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DILUTED AND UNDILUTED MERCOX SEVERELY DESTROY UNFIXED ENDOTHELIAL CELLS. A Light And Electron Microscopic Study Using Cultured Endothelial Cells and Tadpole Tail Fin Vessels

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Abstract

Mercox is a methylmethacrylate-based resin which is widely used for vascular corrosion casting with subsequent scanning electron microscopic analysis. In the present study the effect of undiluted and diluted Mercox (4+1; volume + volume; Mercox: monomeric methylmethacrylate (MMA); 0.02 g catalyst MA/ml Mercox) and methyl-methacrylate with and without catalyst MA (0.625 g/10 ml MMA) on fixed and unfixed endothelial cells was studied. Light microscopy (LM) of cultured capillary endothelial cells (ECs), which were replicated with diluted or undiluted Mercox shows degranulation and membrane perturbation of ECs, while no morphological changes occur in glutaraldehyde-prefixed ECs. Scanning electron microscopy (SEM) of replicas (= resin blocks) polymerized on prefixed ECs reveals unchanged ECs and replicas show many details. Unfixed ECs are destroyed and replicas reveal aberrant features. Transmission electron microscopy (TEM) of prefixed and unfixed ECs (cultured endothelial cells, endothelial cells of perfusion prefixed and of unfixed tadpole tail fin vessels) substantiates LM and SEM findings. Prefixed ECs resist Mercox without fine structural changes, while unfixed cells undergo destruction. It is recommended to fix vessels prior to casting. Extravasations in microvessels are considered to be caused by focal chemical destruction of endothelial cells.

Key Words: Corrosion casting, Mercox, endothelial cells, light microscopy, scanning electron microscopy, transmission electron microscopy.

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Introduction

Since scanning electron microscopy (SEM) of vascular corrosion casts was introduced (Murakami, 1971), a steadily increasing number of studies have utilized the advantages of the technique to analyze microvascular patterns of tissues and organs in three dimensions (for reviews see Hodde and Nowell, 1980; Christofferson, 1988; Hodde *et al.*, 1990; Lametschwandtner *et al.*, 1990; Aharinejad and Lametschwandtner, 1992; Motta *et al.*, 1992).

Mercox (Ladd Research Industries, Inc. Burlington, Vermont, USA) is a frequently used resin in microvascular corrosion castings. Physical and chemical properties (shrinkage, corrosion resistance, replication quality and thermostability) of this methylmethacrylate based resin are known (Weiger *et al.*, 1982, 1986). Although there are indications from literature that Mercox has effects on the blood vessel's wall, few studies have analyzed the effect of diluted or undiluted Mercox on fixed and unfixed endothelial cells (ECs). Recently, Aharinejad and Böck (1993a) found that a Mercox-methylmethacrylate mixture penetrates into endothelial cells, mummifies them, and makes them resistant against maceration. They further found that an unidentified compound escapes from the lumen of injected blood vessels and causes plastic sheets on elastic arteries (Aharinejad and Böck, 1993b). It was also demonstrated by high resolution intravital video-microscopy that undiluted Mercox stimulates vascular wall components, changes vascular permeability and leads to extravasations and filling of nearby lymphatics (Aharinejad *et al.*, 1993a,b).

To further examine the effect of undiluted Mercox, a Mercox-methylmethacrylate mixture (referred to as diluted Mercox) and of methylmethacrylate (with and without catalyst benzoyl peroxide) on fixed and unfixed ECs, we cast cultured ECs ("*in vitro*" assay) and tail fin vessels of tadpoles of *Xenopus laevis Daudin* ("*in vivo*" assay) with those media and studied their effect on ECs and tailfin vessels by intravital phase contrast microscopy, scanning and transmission electron microscopy.

Table 1. Intravital light microscopy (p - phase contrast microscopy: v - video phase contrast microscopy) of fixed and unfixed cultured ECs. n = number of petri dishes studied.

Fixation	diluted Mercox	undiluted Mercox
buffered 1.5% glutaraldehyde	n = 3 (v) n = 2 (p)	n = 2 (v) n = 1 (p)
unfixed	n = 1 (v) n = 2 (p)	n = 2 (v) n = 1 (p)

Table 2. Transmission electron microscopy of fixed and unfixed ECs after casting with diluted and undiluted Mercox with or without catalyst MA, methylmethacrylate monomer (MMA), and methylmethacrylate with catalyst MA. n = number of petri dishes studied.

ECs cast with	fixed	unfixed
not cast (controls)	n = 3	
diluted Mercox with MA	n = 3	n = 3
undiluted Mercox with MA	n = 3	n = 3
undiluted Mercox without MA	n = 3	n = 3
MMA without MA	n = 3	n = 3
MMA with MA	n = 3	n = 3

Materials and Methods

Light microscopy

Replication of cultured endothelial cells. ECs of cerebral capillaries (Tontsch and Bauer, 1989) were seeded in plastic petri dishes (diameter = 33 mm: Cell/cult, Sterilin Limited, Hounslow, UK) and cultured at 37°C until they grew multilayered. Culture medium was poured off the petri dishes and cells were treated as indicated in Table 1.

Endothelial cells were fixed with buffered 1.5% glutaraldehyde (0.1 M sodium cacodylate, pH 7.3, 330 mOsm, 20°C) or remained in the culture medium. After ten minutes fixative or culture medium (unfixed samples) were poured off the dishes and cells were cast with diluted Mercox (4 + 1, dilution with monomeric methyl-methacrylate, volume + volume; 0.02 g catalyst MA/ml Mercox; apparent viscosity: 12 centipoise) or undiluted Mercox (supplemented with 0.02 g catalyst MA/ml Mercox; apparent viscosity: 35 centipoise). The catalyst MA used was supplied by Ladd Research Industries, Burlington, Vermont, USA. The catalyst MA is benzoyl peroxide, the concentration, however, is not specified. Observations were recorded using a video camera (Panasonic CCD, WV-CD 110-E) connected to a Leitz Labovert FS microscope.

