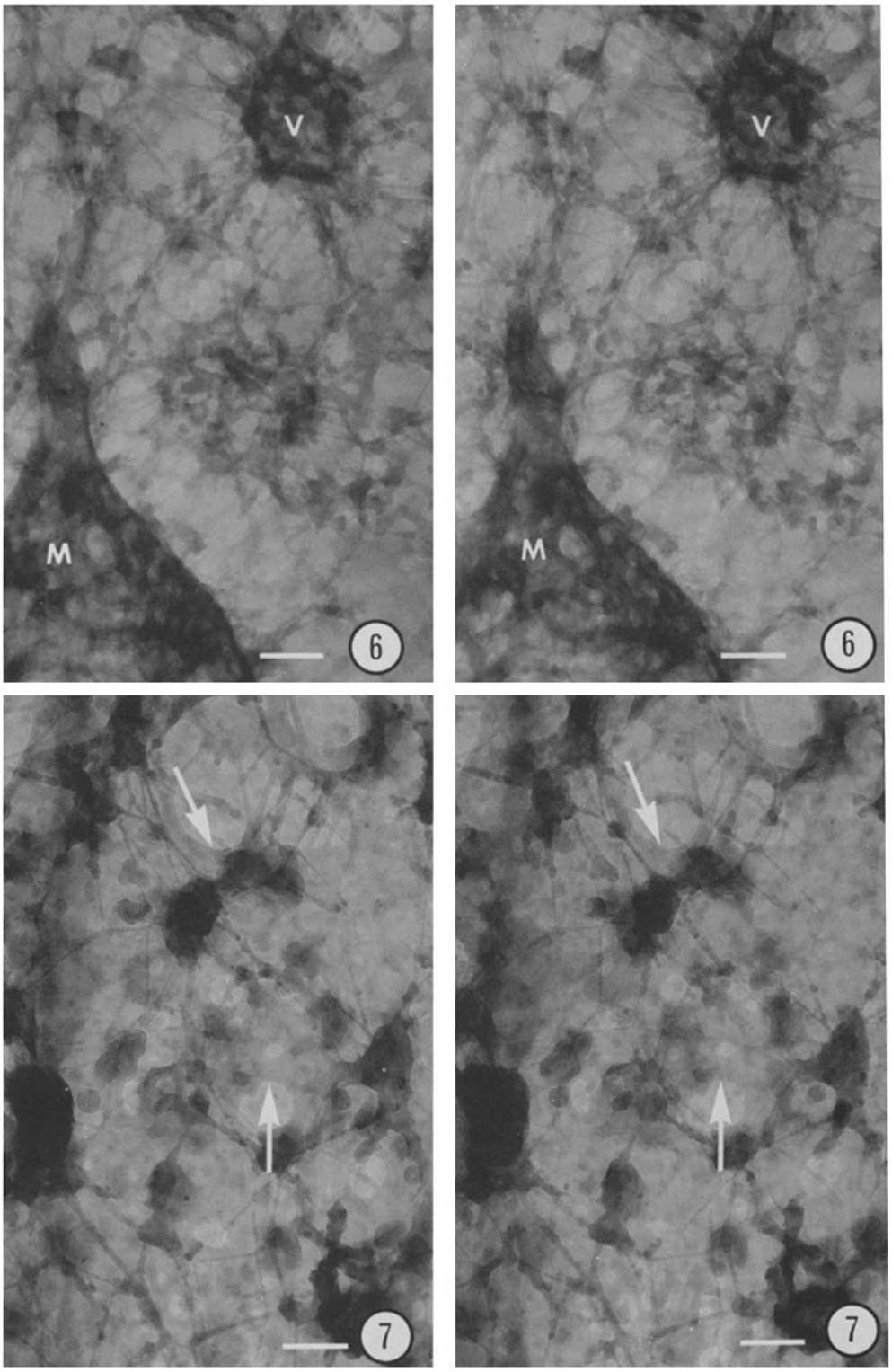


FIGURE 5 WI-38 cell (passage 30) fixed by immersion in 1% OsO_4 for 15 min. As expected, mitochondria (*M*) and lipid-rich inclusions (*L*) are preserved and are prominently stained. The lattice made up of numerous trabeculae is present, as after glutaraldehyde, but not without modifications. To the experienced observer, it is evident that many of the trabeculae are broader and seem to be part of a fenestrated sheet closely associated with the undersurface of the upper cortex (arrows). One notes as well that many trabeculae associated with the stress fibers (*SF*) and elsewhere have free endings (arrowheads) as though caught by the fixative after the strand had broken and started to withdraw toward its two ends. This would reduce the number of trabeculae that traverse the region between the upper and lower cortices. An isolated cisterna of the ER is also shown. 10° tilt. Bar, 0.2 μm. × 48,000.


V
M
6
V
M
6
7
7

product, in part, of the salts retained in the structures. The trabeculae are preserved in their three-dimensional form without evident distortions, although damage from ice crystals could be found in some places, especially in regions within the nucleus and in the thicker parts of cells. Even though the volume of liquid covering the cells on the grid immediately before freeze-fixation is very small (<0.01 ml) and the cells are only 1 μm thick, it is apparent from the internal morphology that the freezing rates (e.g., Freon-12, 2,940°C/s [40]), are too slow to transform the aqueous phase in the cell to noncrystalline ice.

The dimensions of the trabeculae are generally larger than in preparations from other procedures, often 40–50% broader than in glutaraldehyde-fixed, acetone-dehydrated specimens. This suggests to us that glutaraldehyde-OsO_4 and dehydration may preserve the material, but not the dimensions of the trabeculae.

B. Freeze-Drying After Fixation

It seemed important to test whether the MTL might be a product of dehydration and/or critical-point drying after fixation. This was regarded as unlikely, but in the course of the present survey, we decided to fix cells with glutaraldehyde and osmium tetroxide in the usual way, and then freeze them as we had the living cells, and dry *in vacuo*.

PROCEDURES: WI-38 cells, growing on Formvar-coated grids, were fixed with glutaraldehyde and osmium as described under Section I A, and then held in distilled water for 1–2 min. Grids were individually removed from the glass cover slip, flicked free of excess water or blotted to remove the excess water, and immediately plunged into Freon-12 quenched at liquid nitrogen temperatures. The grids were then transferred to a metal grid holder submerged under liquid nitrogen, and transferred quickly to the thermocouple stage of the freeze-drying apparatus and dried for 8 h at 5×10^{-5} Torr. The temperature of the thermocouple stage was −80°C, while the cold trap was maintained at liquid nitrogen temperatures. After 8 h of drying, the specimens were allowed to come to room temperature. The grids were then removed from the drying unit, coated on both sides with a layer of carbon, and stored over a desiccant.