SEM studies of cultured ECs

Endothelial cells (see above) were seeded on cover slips (d = 1 cm) and cultured at 37°C. When cells revealed a multilayered growth they were fixed with buffered glutaraldehyde (1 hour, 20°C; see **Light microscopy**), rinsed in buffer (3 x 10 minutes), dehydrated in a graded series of ethanol (70%, 80%, 90%, 96% and 3 x 100%; 5 minutes each), 100% ethanol:amyl acetate (2:1; 1:1; 1:2), and finally critical point dried via carbon dioxide. Dry cover slips with cells were mounted with conductive silver paste onto specimen stubs, sputtered with a 27 nm thick layer of gold, and examined with a scanning electron microscope (SEM) Stereoscan 250 (Cambridge, UK) operated at an accelerating voltage of 20 kV.

SEM studies of replicas from cultured ECs

Three plastic dishes with ECs, either fixed with buffered 1.5% glutaraldehyde (10 minutes, 20°C) or left unfixed, were cast with 3 ml undiluted or diluted Mercox. Replicated cells were left for 10 minutes at room temperature and then tempered overnight at 60°C. The next day, replicas (with cells attached) were removed from the petri dishes and macerated in 15% KOH at 40°C for 3 hours. Then, the replicas were rinsed in distilled water, frozen in bi-distilled water and freeze-dried. Dry replicas were mounted with conductive silver paint onto specimen stubs (Lametschwandtner *et al.*, 1980), coated with a 27 nm thick layer of gold and examined in a Stereoscan 250 SEM operated at an accelerating voltage of either 5 kV or 10 kV.

TEM Studies on fixed and unfixed replicated ECs

Endothelial cells were seeded onto Transwell-Col filter membranes (Costae, Cambridge, Massachusetts, USA; diameter = 1 mm, pore size = 3 μm) and cultured for 10 days at 37°C. Cells were then treated as outlined in Table 2.

Fixation of cells prior to casting was with 1.5% buffered glutaraldehyde for 15 minutes at 20°C as described above. After casting, ECs were fixed a second time from below the filter membrane with glutaraldehyde, rinsed in buffer solution (3 x 5 minutes, 20°C), and postfixed in 1% aqueous osmium tetroxide (1 hour, 20°C). After three additional rinses in cacodylate buffer, specimens were dehydrated in a graded series of ethanol, and embedded via mixtures of 100% ethanol : Epon 812 (3:1, 75 minutes; 1:1, 5 hours; 1:3, overnight) into Epon 812. Specimens were allowed to polymerize at 40°C (12 hours) and at 60°C (2 days). One micrometer thick semi-thin sections were stained with

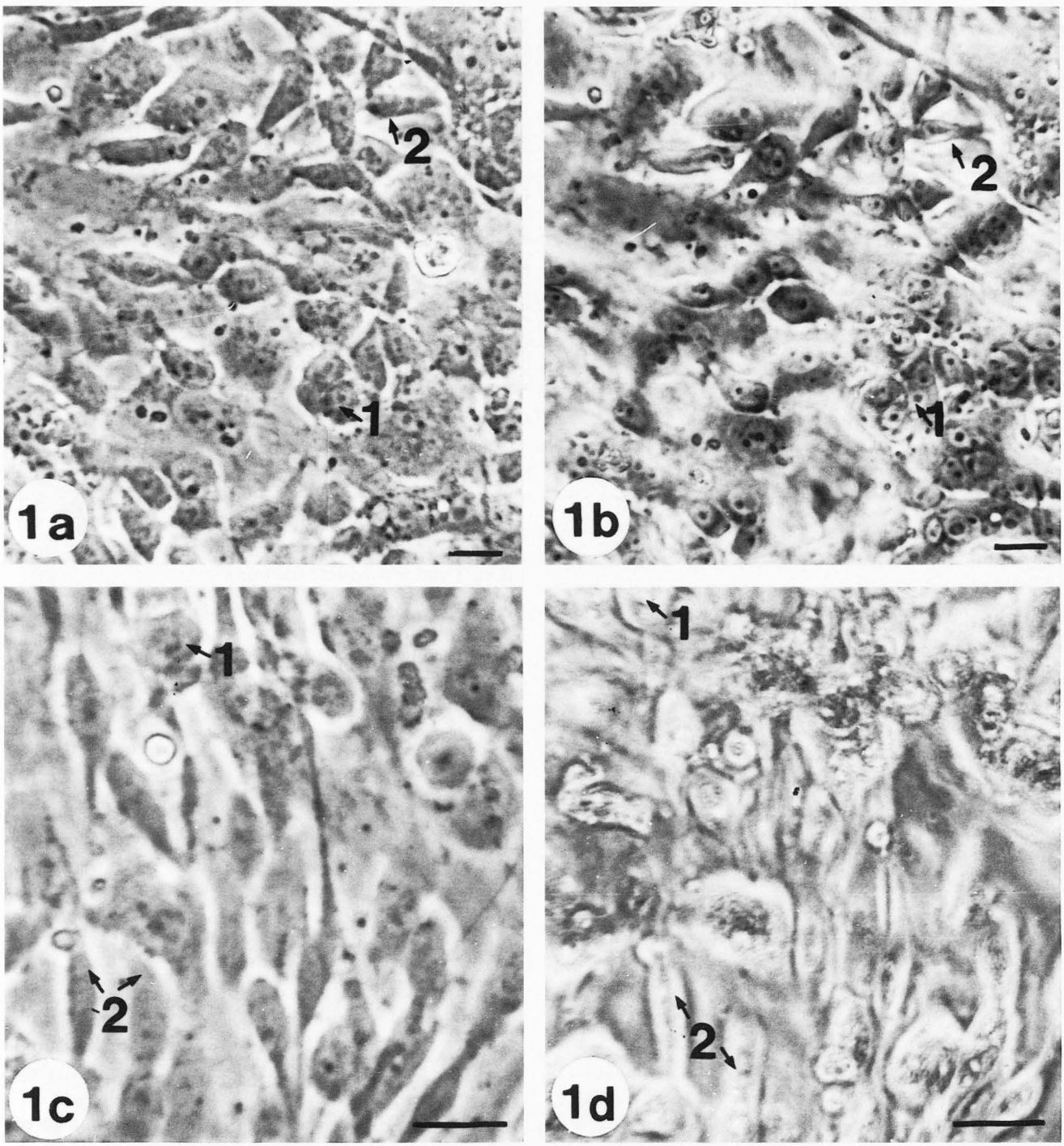

Figure 1. Cultured cerebral capillary endothelial cells (ECs). Phase contrast microscopy. Bar = 20 μm. (**a**). Living (unfixed) cells express two phenotypes (1 = type-I EC, cobblestone-type; 2 = type-II EC, spindle shaped-type). (**b**). Fixed cells (glutaraldehyde) after superfusion with diluted Mercox (4+1). Endothelial cells show no prominent changes. (**c**). Same as in **a**. (**d**). Unfixed ECs after superfusion with diluted Mercox (4+1). ECs degranulate, shrink and EC nuclei become prominent.

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methylene blue; 60-90 nm thick ultrathin sections were stained with uranyl acetate (60 minutes, 40°C) and lead citrate (6 minutes, 20°C). Sections were examined with a Philips 300 transmission electron microscope (TEM)

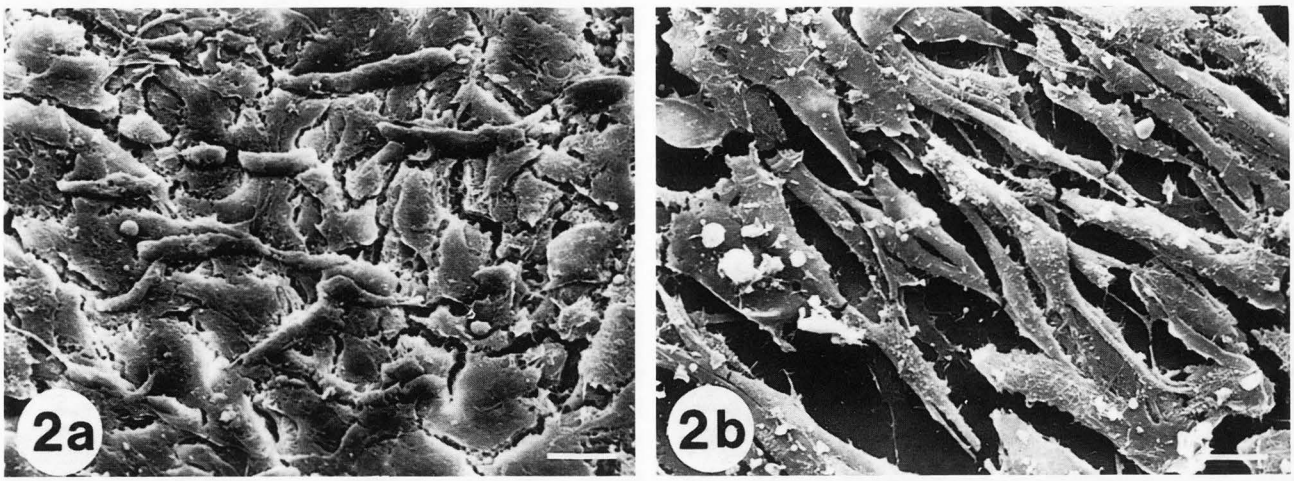


Figure 2. Scanning electron micrograph of cultured ECs from mouse cerebral capillaries. Critical point dried specimens. Bar = 5 μm. (**a**). Type-I ECs form continuous cell layers. (**b**). Type-II ECs remain separated but contact each other via filiform and lobiform protrusions.

operated at an accelerating voltage of 100 kV.

TEM studies on fixed and unfixed and embedded cast tadpole tail fin vessels

Tadpoles of the South African Clawed Toad, *Xenopus laevis Daudin*, stages 52 to 60 (staging according to Nieuwkopp and Faber, 1956) were studied. Animals were raised in the animal facilities of our Institute.

Vascular casting. A total of six tadpoles was studied. Animals were anesthetized in 0.005% MS 222 (ethyl-m-paraaminobenzoate, Sigma Chemicals, St. Louis, Missouri, USA), the right systemic arch was exposed, opened, a glass cannula was inserted with a micromanipulator and ligatured in place. After a short rinse with Ringer solution (Adam and Czihak, 1964) and clear efflux from the proximal stump of the right systemic arch, 1 ml resin {0.8 ml undiluted Mercox and 0.2 ml monomeric methyl methacrylate (Fluka, Basel, Switzerland) supplemented with 0.02 g catalyst MA} was injected at a flow rate of 5-8 ml per hour.

Tadpole #1 was immersed immediately after the injection of the resin into buffered 1% glutaraldehyde (40°C, 2 hours). After 3 rinses (5 minutes each) in buffer solution, the tail of the tadpole was cut into smaller pieces and postfixed in a 1% aqueous solution of osmium tetroxide (20°C, 2 hours).

Tadpoles #2-#6 were perfused via a glass cannula ligatured into the arterial trunk with the venous sinus opened for efflux.

Tadpole #2 was immediately fixed after casting at room temperature in buffered 2% glutaraldehyde and treated like tadpole #1.

Tadpoles #3-#6 were fixed prior to casting via vascular perfusion with buffered 2% glutaraldehyde. After casting animals were immersed in fixative (20°C, 4 hours) and treated like tadpole #1.

Specimens from tadpoles #1-3 were dehydrated in a graded series of ethanol and treated as described above, while those from tadpoles #4-#6 were directly embedded in Epon 812. The specimens were further processed as described above.

Results

Light microscopy

Endothelial cell cultures grow multilayered and, depending on culture conditions, express two phenotypes, i.e., type-I ECs (cobblestone-type) and type-II ECs (spindle shaped-type) described by Tontsch and Bauer (1989; Figs. 1a and 1c).

Fixation of cells with buffered 1.5% glutaraldehyde results in no significant structural changes at the light microscopical level (Fig. 1b). Casting fixed cells with diluted or undiluted Mercox causes no structural changes. Casting of unfixed ECs with diluted Mercox or with undiluted Mercox causes severe EC degranulation and cell membrane perturbations. The nuclei of ECs become prominent because the cell size decreases (Fig. 1d).