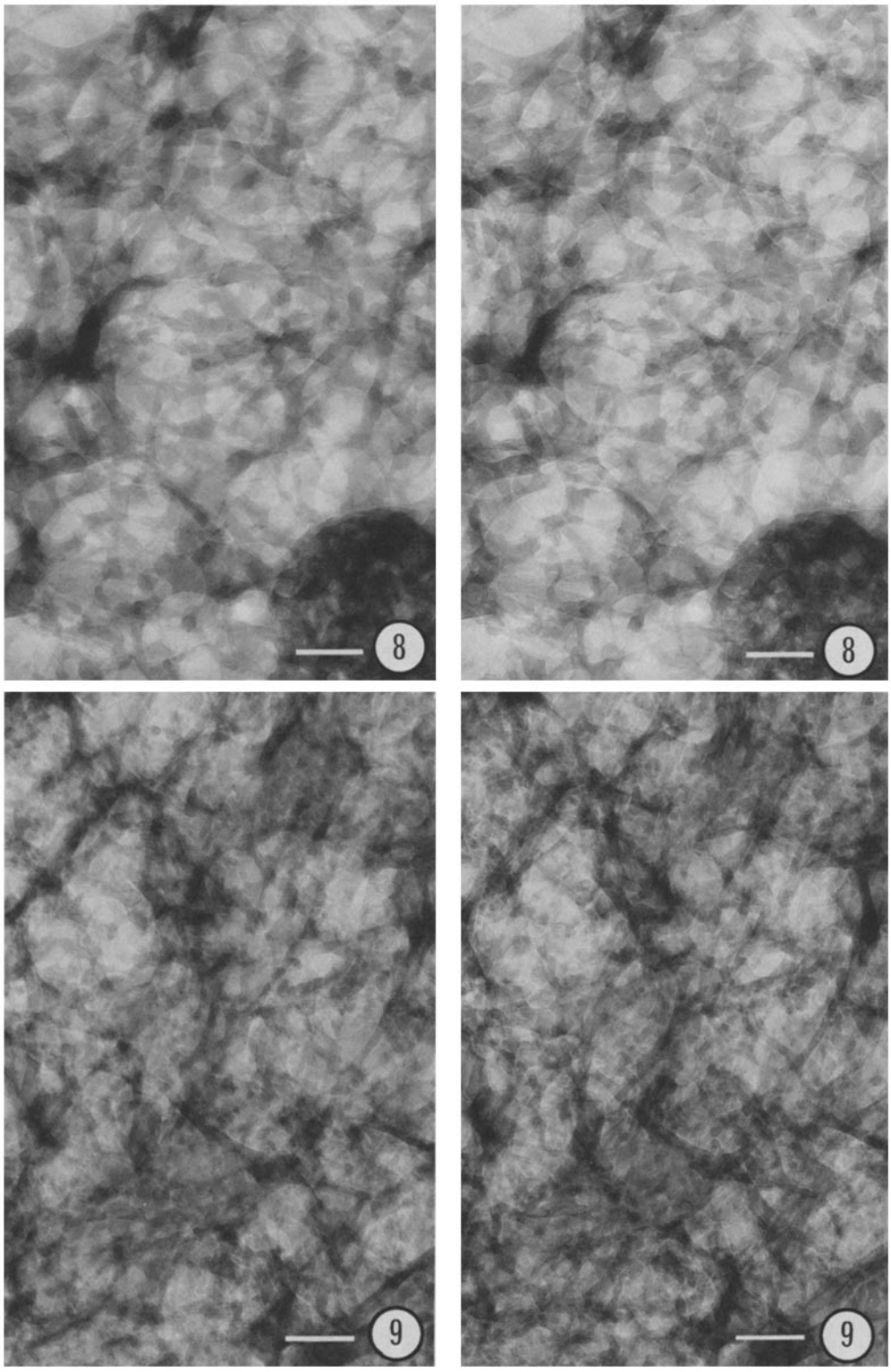
OBSERVATIONS: The important observation from this experiment is that the microtrabecular lattice is still observed and displays the general characteristics normally observed after fixation with the standard glutaraldehyde-osmium fixation, dehydration with solvents, and critical-point drying (Fig. 9). However, the individual trabeculae are as large as in cells frozen-dried without previous fixation (Section II A), which suggests that drying by acetone and/or critical-point drying from CO_2 does cause some shrinkage in the dimensions of the trabeculae, especially at their midpoints. The contours of the trabeculae in these fixed and frozen-dried cells are not so smooth and uniform as in nonfixed frozen-dried cells (Fig. 8).

C. Freeze-Substitution

Another important and accepted technique for the preservation of biological ultrastructure is

FIGURE 6 Stereo image of an intermediate passage WI-38 cell exposed to osmium vapors for 1 min. The lattice microtrabeculae are preserved adequately by short vapor-phase fixation, although there are few microtrabeculae in cells fixed in this way and the cells tend to have smoother contours than cells fixed by immersion. Note that the cortical regions are denser and less homogeneous than in Fig. 5. It is also evident that many of the trabeculae have withdrawn to the larger masses in the cytoplasm such as vesicles of the ER (*v*) or polysomes. These phenomena may be the early stage of deformations induced by inadequate or slow fixation with osmium vapors. The end of a mitochondrion (*M*) is at the lower left. 12° total tilt. Bar, 0.2 μm. × 48,000.

FIGURE 7 Stereo image of a portion of a WI-38 cell fixed for 2 h over vapors of OsO_4. The failure of the OsO_4 to preserve the microtrabeculae in the usual form is even more evident than in either of the preceding images. It is certain that the upper and lower plasma membrane of this cell are fixed, as are all the membranes, but the ground substance or matrix of the cytoplasm, represented in these images by the microtrabeculae, has decayed or is not preserved in its polymerized form. Evidence of this failure increases with time of exposure to the OsO_4 vapors. The "lakes" of dense materials associated with the upper and lower surface of the cytoplast are thick and continuous (arrows), as though the process initiated in the shorter exposure (Fig. 6) had continued. This leads eventually to a total deformation of the finely divided microtrabeculae. Total tilt 15°. Bar, 0.2 μm. × 48,000.


8
8
9
9

freeze-substitution (20, 40). In these procedures, cells are rapidly frozen from the living state and subsequently, while still locked in ice, fixed with OsO_4 as the ice dissolves in cold acetone. For small samples, such as thin whole cells in culture, the substitution process is rapid, though with blocks of tissue the substitution requires considerably longer.

PROCEDURES: Cells on grids were frozen as described immediately above, then transferred to solvent-resistant vials containing 10% osmium tetroxide in absolute acetone maintained at liquid nitrogen temperatures. The 10% osmium solution (wt/vol) was prepared by adding crystalline osmium to 100% reagent grade acetone that was almost completely frozen by liquid nitrogen. This mixture was allowed to freeze, and liquid nitrogen was added to the top of the frozen acetone-osmium mixture. The grids were transferred to this mixture, then to a −70°C freezer for 8–10 h, then to a −25°C freezer for 8 h, then to 0°–4°C for 4 h, and lastly allowed to come to room temperature. At that point, the grids were placed in a multiple grid holder (while submerged in 100% acetone) and dried by the critical-point method. The grids were then carbon-coated and stored in a desiccator.

OBSERVATIONS: The microtrabeculae are preserved (Fig. 10) in dimensions comparable to those in glutaraldehyde-fixed cells. Damage from ice crystals is frequent in the thicker regions of the cells in these preparations. Nonetheless, it is significant that the typical 3- to 6-nm trabeculae of the lattice are present.

III. Model Systems Tested

Model systems have been used to test the tendency of this or that procedure to produce artifacts (37). We chose two distinctly different models in which to search for evidence of trabeculae-like structures after fixation.

A. Whole Erythrocytes

PROCEDURES: ½ ml of circulating human blood was suspended in 10 ml of isotonic saline, centrifuged gently, and washed in two additional aliquots of saline. Drops of resuspended erythrocytes were placed on Formvar-coated grids to which polylysine had been added (27) and allowed to settle for 5 min in a warm (37°C), moist chamber. The erythrocytes were subsequently fixed and processed as described under Section I A for cultured cells.

OBSERVATIONS: The biconcave erythrocyte is too thick and dense for even high energy electrons to penetrate except at its center. Micrographs of this region show the structure to be homogeneous and without evidence of any MTL. A few leukocytes were located on these grids, and, as with all nucleated cells examined thus far, they exhibited in their cytoplasms a typical trabecular structure.

B. Bovine Serum Albumin

PROCEDURES: Solutions of crystalline bovine serum albumin (20% wt/vol in H_2O) were mixed with 2% glutaraldehyde at ratios of 1:1, 2:1, 3:1, 4:1, 5:1, and applied immediately to Formvar-coated grids which were then placed in a moist chamber at 37°C for 30 min. The grids were subsequently processed as described in Section I A, including acetone dehydration and critical-point drying.

OBSERVATIONS: Although a single protein such as bovine serum albumin admittedly does not

FIGURE 8 Stereo image of a portion of the cytoplasm of a frozen-dried WI-38 cell. Of particular interest there is that the microtrabeculae in these preparations range from 9 to 30 nm, which is thicker than those observed in cells fixed with glutaraldehyde/osmium. Contours of the microtrabeculae are smoother, and the spaces outlined by the trabeculae are more uniform than in chemically fixed and acetone-dehydrated specimens. Some membranes are visible, probably vesicles of the ER. As in other preparations the microtrabeculae show continuities with the surfaces of inclusions (probably secondary lysosomes), the ER, and the other microtrabeculae. The mass of the cell's components, the salts from the tissue culture medium trapped in the trabeculae, and a small (20 μm) aperture all contribute to the contrast in this image of a frozen-dried specimen. 7° total tilt. Bar, 0.2 μm. × 50,000.

FIGURE 9 Stereo image of a portion of a WI-38 cell frozen-dried after glutaraldehyde/osmium fixation. The morphology of the microtrabecular lattice in this specimen resembles more closely that of glutaraldehyde/osmium-fixed, solvent-dehydrated cells than that in Fig. 8. It retains, however, some of the attributes of the structure in Fig. 8 and substantiates at the very least that the microtrabecular lattice is not the product of ethanol-acetone dehydration and critical-point drying. 9° total tilt. Bar, 0.2 μm. × 51,000.