Scanning electron microscopy (SEM)

Scanning electron microscopy confirms that the two EC types form several layers and contact each other by filiform or lobular cytoplasmic protrusions (Fig. 2).

Replicas (3.5-4 mm thick) of ECs fixed prior to casting (replication) reveal clear impressions of endothelial cell borders, endothelial cell nuclei and cytoplasmic

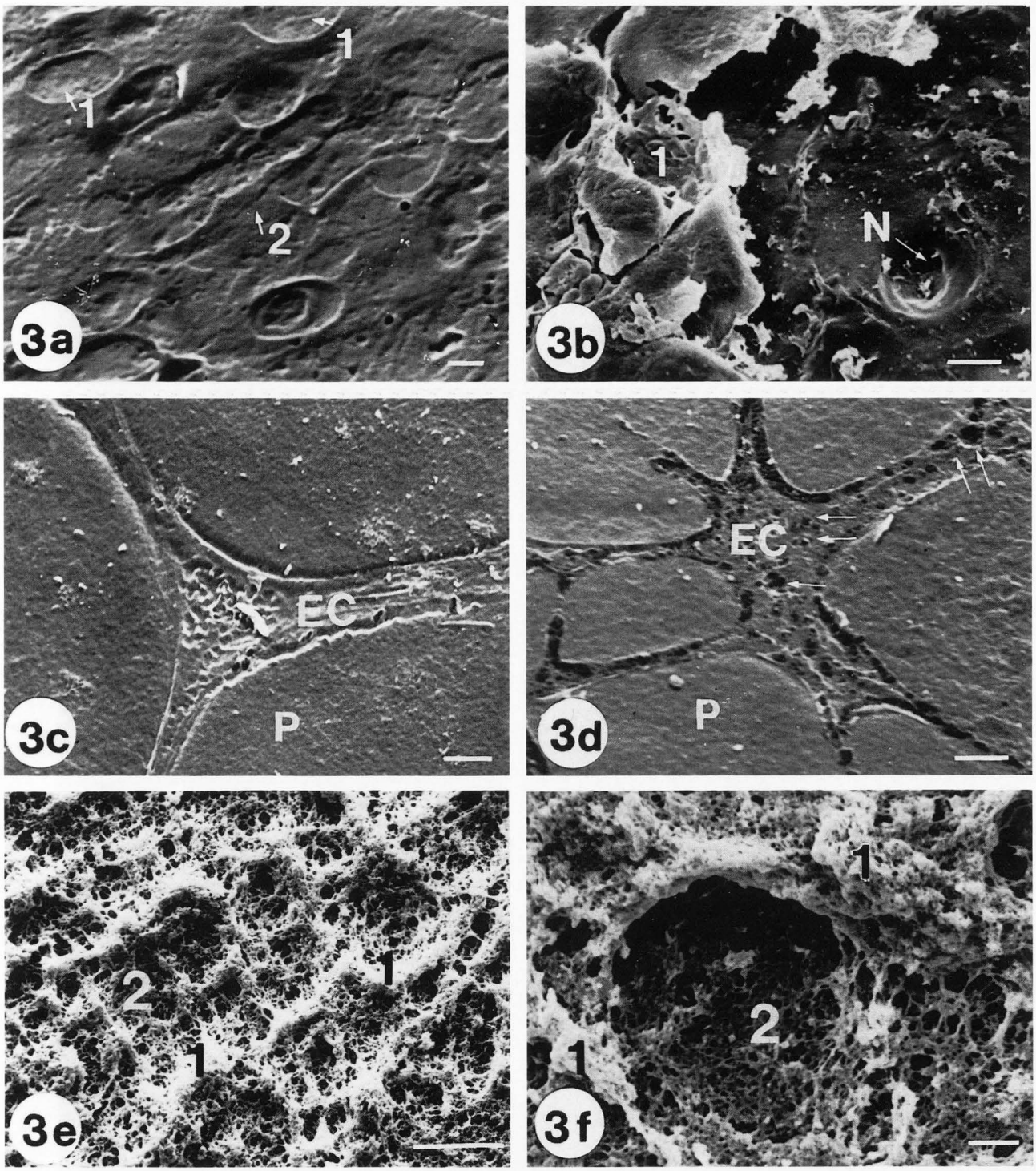
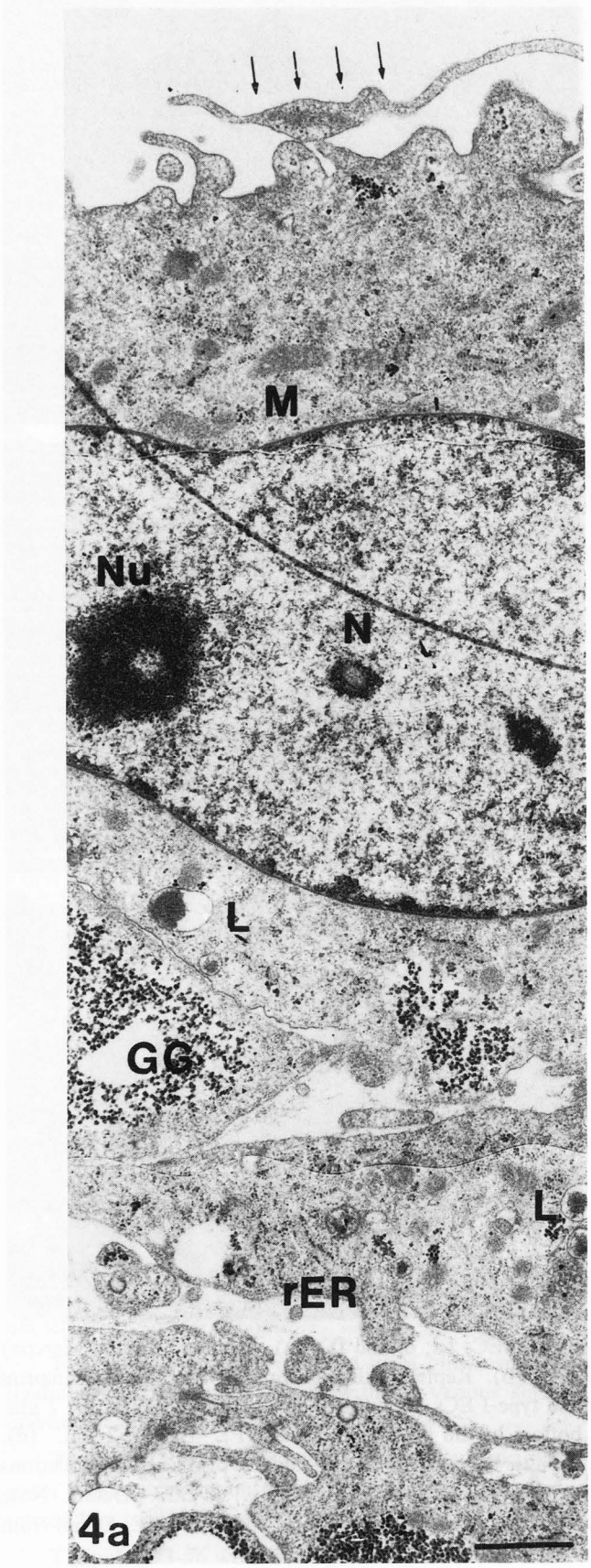