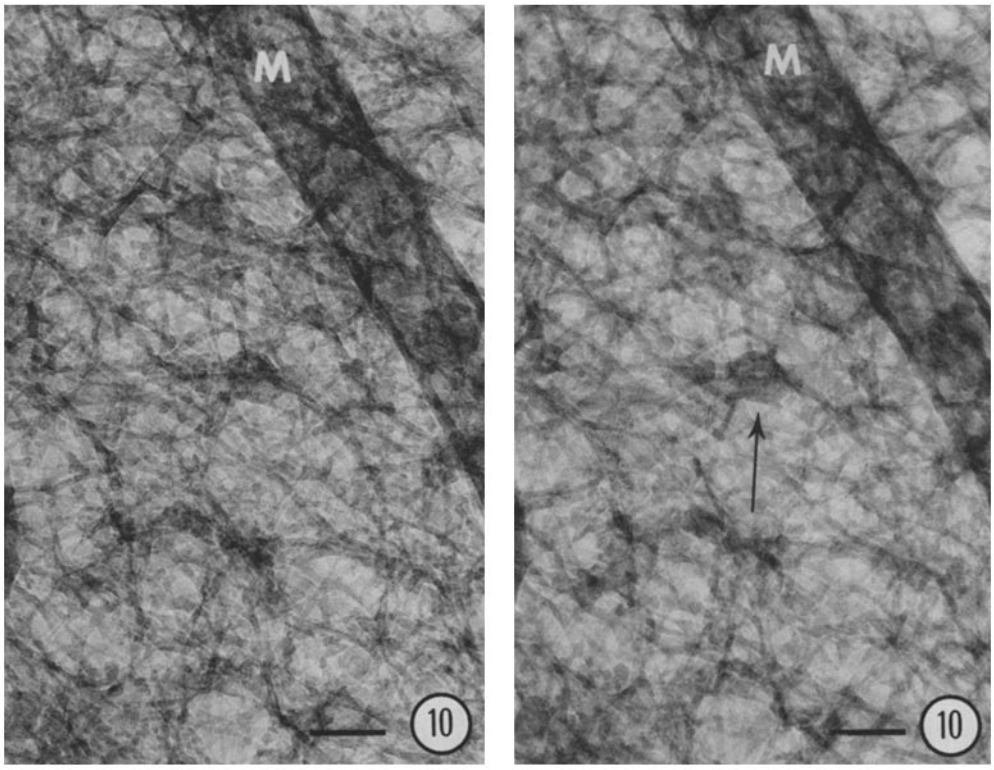


FIGURE 10 Stereo image of a WI-38 cell prepared by the freeze-substitution-OsO_4 method. Differences in the fine structure are evident compared with the frozen-dried cells. The microtrabeculae are more uniform in size than in frozen-dried and glutaraldehyde/osmium-fixed cells. Most microtrabeculae here are 4–6 nm thick though a few are <4 nm. Filaments thicker than 7 nm are abundant. Interestingly, the dimensions of many of the intertrabecular spaces are similar to those seen in cells prepared by other techniques. It is most significant that the trabeculae are preserved in their three-dimensional lattice configuration. A portion of a mitochondrion (*M*) and a small vesicle (arrow) are shown in this image. 5° total tilt. Bar, 0.1 μm. × 100,000.

duplicate the cytoplasmic ground substance, it has been used repeatedly to investigate the coagulating effects, if any, of chemical fixatives (37). In this latest trial, it became fixed in the sense of becoming gelatinous, but it did not show in its structure, even at × 20,000, any evidence of a microtrabecular lattice.

IV. Images of Sections of Cultured Cells

All of the observations reported up to this point were made on cells prepared without any embedding matrix such as Epon-Araldite. In such cells, the spaces normally occupied by such matrices are, in the vacuum of the microscope, also in vacuum. Accordingly, differences would be expected between micrographs of whole, unembedded cells and sections of similar cells. One would expect, nevertheless, that the structures in one should be identifiable in the other, and we have sought to make appropriate comparisons with this in mind. We have accordingly made stereo pairs of thin and thick sections, at both low and high voltages. Additionally, and especially to monitor the action of liquid CO_2, we have examined cells that were dried by the critical-point method and subsequently embedded and sectioned. The purpose was to detect differences, if any, between the appearances of cells that had and had not been exposed to critical-point drying.

A. Thin Sections of Conventional Preparations

PROCEDURES: Cells for conventional transmission electron microscopy were plated onto heat-sterilized,

carbon-coated glass slides and maintained in culture as previously described (56). When ready for fixation, the cells on the slides were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate at 37°C for 15 min. Fixed cells still on the slides were rinsed in 0.2 M sodium cacodylate, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2–7.4) for 10–15 min, washed briefly in distilled water (1–2 min.), stained en bloc in 0.5% uranyl acetate in s-collidine for 1 h, dehydrated in increasing concentrations of reagent grade acetone, infiltrated for 1 h in Epon-Araldite, and finally embedded in this resin. Embedding was accomplished by inverting the glass slide (cells down) onto another slide coated with a fluorocarbon (Spraymate, Minnesota Mining and Manufacturing Co. St. Paul, Minn.). This sandwiched preparation was then hardened at 60°C for 48 h, after which the slides were separated with a razor blade, leaving the embedded cells in a thin layer of resin. Individual cells or groups of cells were selected, cut out of the waferlike embedment with a razor blade, and mounted on Epon blanks for sectioning. Sections were spread on Formvar-coated grids, stained again with aqueous uranyl acetate and lead citrate, and viewed in a Philips 300 electron microscope operated at 80 kV.

OBSERVATIONS: Examination at suitably high magnifications of thin sections of conventionally prepared, plastic-embedded material reveals, as it has for other investigators (6, 8, 9, 15, 21, 26, 39, 44, 45, 51, 52, 59), a considerable amount of "flocculent" material associated with microtubules, microfilaments, free ribosomes, membranes of the endoplasmic reticulum (ER), etc. (Figs. 11 and 12). None of these structures has "clean," or smooth surfaces: in some places, the flocculent material forms strands which extend from one to another of the better defined structures (see arrowheads in circular inserts); in other places, there are small patches of what can be called a lattice. The dimensions of the strands vary greatly (e.g., 2.5–6.5 nm diameter), as do those of the microtrabeculae in stereo images of whole cells, but their appearance here is very different in that they are feathery and without distinct margins. If we assume that the two are equivalent, then we have to account for the discrepancy in their appearance.

At least two explanations come to mind. The most obvious is that any one thin section, ~60 nm thick, includes only fragments of the lattice microtrabeculae and these will therefore appear mostly as bits and pieces in the section. Where a microtubule is present in the section over some part of its length, more fragments of the lattice attached to its surface will also appear in the section. The same is evident, though less strikingly so, with the 6–7 nm and 9- to 10-nm microfilaments (14, 15, 22). Where the 6- to 7-nm actin-containing filaments are bundled together to form a stress fiber, the "flocculent matrix material" fills the spaces between the filaments (Fig. 12).

Another explanation we find reasonable is that, in sections, we are looking at a uranyl-stained component of the trabeculae that scatters electrons more than the bulk of the materials making up the lattice. These latter, having, we assume, the same scattering properties as the Epon-Araldite, fail to be visualized at all. The space occupied by Epon-Araldite in sections is, in whole cells, continuous with the vacuum of the microscope and in failing to scatter electrons enhances the contrast of the trabeculae, which do.

B. Thin Sections of Cells First Dried by Critical-Point Method

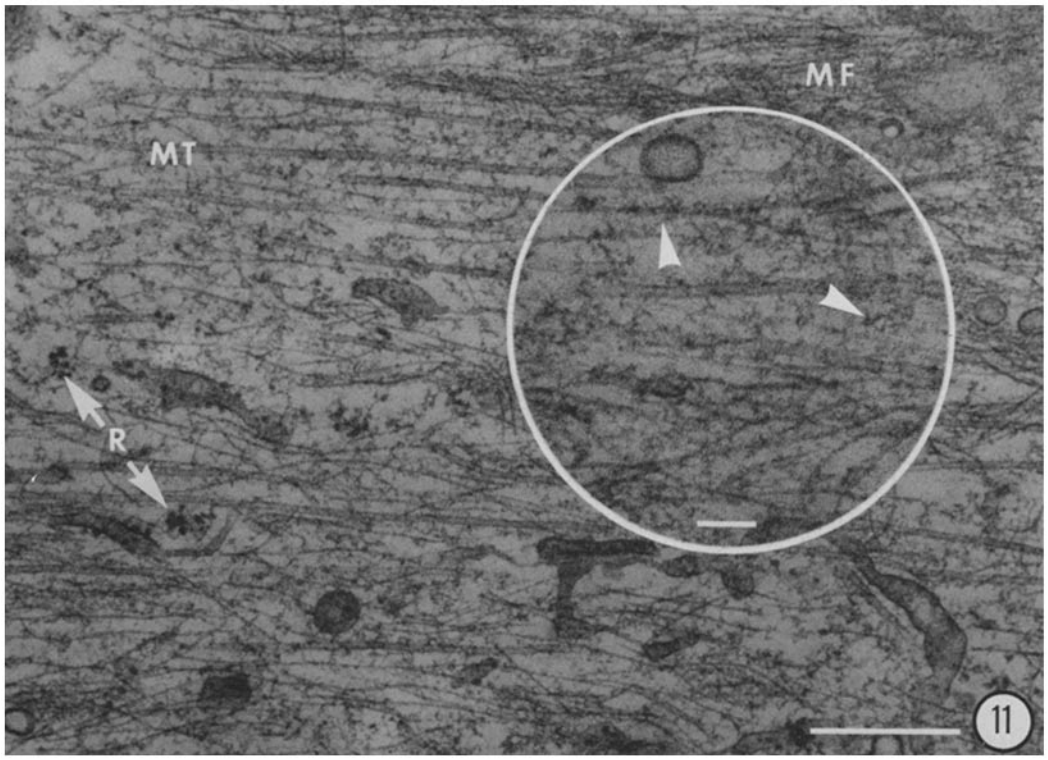
PROCEDURES: WI-38 cells were fixed and dried by the critical-point method as though prepared for whole-cell microscopy (Section I A). They were then infiltrated for 1 h in Epon-Araldite and finally embedded in this mixture. Subsequent handling repeated exactly the procedures followed (Section IV A) for preparing cultured cells for thin sectioning.

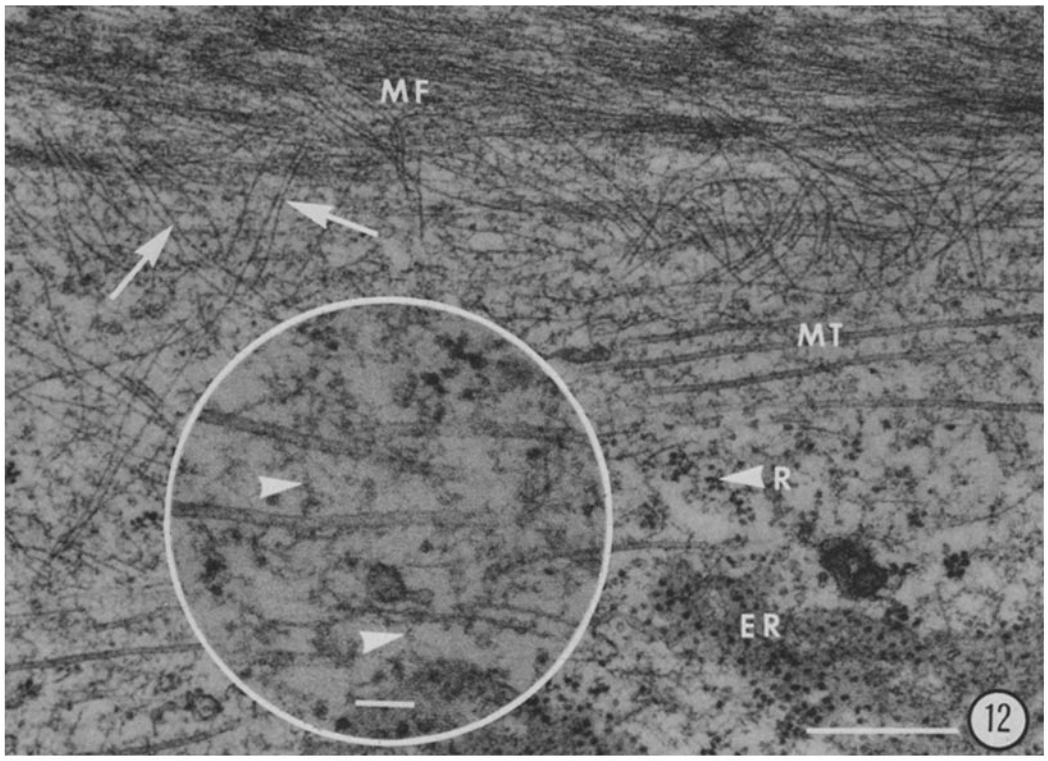
OBSERVATIONS: Comparisons of the images shown in Figs. 11 and 12 reveal no significant differences: the flocculent material equated with the trabecular lattice is distributed in exactly the same manner in both. Association of the material with ribosomes, filaments, and microtubules appears the same in each of the images (see arrows in enlarged areas). It has to be concluded, therefore, that the act of drying the cell through liquid CO_2 (Fig. 12) has no major effect on the fine structure.

C. Thick Sections

PROCEDURES: Epon-Araldite-embedded cultured cells were sectioned on glass knives at various thicknesses (0.25–0.75 μm) and mounted on Formvar-coated copper grids. Before embedding, they were stained for 18 h in 0.5% uranyl acetate. After sectioning, the preparations were stained for 3 h with 4% aqueous uranyl acetate and 15 min in lead citrate at 60°C in a moist chamber. After staining, the grids were stabilized by evaporating a layer of carbon on each side and stored in a desiccator.

OBSERVATIONS: It was assumed in this experiment that stereo viewing of a thicker section (Fig. 13) should demonstrate a greater level of continuity in the flocculent material than is evident in thin sections. In several places, and especially between polysomes or between closely as-


MF
MT
R
11


MF
MT
R
ER
12

sociated microtubules, there is reasonable evidence of a fine lattice which is interpreted as the stainable portions of the lattice evident in the whole-cell equivalent. In other places, however, there are large voids (asterisk) which suggest that somewhere (either in the en bloc staining for 18 h in uranyl acetate, or in the process of embedding) portions of the ground substance were extracted from the cell.

To provide an explanation for this latter point, we have in Fig. 14 represented in stereo images (at the same magnification as in Fig. 13) a portion of a whole cell also stained for 18 h in uranyl acetate and dried by the critical-point method. Clearly, it is not changed appreciably from the structure observed in other stereo images of whole cells (e.g., Fig. 3). A careful comparison of Figs. 13 and 14 reminds us that in whole cell image there are large intertrabecular spaces which match in size the voids seen in the sectioned image. This leaves the flocculent or feathery appearance of the lattice "trabeculae" in the sectioned image to be explained, and for this we have already suggested that what one sees in the sectioned image is only the stainable portion of the trabeculae; the rest, while still there, is lost to view because there is insufficient differential in scattering properties between it and the matrix.

V. Test for the Integrity of the Cell Surface

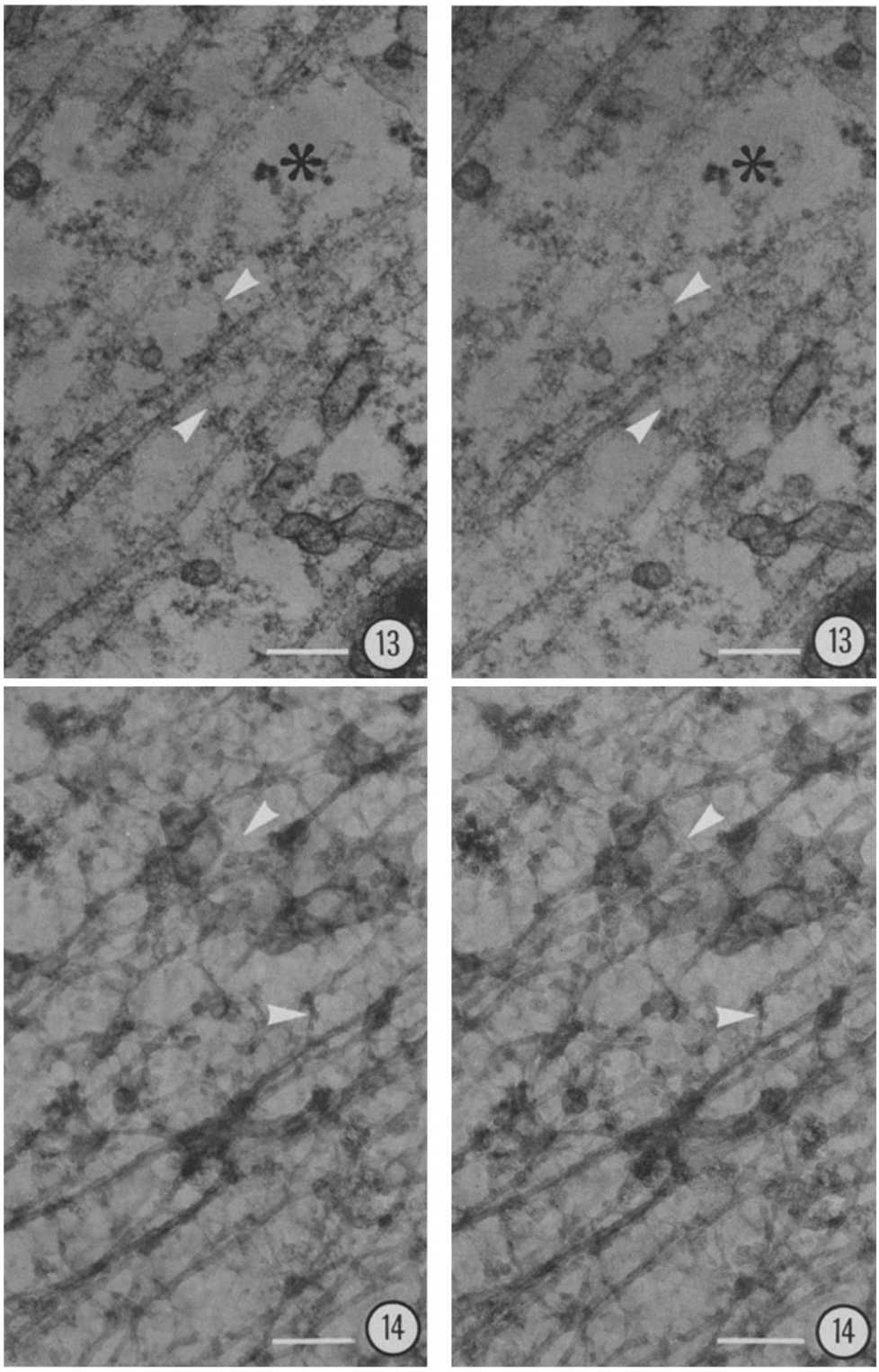
It is difficult in stereo views of whole cells to identify the upper surface of the cell. This difficulty is less significant if a marker is included in or on the membrane. That the surface is in fact present and intact is indicated by images of vertical sections through cultured cells as well as by images of cells similarly treated and examined by scanning microscopy. Nonetheless, in the unlikely possibility that the high voltage beam might have damaged or destroyed the integrity of the upper free surfaces of these cells, it was decided to identify the surface with a marker and to prepare images for stereo viewing. To demonstrate that this thin surface layer of the cell has withstood the rigors of fixation, dehydration, and critical-point drying is also to make more believable the preservation of the underlying morphology of the cytoplasmic ground substance after the same treatment.