Figure 3. Replicas of prefixed (**a**, **b**, and **c**) and unfixed cultured ECs (**d**, **e**, and **f**). (**a**). Replica (diluted Mercox) with imprints of EC nuclei (1) and EC borders (2). Bar = 2 μm. (**b**). Replica (diluted Mercox) with a nuclear imprint (N) of a type-I EC (right half of micrograph) and non-corroded type-I ECs (1; left half of micrograph). Bar = 2 μm. (**c**). Replica (undiluted Mercox) of a type-II EC and the bottom of the plastic Petri dish (P). Bar = 2 μm. (**d**). Replica (undiluted Mercox) of an unfixed type-II EC. Note the altered shape of the cell and the prominent granulations (arrows). P: replicated bottom of Petri dish. Bar = 2 μm. (**e**). Replica (undiluted Mercox) of ECs. Detail. Note the cotton-wool-like structure of the replica. No cellular structures can be identified. Bar = 5 μm. (**f**). Detail from **e**. Cell detritus forms ridges (1) with valleys (2) between. Bar = 1 μm.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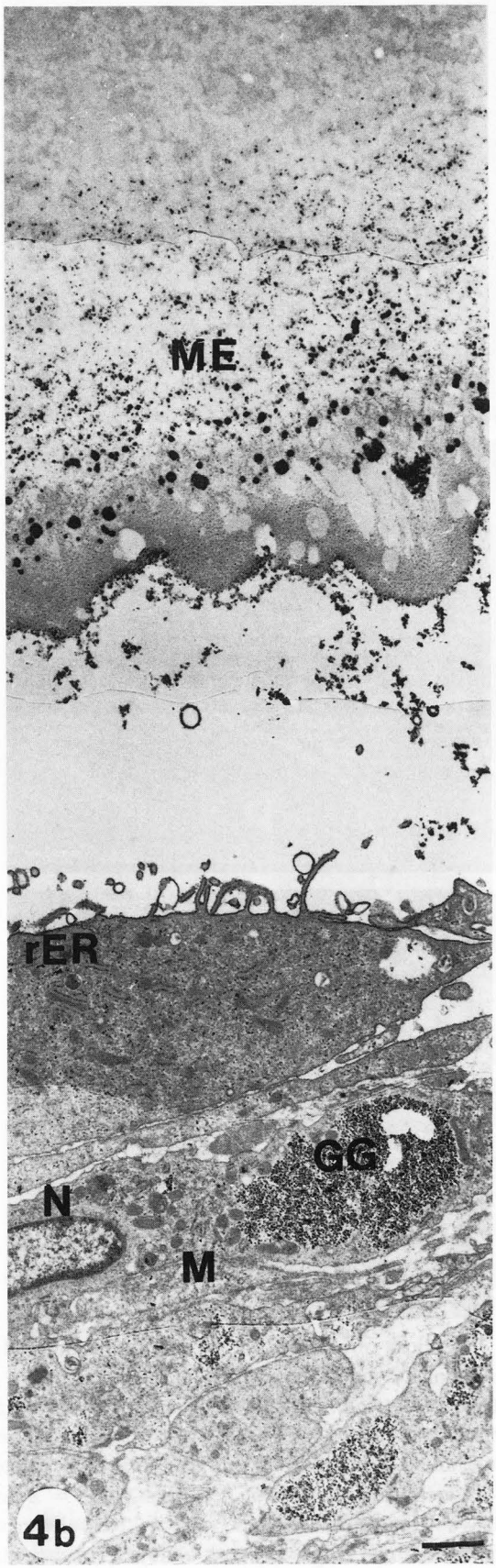
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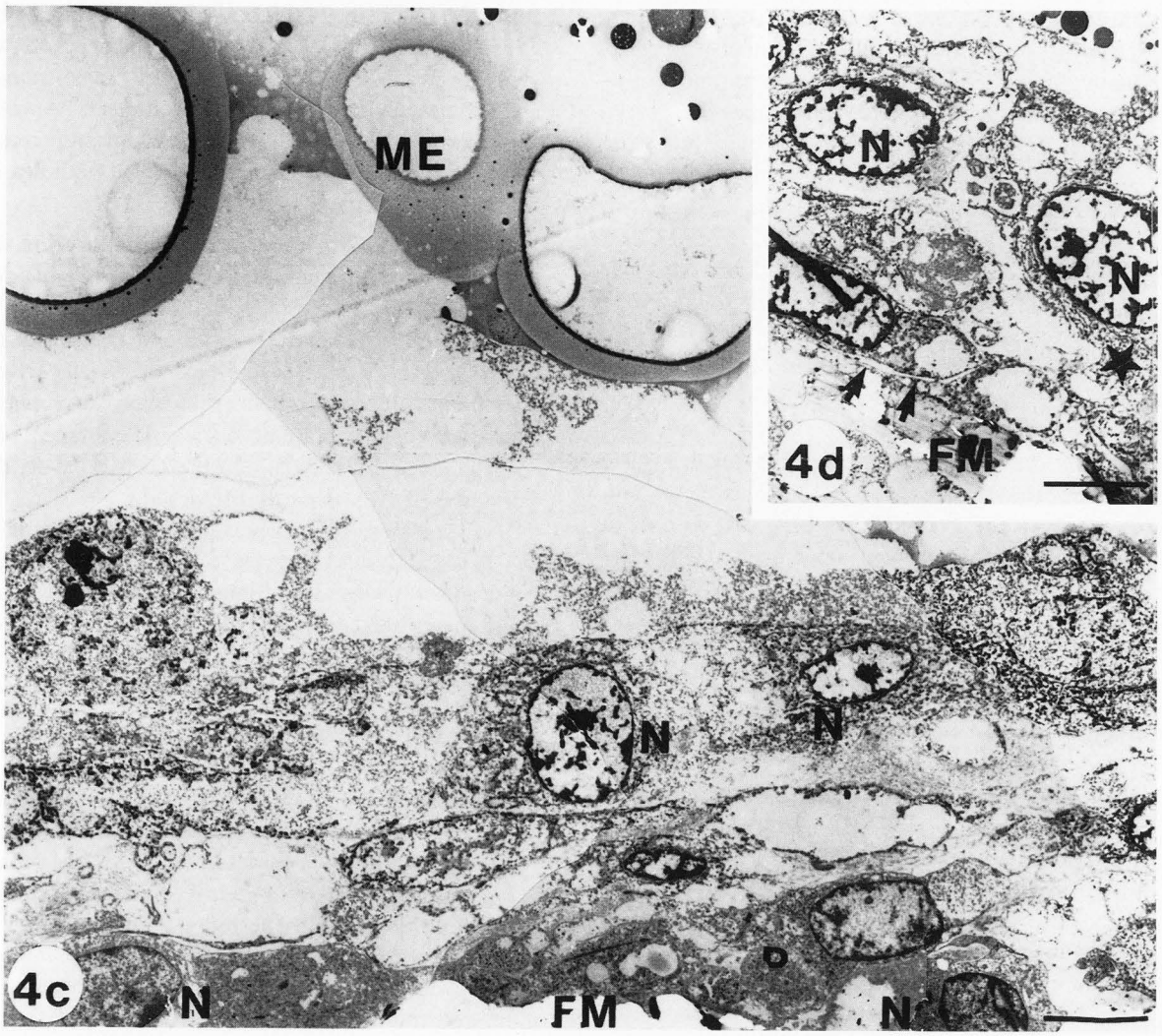
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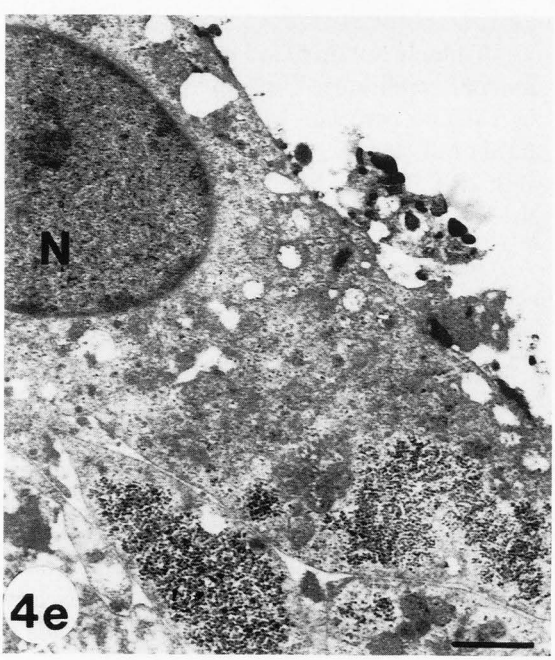