PROCEDURES: Cells were fixed with glutaraldehyde plus osmium tetroxide as previously described (Section I A). After dehydration and critical-point drying, the preparations were placed in a metal evaporator and coated with 20–30 Å of platinum-palladium.

OBSERVATIONS: It is evident that the evaporated platinum-palladium renders the cell surface visible by giving it a finely granular appearance (Fig. 15). One has the impression of looking through a partially opaque (frosted) glass at the subjacent morphology of the cytoplasmic ground substance. It is thus possible to conclude that the upper free surface is intact and smooth and not a part of the lattice structure clearly evident beneath.

FIGURE 11 Thin section of a cultured WI-38 cell fixed and processed for electron microscopy by conventional methods. Microtubules (*MT*), microfilaments (*MF*), and ribosomes (*R*) appear in the expected forms and dimensions. This image and the inset at high magnification are included to illustrate the appearance of what has to be interpreted as the microtrabeculae in thin sections. The microtrabeculae have dimensions similar to those in the whole-cell preparations, but they are far less distinct and look as one might expect remnants or fragments of the trabeculae to look. They are obviously attached to the surfaces of the microtubules and ER vesicles and are confluent with filaments in the stress fibers. Bar, 0.5 μm. × 40,000. *Inset:* Bar, 0.1 μm. × 80,000.

FIGURE 12 Thin section of a WI-38 cell that was fixed and processed as a whole cell (including critical-point drying) before being embedded in Epon-Araldite and sectioned. The section, which includes microfilaments (*MF*) just under the cell surface, has the same morphology as that of the cell in Fig. 11 which was not subjected to critical-point drying. The preservation of the microtrabeculae may be better than in Fig. 11. Again the microtrabeculae are identified as strands having the size and associations, but lacking the crispness of those in the whole-cell images. The microtrabeculae seem to fill the space between the 6-nm actin filaments of the stress fibers (*MF*). (See text for further interpretation of images.) Microtubules (*MT*) and more randomly oriented 10-nm filaments (arrows), as well as the cisternae of the endoplasmic reticulum (*ER*) and ribosomes (*R*), are included in the micrograph. Bar, 0.5 μm. × 40,000. *Inset:* Bar, 0.1 μm. × 80,000.


13
13
14
14

DISCUSSION

The Artifact Controversy

The question of what is artifact and what is not is a persistent one in electron microscopy, especially where micrographs depict what are essentially new or unexplored structures. In the strictest sense, of course, the content of the images is all artifact where the usual procedures are employed.

The question is one of equivalence. To what extent do the images represent what was in the ground substance when the fixative was applied, and to what extent may these images be used to investigate the form and function of this part of the cell? Obviously, after examining the results of these variations in preparation, we conclude that what is seen consistently and reproducibly in our whole cell images is useful for studies of the nonrandom organization and nonhomogeneous nature of the cytoplasmic ground substance and the phenomena in which the ground substance is involved. Since there are few alternatives to electron microscopy for examining structures smaller than 1,000 Å, we have little choice but to make the most of this one.

It would be generally conceded that most of the cell structures revealed by electron microscopy and frequently questioned have been demonstrated to exist before fixation. This has been done by light optical methods on living materials, by cytochemical procedures, and by freeze-fracture and replication studies. Thus, the structure of the plasma membrane is no longer questioned. Although fragile and thin in dimension, it appears to retain its integrity and to withstand the rigors of chemical fixation and dehydration surprisingly well. The same may be said for the endoplasmic reticulum, as evidenced by the form of the derived microsomes (32).

Ribosomes have without doubt the morphology depicted in thin sections and substantiated by negative staining. And microtubules, especially in ordered arrangements (as in axonemes), are depicted as joined by slender bridges or links (48). In cilia, the ordered disposition of dynein arms and the slender spokes as seen in sections has been verified by negative staining of tubules freshly isolated (50) and in critical-point-dried cilia (42). Similar arms and bridges observed in micrographs of microvilli and axostyles, have been recognized in replicas of freeze-etch preparations of these same structures (4, 28, 29). Microfilaments in bundles (stress fibers), even though individually only 6–7 nm in diameter, display form birefringence and have been identified as to location, form, and composition by immunochemical procedures. These several examples of fine details of morphology are readily accepted. Thus, it is reasonable to believe that structures of similar dimensions, for several reasons not previously observed, are also faithfully preserved by glutaraldehyde-osmium tetroxide fixation. Yet, there could be exceptions, especially where the relative disorder of the observed structural elements increases the appearance of artifact.

So, we have attempted in this study to explore again in the preservation of cultured cells a possible role of fixation and dehydration in the generation of artifacts. More specifically we are asking whether these preparation procedures produce the three-dimensional lattice or meshwork of slender strands (microtrabeculae) that is being depicted in

FIGURE 13 Stereo image of a thick section (0.25 μm) of a WI-38 cell stained en bloc in uranyl acetate for 18 h. The "fuzzy" material associated with the microtubules and especially evident in this micrograph is taken to represent the sectioned equivalent of the microtrabeculae of the whole-cell image. The same material (arrowheads) is observed in association with polysomes, microfilaments, and surfaces of ER cisternae. When observed stereoscopically it forms an incomplete microtrabecular network similar, but not identical, to that present in the whole-cell images. Large voids (asterisk) suggest that portions of the ground substance were extracted from the cell. Tilt ±4°. Bar, 0.2 μm. × 60,000.

FIGURE 14 Stereo image of a portion of the cytoplasm of a whole, critical-point-dried cell (WI-38) stained en bloc for 18 h in uranyl acetate. This experiment tests the assumption that uranyl acetate (pH 4.5) extracts material from the slender microtrabeculae and provides images for comparison with those derived from thin sections. The variations in the size of the slender microtrabeculae (arrowheads) is evident as in other whole-cell preparations. It appears, subjectively, that the microtrabeculae have lost density compared with cells exposed to uranyl acetate for short periods. Tilt 5°. Bar, 0.2 μm. × 60,000.