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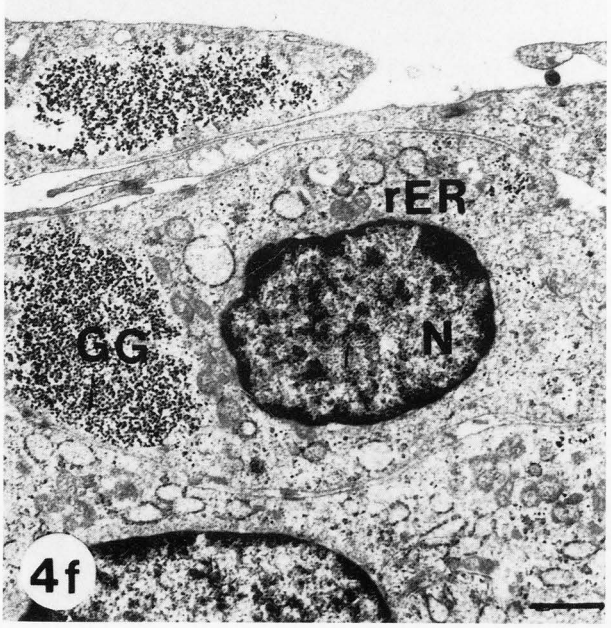
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Figure 4 (on preceding pages 726-727). Fine structure of cultured ECs. Transverse sections. (**a**). Fine structure of multilayered growing ECs after superfusion (fixation) with glutaraldehyde. Luminal cytoplasmic protrusions (arrows), mitochondria (M), lysosomes (L), glycogen granules (GG) and rough endoplasmic reticulum (rER) are found. Nu: nucleolus; N: nucleus. Bar = 1 μm. (**b**). Fine structure of fixed ECs after casting with diluted Mercox (ME). Bar = 1 μm. (**c**). Unfixed ECs cast with undiluted Mercox. Note the damaged upper cell layer and the appearance of the overlaying Mercox (ME). FM: filter membrane; N: nucleus. Bar = 5 μm. (**d**). Unfixed ECs cast with undiluted Mercox without catalyst MA. The fine structure of ECs is damaged. FM: filter membrane; N: nucleus. Bar = 5 μm. (**e**). Unfixed ECs cast with monomeric methylmethacrylate acid without catalyst MA. The EC nucleus (N) is preserved. Bar = 1 μm. (**f**). Unfixed EC cast with monomeric methylmethacrylate acid supplemented with catalyst MA. The structure of the EC is preserved. GG: glycogen granules; N: nucleus; rER: rough endoplasmic reticulum. Bar = 1 μm.

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protrusions. Type-I and type-II ECs can be clearly differentiated (Fig. 3a). Size and shape of cells correspond to the *in vivo* situation. Replicas made from diluted and undiluted Mercox-CL reveal many details (Figs. 3c and 3d).

Interestingly, in one casting experiment, fixed EC layers floated off the bottom of the Petri dish and thus Mercox enclosed the cells from both sides preventing them from maceration. During manipulation of the freeze-dried specimen, the thin layer of Mercox covering the bottom layer of ECs broke away and a layer of preserved type-I ECs (Fig. 3b, left half of micrograph) and a replica with imprints of basal EC surfaces (Fig. 3b, right half of micrograph) became available for SEM inspection.

Replicas of unfixed ECs reveal no identifiable imprints of cells. The replicas reveal a fluffy, cotton wool-like texture with local clumpings of globular structures interconnected by fine thread-like material (Figs. 3e and 3f). Replicas of nearby cell-free areas reveal the smooth surface of the bottom of the plastic Petri dish (Figs. 3c and 3d).

Transmission electron microscopy (TEM)

Controls. Endothelial cells cultured on filter membranes grow multilayered (Fig. 4a). Cells reveal an oval nucleus and interdigitate by cytoplasmic processes. Junctional complexes sometimes connect adjacent cells. Mitochondria are found around the nucleus. Rough endoplasmic reticulum, glycogen granules, lysosomes and coated vesicles are present.

Fixed ECs. There are no prominent structural changes found in ECs fixed prior to casting with undiluted or diluted Mercox, methylmethacrylate with or without catalyst MA (Fig. 4b). Cell nuclei remain homogenous, the nucleolus stays prominent. Apical cytoplasmic processes of cells, rough endoplasmic reticulum, mitochondria, lysosomes and glycogen granules remain unchanged.

Unfixed ECs. In unfixed ECs, replication with diluted (Fig. 4c) or undiluted Mercox (Fig. 4d) destroys cells of the uppermost layer. Cell membranes disappear, and cytoskeletal elements and cytoplasm mix up with casting media. In the next deeper layer, nuclear membranes already remain; however, karyoplasm and chromatin agglomerate on nuclear membranes. All cell organelles are destroyed. In the deepest layer, the fine structure of ECs is partly preserved.

ECs which were replicated with methylmethacrylate without catalyst MA reveal a homogenous cell nucleus with intact nuclear membrane (Fig. 4e). ECs replicated with methylmethacrylate with catalyst MA reveal an almost intact fine structure with the cytoplasm slightly granulated and the endoplasmic reticulum dilated (Fig. 4f).

Tadpole tail fin blood vessel endothelium. Controls. The endothelium of the tadpole tail fin blood vessels is 0.4 μm thick (average). It contains many vesicles (diameter = 0.09 μm) attached to the plasmalemma (Fig. 5a). The endothelium is of the continuous type with tight junctions. The luminal surface reveals cytoplasmic protrusions. The nuclei of ECs are homogenous and have a nucleolus. Lysosomes, endoplasmic reticulum and mitochondria are present. For further details we refer to Rhodin and Lametschwandtner (1993).

Tadpole tail fin blood vessel endothelium. Experimental conditions. Tail fin vessels which were fixed by vascular perfusion before they were cast with undiluted or diluted Mercox-Cl reveal an intact endothelium (Fig. 5b). Casting of unfixed vessels damages endothelial cells (Fig. 5c) and leads to focal ball-shaped extravasations of the injected resin (Fig 5d).

Discussion

Scanning electron microscopy of microvascular corrosion casts enables us to study three dimensional arrangements of minute blood vessels, assists to localize and analyze functional structures like sphincters, venous valves, intimal cushions, atherosclerotic lesions, and visualizes nuclei of ECs and highlights details of the luminal surface by their negative imprints on the casts (for reviews, see Aharinejad and Lametschwandtner, 1992; Motta *et al.*, 1992). Despite the wide range of

applications of the technique, only a few studies deal with the physico-chemical properties of available resins and their impact on qualitative and quantitative aspects of vascular casts (Weiger *et al.*, 1982, 1986; Christofferson, 1988). Very recently, first studies were designed to investigate interactions between casting materials and the vascular wall components (Aharinejad *et al.*, 1992, 1993a,b; Aharinejad and Böck, 1993a,b).

The present study, for the first time, focusses on the effect of Mercox (undiluted, diluted) and methylmethacrylate (with and without catalyst paste MA) on cultured (cerebral) ECs and analyzes them by phase contrast video-microscopy, scanning and transmission electron microscopy. The study convincingly demonstrates that Mercox destroys unfixed ECs, but does not alter fixed endothelial cells. This implies to recommend a prefixation before casting in those experiments, where fine surface details of the vascular endothelium are in the focus of the study. Prefixation should be mild to prevent extremely long periods of tissue maceration (for optimal tissue maceration, see Sims and Albrecht, 1993).

In respect to replication of EC surface structures the nature of the interface resin-luminal surface of the endothelium becomes of interest. At the moment, controlled studies are still lacking which define whether and to what extent the amount and nature of material (e.g., plasma proteins) adsorbed to the plasma membrane of ECs and physico-chemical properties of the resin (e.g., viscosity, grade of hydrophobicity/hydrophilicity) influence interactions between resin and ECs.

It is interesting to recall that methylmethacrylic acid with or without catalyst MA did not dramatically change the morphology of ECs as observed by phase contrast video-microscopy and thus behaved totally different from Mercox. From this observation, we conclude that some yet unidentified component(s) of Mercox, other than the methylmethacrylate compound, causes endothelial breakdown. Whether the observed destruction of ECs after plating of unfixed ECs or after casting of unfixed tadpole tail fin vessels with undiluted or diluted (4+1) Mercox (Hodde, 1981) results from lysis of the cell membranes with subsequent collapse of the cell or whether it is an osmotic phenomenon, remains to be studied.