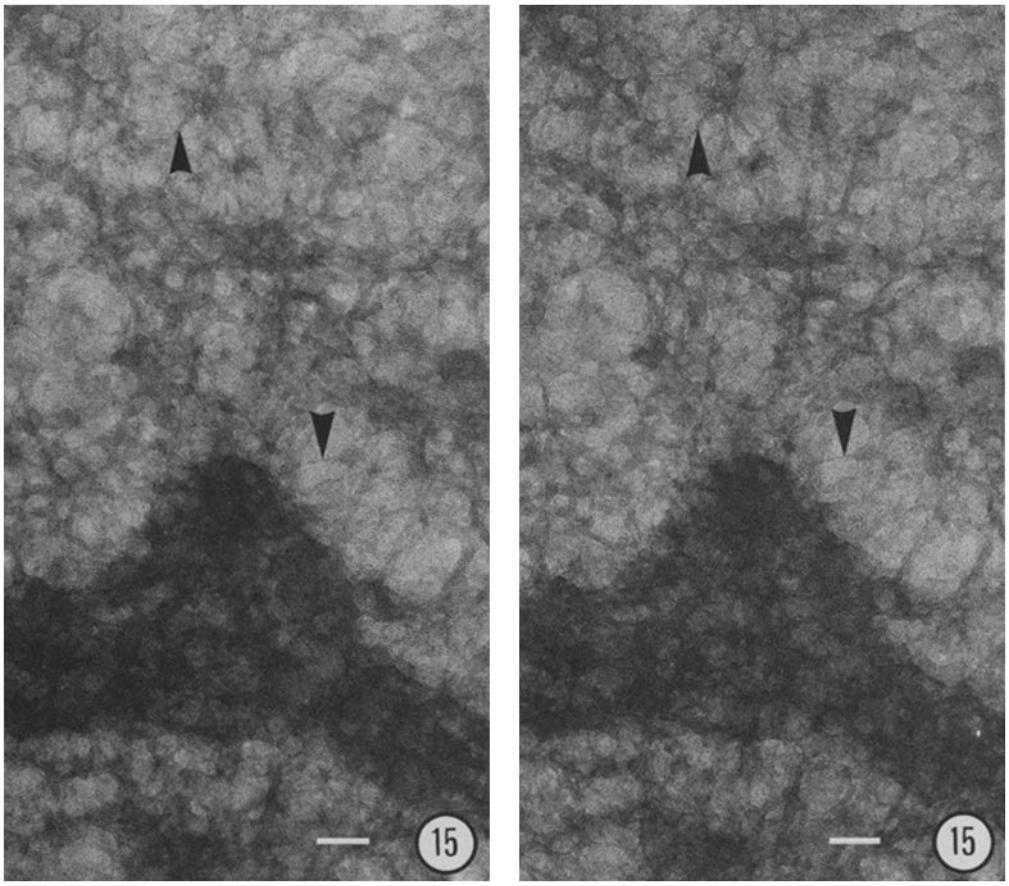


FIGURE 15 Stereo micrograph of a whole, critical-point-dried cell (WI-38) shadowed lightly with platinum-palladium to render visible the upper surface of the cell. This procedure was performed to establish the integrity of the plasma membrane after exposure to fixation, staining, dehydration, and drying by the critical-point method. Viewed in stereo, the image includes a uniform layer of granular material on the surface, a layer through which one looks to see the lattice beneath. There are no apparent interruptions in the continuity of the surface. The granularity is due to the action of the beam on the thin (2- to 3-nm) layer of metal deposited during shadowing. Even though the trabeculae are not so clearly imaged as in the unshadowed preparations, they are there and appear as if viewed through frosted glass (black arrowheads). Tilt, 9°. Bar, 0.1 μm. × 70,000.

all of the cells in this study (5, 7, 10, 55, 56) with the exception of erythrocytes. In cultured cells fixed with glutaraldehyde and dried by the critical-point method, the lattice comprises microtrabeculae that are usually 3–6 nm in "diameter" and 100 or more nm in length. These outline intertrabecular spaces that vary greatly in size but are in the range between 50 and 150 nm across. It is apparent in the three-dimensional images provided by stereo microscopy that the microtrabeculae are continuous with the inner surfaces of the plasma membrane (cytoplasmic cortex), the microtubules and microfilaments, and the cytoplasmic surfaces of the ER. All of these latter surfaces as well as the ribosomes are coated with thin layers of a material that is continuous with that of the trabeculae. Other details of the structure of the cytoplasmic ground substance or matrix are discussed below and can be observed as well in publications of Buckley (5), Buckley and Porter (7), and Wolosewick and Porter (55).

Chemical Fixation

Although many attempts have been made since 1963 (when glutaraldehyde was introduced) to "improve" fixation methods, nothing very signifi-

cant has been reported as a replacement for the now-standard glutaraldehyde-OsO_4 formula. Buffers, pH optima, tonicity levels, and times of exposure have been varied for different tissues, yet the fine structure that has emerged has remained very much the same. Additionally, glutaraldehyde has been used simultaneously or sequentially with OsO_4 without significant variations in the results (11). Since the product of these procedures is widely accepted as satisfactory by most practitioners, it has seemed appropriate to accept it here as the standard against which the product of other procedures may be evaluated.

With OsO_4

Observations growing out of the fixation with OsO_4 are of interest in this context. Where cells were exposed to solutions of osmium tetroxide for a period of only 15 min, the lattice has most of the features shown after fixation with glutaraldehyde and osmium. This represents the initial response, a cessation of all motion in the cells. Over the longer period of exposure to OsO_4 the lattice structure undergoes a progressive decay; the microtrabeculae, which normally traverse the space between the upper and lower surfaces of the cell, disappear and the image includes mostly dense gobbets of material which are interpreted as the remnants of lattice material. At the same time, the upper and lower cortices of the cytoplast increase in density and probably in thickness. From these observations we conclude that OsO_4 fixation of the lattice is a transient phenomenon resulting in a progressive decay of the preserved form and concomitant solubilization of the polymer.

Why, when allowed continued contact with the cell, OsO_4 acts to destroy the initial fixation is not known. It is, however, an effect that is not surprising, for as early as 1948 it was recognized that extended exposure to this reagent increases the visibility of membrane-limited structures, apparently by solubilizing and eluting the surrounding cytoplasmic ground substance, while stabilizing the membranes (38). Apparently, in the studies reported here, we are observing in detail the early stages in this dissolution of the cytoplasmic ground substance. The phenomenon parallels the failure of OsO_4 to fix the so-called intercellular cement with its high content of mucopolysaccharides (49). This becomes especially interesting in the light of reports that tests with ruthenium red and lanthanum hydroxide indicate the presence of acid mucopolysaccharides in the trabeculae of the lattice (9, 47).

It is evident as demonstrated some time ago (31) that OsO_4, even where appropriately buffered, is not a reliable fixative for anything except membranes. More recently, Pollard et al. (33) have reported the instability of actin filaments in model (in vitro) preparations exposed to OsO_4.

We have not noticed such deleterious effects of osmium tetroxide on cells fixed initially with glutaraldehyde. Within the time periods used, in any case, there were none of the changes observed with OsO_4 alone as the fixative. The striking fact that polymers such as cytoplasmic microtubules disappear completely in some instances after only a few minutes exposure to OsO_4 should alert one to the extraordinary differences in the action of these two fixatives.

That the lattice appears after paraformaldehyde fixation in a variety of cell types[1] and in a form identical to that after glutaraldehyde, should not be overlooked as evidence that the system occurs naturally.

Fixation by Freezing

That the trabeculae which make up the MTL are preserved by all of the freezing techniques employed is also meaningful, although in each instance their appearance is slightly different from that following glutaraldehyde. The variation is, we believe, related in some measure to the form that ice assumes during the freezing process or the degree to which freezing extracts water from the trabeculae. That is, whether amorphous or crystalline, the ice can determine the degree of damage caused in these preparations, especially in the size of intertrabecular spaces. Where the freezing rates are very fast, as in the case of liquid nitrogen or Freon-12 applied to the thin margins of cultured cells, the aqueous environment could be transformed into a solid vitreous state.

However, for thick samples this probably is not the case. Furthermore, the transition from amorphous to crystalline ice can occur between −120°

[1] In the time that has elapsed since this paper was initially submitted for publication, several other cell types (platelets, myoblasts, thyroid epithelial cells, neutrophiles, and neuroblastoma cells) have been observed. In each instance the MTL is present but with variations in form with cell type that are striking (K. R. Porter and K. Anderson, unpublished observations).

and −80°C during lyophilization, depending on the concentration of solutes (3). The higher the concentration of solute, the higher the transition point, and consequently, the lower the chance for damage by ice crystals. In our freezing procedures, the thicker parts of the cells probably were not frozen in the amorphous condition. This is not unexpected since cryo-protectants, which raise the concentration of solutes, were not used.

These considerations make it seem highly probable that the images we have collected of cells frozen quickly and thereafter dried while still frozen reflect what was present at the time of freezing (fixation) and not merely damage by ice crystals. Thus it is significant that the stereo micrographs of the cytoplasmic ground substance of frozen cells show a three-dimensional lattice similar to glutaraldehyde-fixed cells, dehydrated by alcohol or acetone, and dried by the critical-point method. It is true that the trabeculae of the frozen-dried cell are thicker on the whole than those in the standard, and we assume that the finer form of the latter is a product of dehydration in alcohol and acetone.

As illustrated in Fig. 9, the same morphology emerges from stereo images of cells frozen and dried after fixation as from those unfixed. This obviously supports the view that chemical fixation is not generating the observed structure, and that in avoiding the use of dehydrating agents and critical-point drying, we have not altered the standard image appreciably. Trabeculae in preparations treated in this way resemble more closely in their dimensions those of frozen-dried cells than those of cells exposed to alternative procedures of drying. This suggests that the usual procedures do indeed have a shrinking effect on the dimensions of the trabeculae and may distort somewhat the form of the lattice.

Other experiments done to test the benefits of holding in place, by freezing, the substructures of the ground substance while they are fixed with OsO_4 in acetone (known as freeze-substitution) likewise yielded images similar to those of the standard. The microtrabeculae appear more slender and uniform along their length, which may reflect the postfixation effects of dehydration in acetone mentioned above.

There are observations from freeze-fracture studies of cells that are pertinent in the present context. Ordinarily, the fracture plane follows membranes and the replicas reflect the particulate or molecular components of the lipid bilayer. In other places, however, the fracture moves through the cytoplasmic ground substance or some extension of it (microvilli) and should therefore expose the MTL to view. We have examined published micrographs (12) of freeze-fracture replicas and have found in most instances some evidence of irregularities in the surface which in their size and disposition could easily represent replicas of trabeculae. That structures in the same size range and of a similar nature can be seen in fracture replicas, was intentionally demonstrated by Bloodgood and Miller (4) in fractures taken through the axostyles of *Saccinobaculus*. Likewise, in replicas of fracture planes through microvilli, Mooseker and Tilney (29) and McNutt (28) have identified bridges, which we equate with microtrabeculae, between the central bundle of actin filaments and the under-surface of the plasmalemma. This miscellany of observations can be taken to indicate that both in specimens frozen from the living state, and after fixation with glutaraldehyde, it is possible to identify units having many of the features of trabeculae observed in the standard image. Most impressive in this regard, however, are the replicas of deeply-etched fracture surfaces from cells frozen at liquid helium temperatures. Here without doubt the MTL is evident (19).

Other Considerations

MODELS: ERYTHROCYTES AND BOVINE SERUM ALBUMIN: As noted above (III A and B), aldehyde fixation of mature circulating erythrocytes reveals little evidence of a trabecular structure.[2] If the lattice were a precipitation artifact, protein-rich erythrocytes (or more so, platelets) should show a meshlike appearance similar to other whole cells. This reasoning is applicable as well to intracellular organelles as first noted by Wessells et al. (52), Buckley (5), Wolosewick and Porter (55). That is, cisternae of the ER, Golgi, and mitochondria probably contain concentrations of proteins or macromolecules as high or higher than those in the cytoplasm. Yet, examination of whole cells and sections generally fail to show structured lattices within these organelles and systems.

Additionally, in these studies, we have tested various chemical fixatives on thin films of serum

[2] Some platelets are also devoid of the lattice or any equivalent in their thinner margins (R. Steele, personal communication).

albumin as have others for the same purpose. In no instance have we found glutaraldehyde to produce in these films anything like the trabecular structure of the cytoplasmic ground substance.

The Lattice in Sections

There is clearly a problem in relating the whole-cell image of the trabecular lattice to the thin-section image available from these studies as well as from the literature. Part of this problem arises from the fact that the thin section includes only fragments of the trabeculae; that is easily understood. It is less easy to account for the differences between the feathery character of the material attached to the larger components of the cytoplasm and the thicker trabeculae bearing the same relationship to these components. We have suggested that the epoxy resin that surrounds every component in the sections has electron-scattering properties not dissimilar from those of the lattice elements. As one consequence of this, the true margins of the trabeculae are not apparent. But this explanation does not tell us what it is that we are seeing as feathery material in the thin section, unless it represents the residue seen after staining with uranyl acetate. We sought to test this idea by exposing whole cells to uranyl acetate for extended periods before dehydration and critical-point drying. The results suggest that, rather than adding more contrast to the lattice, prolonged exposure to uranyl acetate (and at low pH) can actually extract some of the lattice components.

This is a problem that needs the investigations we are currently conducting. It should be noted finally that the appearance of the trabeculae in sections is essentially the same in all cases except where ruthenium red and lanthanum hydroxide, compounds which bind to acid mucopolysaccharides, have been used to stain the microtubule-associated material. In such preparations the "fuzzy" or "feathery" material is much more substantial in its dimensions, and also more dense (9, 21, 47).

Summary and Conclusions

Throughout the course of this discussion we have concentrated on the actual or artifactual nature of the lattice, i.e., its presence, and hence its expression, as a structural entity in the living cell. From the various experiments described above, we conclude that the microtrabecular lattice must exist in a form not too dissimilar from that depicted in the stereo micrographs. The general reliability of glutaraldehyde in preserving other features of fine structure, identical in their dimensions to microtrabeculae, supports this conclusion. We have in mind, for example, the so-called spokes (5-nm radial links) within the cilium (50) which are distributed in a perfect repeating pattern and could scarcely represent an artifact. Recall also the regular arrangement of bridges depicted in micrographs of microvilli pretreated with 15 mM $MgCl_2$ (29) before fixation. These bridges have precisely the form and appearance of microtrabeculae observed in other parts of the cell in more random arrangements.

Perhaps most compelling in convincing us that the system under observation exists in the living cell is a study on its structural changes during cell function. The rapid transitions displayed by the lattice during the aggregation and dispersion of pigment in chromatophores are dramatic and cannot be interpreted as a product of fixation without an extraordinary strain on the imagination (10). The same must be said for the observed changes which take place in response to low temperatures. Although not so sensitive to chilling as the contained microtubules, the MTL deforms as though certain bonds within its structure are weakened while others are retained. This restructing of the lattice when normal temperatures are restored is both prompt and dramatic (57).

The nonrandom distribution of the microtrabeculae relative to other structures and surfaces of the cytoplasm makes these cytoskeletal elements integral components of an organized cytoplast; they evidently divide the cytoplasm into two phases, one water-rich and the other protein-rich.

The elements of the lattice are not free-flowing in the living cell. If they were, we suggest that would mean transitions between a structured gel state and a homogeneous sol—transitions we have not observed. Alterations in structure occurring either naturally (e.g., ruffling lamellipodia, pigment translocation) or experimentally induced by low temperature, should be regarded as the result of a graded deformation or reformation of a viscoelastic polymerized unit. In other words, there is probably a shift or continual flux between a random and nonrandom state without the loss of trabecular structure.

Experiments in progress with colchicine, low temperature, and cytochalasins further strengthen the case for the existence of the MTL by inducing local and unique alterations in the structure of the

lattice. In other words, all these agents impart randomness to the lattice, but probably in different ways.

Finally, if the evidence just discussed were weaker, one could fall back on the thought that the methods we use are the most reliable means available for visualizing cell fine structure and we therefore find it excusable to make the most of them. Again, we repeat, we are dealing with a question of equivalence—to what degree is the structure observed equivalent to the native, unfixed structure of the ground substance? It will doubtless require further study and experimentation to answer this question to the satisfaction of all.

We would like to thank Mr. George Wray of the High Voltage Electron Microscope installation in Boulder, Colo. for maintaining the 1 MeV electron microscope in excellent condition throughout the course of this study. The superb technical assistance of Ms. Judy Fleming and Ms. Joyce Albersheim are gratefully acknowledged.

This study was supported in part by National Institutes of Health (NIH) grants RR-00592 and AG-00310 to K. R. Porter and NIH postdoctoral fellowship award 1F32-GM-00138 to J. J. Wolosewick.

Received for publication 28 April 1978, and in revised form 20 February 1979.

REFERENCES

1. AARONSON, S. A., and C. WEAVER. 1971. Characterization of murine sarcoma virus (KIRSTEN) transformation of mouse and human cells. *J. Gen. Virol.* **13**:245–252.
2. ANDERSON, T. 1951. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans. N. Y. Acad. Sci.* Ser. II. **13**:130–134.
3. BLACKMAN, M., and N. D. LISGARTEN. 1958. Electron diffraction investigations into the cubic and other structural forms of ice. *Adv. Phys.* **7**:189–198.
4. BLOODGOOD, R. A., and K. R. MILLER. 1974. Freeze-fracture of microtubules in motile axostyles. *J. Cell Biol.* **62**:660–671.
5. BUCKLEY, I. K. 1975. Three-dimensional fine structure of cultured cells: Possible implications for subcellular motility. *Tissue Cell.* **7**:51–72.
6. BUCKLEY, I. K., and K. R. PORTER. 1967. Cytoplasmic fibrils in living cultured cells. A light and electron microscopic study. *Protoplasma (Berl.).* **64**:340–380.
7. BUCKLEY, I. K., and K. R. PORTER. 1975. Electron microscopy of critical point dried cultured cells. *J. Microsc.* **104**:107–120.
8. BUNGE, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56**:713–735.
9. BURTON, P. R., and H. L. FERNANDEZ. 1973. Delineation by lanthanum staining of filamentous elements with the surfaces of axonal microtubules. *J. Cell Sci.* **12**:567–583.
10. BYERS, H. R., and K. R. PORTER. 1977. Transformations in the structure of the cytoplasmic ground substance in erythrophores during pigment aggregation and dispersion. I. A study using whole-cell preparations in stereo high voltage electron microscopy. *J. Cell Biol.* **75**:541–558.
11. CHANG, J. H. T. 1972. Fixation and embedment, *in situ*, of tissue culture cells for electron microscopy. *Tissue Cell.* **4**:561–574.
12. ELLISMAN, M. H., J. E. RASH, L. A. STAEHELIN, and K. R. PORTER. 1976. Studies of excitable membranes. II. A comparison of specializations at neuromuscular junctions and nonjunctional sarcomas of mammalian fast and slow twitch muscle fibers. *J. Cell Biol.* **68**:752–774.
13. GERSHENBAUM, M. R., J. W. SHAY, and K. R. PORTER. 1974. The effects of cytochalasin B on Balb/3T3 mammalian cells cultured *in vitro* as observed by scanning and high voltage electron microscopy. *In* Scanning Electron Microscopy, part III. IIT Research Institute, Chicago, Ill. 589–590.
14. GOLDMAN, R. D. 1975. The use of heavy meromyosin as an ultrastructural cytochemical method for localizing and determining the possible functions of actin-like microfilaments in non-muscle cells. *J. Histochem. Cytochem.* **23**:529–542.
15. GOLDMAN, R. D., J. A. SCHLOSS, and J. M. SANGER. 1976. Organizational changes of actin-like microfilaments during cell movement. *In* Cell Motility, Book A. Cold Spring Harbor Conference on Cell proliferation, Vol. 3. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 217–245.
16. GRAY, E. G. 1975. Synaptic fine structure and nuclear, cytoplasmic and extracellular networks. *J. Neurocytol.* **4**:315–339.
17. HAYAT, M. A. 1970. Principles and techniques of electron microscopy: Biological applications. Vol. 1. Van Nostrand Reinhold Co., New York. 412.
18. HAYFLICK, L., and P. S. MOOREHEAD. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585–621.
19. HEUSER, J. E. 1978. Membrane surfaces viewed by transmission EM of tissues quick-frozen, deep-etched, and rotary replicated: a simple alternative to high resolution scanning EM. *J. Cell. Biol.* **79**(2, Pt. 2):224*a*. (Abstr.).
20. HEUSER, J. E., T. S. REESE, and D. M. LANDIS. 1976. Preservation of synaptic fine structure by rapid freezing. *Cold Spring Harbor Symp. Quant. Biol.* **40**:17–24.
21. HINKLEY, R. E. 1973. Axonal microtubules and associated filaments stained by alcian blue. *J. Cell Sci.* **13**:753–761.
22. ISHIKAWA, H. R., R. BISCHOFF, and H. HOLTZER. 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* **43**:312–328.
23. KILARSKI, G., and H. KAPROWSKI. 1976. Observation of whole, cultured-human brain cells using 100 kilovolts electron microscopy. *J. Microsc. Biol. Cell.* **25**:73–80.
24. LE BEUX, Y. J., and J. WILLEMOT. 1975. An ultrastructural study of the microfilaments in rat brain by means of heavy meromyosin labeling. I. The perikaryon, the dendrites and the axon. *Cell Tissue Res.* **160**:1–36.
25. LE BEUX, Y. J., and J. WILLEMOT. 1975. An ultrastructural study of the microfilaments in rat brain by means of E-PTA staining and heavy meromyosin labeling. II. The synapses. *Cell Tissue Res.* **160**:37–68.
26. LENK, R., L. RANSON, Y. KAUFMANN, and S. PENMAN. 1977. A cytoskeletal structural associated with polysomes obtained from HeLa cells. *Cell.* **10**:67–78.
27. MAZIA, D., G. SCHATTEN, and W. SALE. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *J. Cell Biol.* **66**:198–200.
28. MCNUTT, N. S. 1978. A thin-section and freeze-fracture study of microfilament membrane attachments in choroid plexus and intestinal microvilli. *J. Cell Biol.* **79**:774–787.
29. MOOSEKER, M. S., and L. G. TILNEY. 1975. Organization of an actin filament complex. *J. Cell Biol.* **67**:725–743.
30. PALADE, G. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* **95**:285–298.
31. PALADE, G. E. 1956. The fixation of tissues for electron microscopy. *In* The Proceedings of the Third International Conference on Electron Microscopy, 1954. R. Ross, editor. Royal Microscopical Society, London. 129–141.
32. PALADE, G. E., and P. SIEKEVITZ. 1956. Liver microsomes, an integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2**: 171–200.
33. POLLARD, T. D., K. FUJIWARA, R. NIEDERMAN, and P. MAUPIN-SZAMIER. 1976. Evidence for the role of cytoplasmic actin and myosin in cellular structure and motility. *In* Cell Motility, Book B. Cold Spring Harbor Conference on Cell Proliferation, Vol. 3. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 689–724.
34. PORTER, K. R. 1953. Observations on a submicroscopic basophilic component of cytoplasm. *J. Exp. Med.* **97**:727–750.
35. PORTER, K. R. 1976. Introduction: Motility in cells. *In* Cell Motility Book A. Cold Spring Harbor Conference on Cell Proliferation. Vol. 3. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1–28.
36. PORTER, K. R., A. CLAUDE, and E. F. FULLAM. 1945. A study of tissue cells by electron microscopy. Methods and preliminary observations. *J. Exp. Med.* **81**:233–246.
37. PORTER, K. R., and F. KALLMAN. 1953. The properties and effects of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy. *Expt. Cell Res.* **4**:127–141.
38. PORTER, K. R., and H. P. THOMPSON. 1948. A particulate body associated with epithelial cells cultured from mammary carcinomas of mice of a milk-factor strain. *J. Exp. Med.* **88**:15–24.

39. REAVEN, E. P., and S. G. AXLINE. 1973. Subplasmalemmal microfilaments and microtubules in resting and phagocytizing macrophages. *J. Cell Biol.* **59**:12–27.
40. REBHUN, L. I. 1972. Freeze substitution and freeze drying. *In* Principles and Techniques of Electron Microscopy, Vol. 2. M. A. Hayat, editor. Van Nostrand Reinhold, New York. 3–49.
41. SABATINI, D. D., K. BENSCH, and R. J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19–58.
42. SALE, W. S., and P. SATIR. 1976. Splayed *Tetrahymena* cilia: a system for analyzing sliding and axonemal spoke arrangement. *J. Cell Biol.* **71**: 589–605.
43. SCHULTZE, M. 1865. Sur Kenntniss der leuchtogane von Lampyris splendidula. *Arch. Mikr. Anat.* **1**:124–137.
44. SMITH, D., and V. JARLFORS. 1970. Organization of the synaptic axoplasm in the lamprey (*Petromyzon marinus*) central nervous system. *J. Cell Biol.* **46**:199–219.
45. SPOONER, B. S., K. M. YAMADA, and N. K. WESSELLS. 1971. Microfilaments and cell locomotion. *J. Cell Biol.* **49**:595–613.
46. STRANGEWAYS, T. S. P., and R. G. CANTI. 1927. The living cell *in vitro* as shown by darkground illumination and the changes induced in such cells by fixing reagents. *Q. J. Micros. Sci.* **281**:1–14.
47. TANI, E., and T. AMETANI. 1970. Substructure of microtubules in brain nerve cells as revealed by ruthenium red. *J. Cell Biol.* **46**:159–165.
48. TILNEY, L. G. 1971. How microtubule patterns are generated. The relative importance of nucleation and bridging of microtubules in the formation of the axoneme of *Radiophrys*. *J. Cell Biol.* **51**:837–854.
49. VIAL, J., and K. R. PORTER. 1975. Scanning electron microscopy of dissociated tissue cells. *J. Cell Biol.* **67**:345–360.
50. WARNER, F. D. 1974. The fine structure of the ciliary and flagellar axoneme. *In* Cilia and Flagella. M. A. Sleigh, editor. Academic Press, Inc., London. 11–38.
51. WEISS, P. 1967. Neuronal dynamics. *Neurosci. Res. Program Bull.* **5**: 371–400.
52. WESSELLS, N. K., B. S. Spooner, and M. A. Luduena. 1973. Surface movements microfilaments and cell locomotion. *In* Locomotion of Tissue Cells, Ciba Foundation Symposium 14. K. R. Porter, and D. W. Fitzsimons, editors. Associated Scientific Publishers, Amsterdam. 53–82.
53. WOLOSEWICK, J. J., and K. R. PORTER. 1975. High voltage electron microscopy of WI-38 cells. *Anat. Rec.* **181**:511–512.
54. WOLOSEWICK, J. J., and K. R. PORTER. 1975. High voltage electron microscopy of the ground substance of cultured WI-38 cells. *J. Cell. Biol.* **67**(2, Pt. 2):460*a*. (Abstr.).
55. WOLOSEWICK, J. J., and K. R. PORTER. 1976. Stereo high voltage electron microscopy of whole cells of the human diploid cell line WI-38. *Am. J. Anat.* **147**:303–324.
56. WOLOSEWICK, J. J., and K. R. PORTER. 1977. Observations on the morphological heterogeneity of WI-38 cells. *Am. J. Anat.* **149**:197–226.
57. WOLOSEWICK, J. J., and K. R. PORTER. 1977. Effect of low temperatures on the ground substance of cultured cells. *J. Cell. Biol.* **75**(2, Pt. 2):275*a*. (Abstr.).
58. WOLOSEWICK, J. J., K. R. PORTER, K. LUBY, K. ANDERSON, J. L. MEEK, and J. ANDREOZZI. 1976. The structure of the cytoplasmic ground substance. A study of its real or artifactual nature. *J. Cell. Biol.* **70**(2, Pt. 2):400*a*. (Abstr.).
59. YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49**:614–635.