The observation that nuclei of ECs resist longest may explain, why EC nuclear imprints are found at all on vascular casts, and why they are found more often on casts of large vessels. Generally, the thicker wall of larger vessels will act as confinement and keeps the EC nuclei in place. Because the extent to which EC nuclei in unfixed vessels become destroyed cannot be defined at the moment, quantification of EC nuclear imprints is not yet reliable.

When controlling the casting process during tadpole tail fin vascular injections under video light microscopy, we gained convincing evidence that extravasations found in microvascular corrosion casts at the capillary level occur also by a (multi)focal "chemical destruction" of the ECs in unfixed capillaries by hitherto unspecified compound(s) of Mercox. We think that the occurrence of the focal "chemical destruction" depends on many factors, the most important ones being the state of the resin (state and patterns of phase separation, viscosity, extent and duration of contacts of hydrophilic or hydrophobic phases with the endothelium) and of the endothelium (absorption of plasma protein, etc.) and its perivascular environment (heat transfer). These factors, of course, cannot be controlled sufficiently in whole body (organ, tissue) vascular castings, but using the tadpole tail fin as a model (Clark, 1918; Rhodin and Lametschwandtner, 1993) casting conditions could be controlled to a much higher degree than done so far in any other system. Future studies in this model should make valuable contributions to our understanding of the various forms of vascular corrosion casts shown by scanning electron microscopy of casts (Aharinejad and Böck, 1994).

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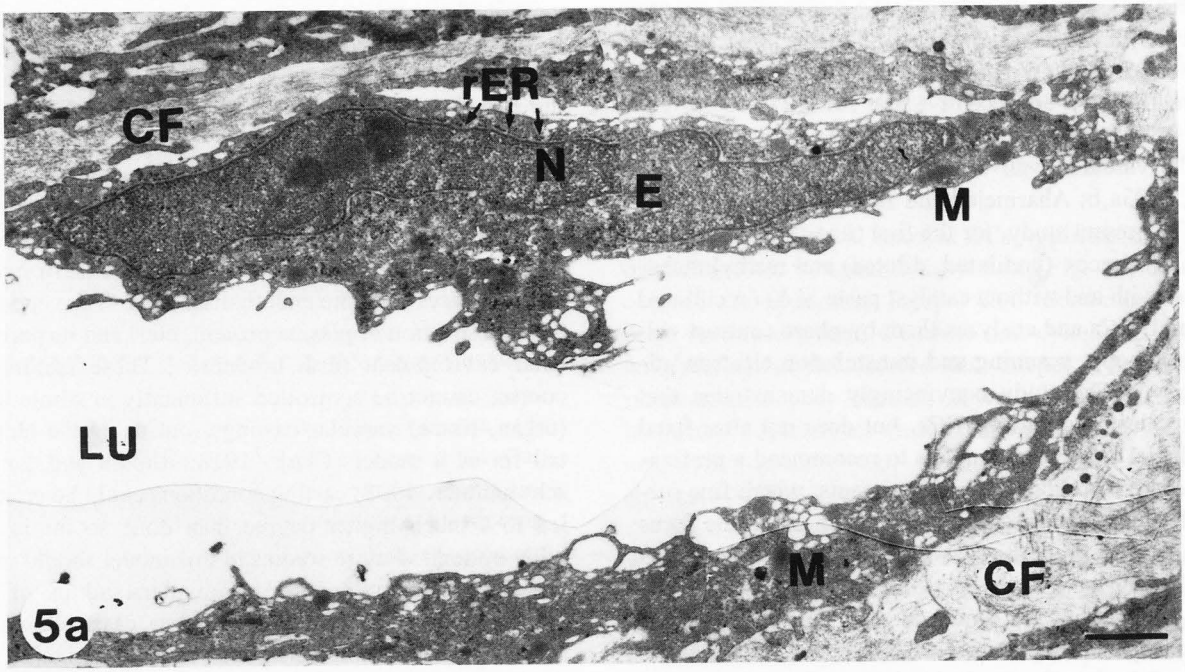
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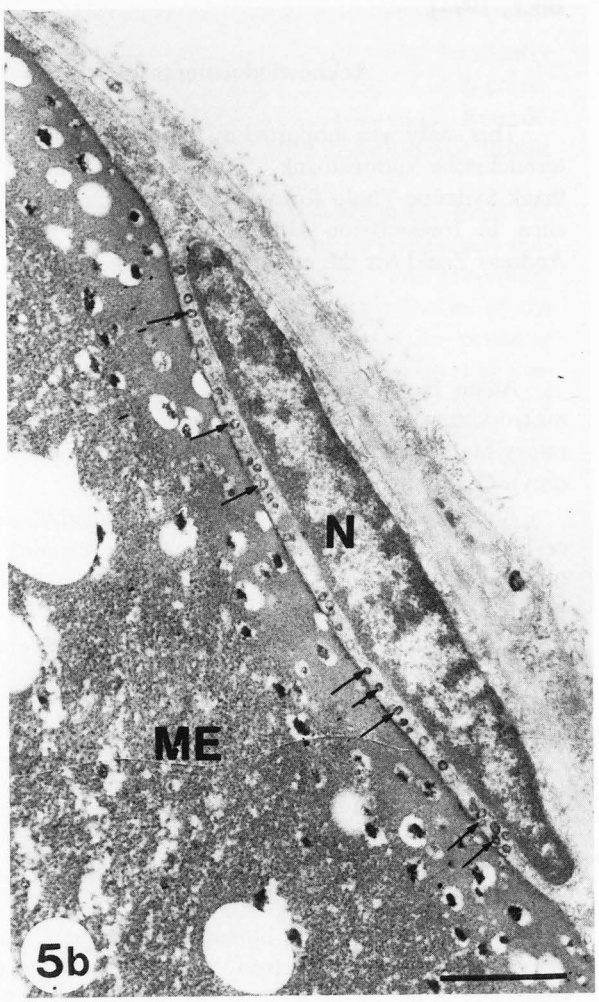
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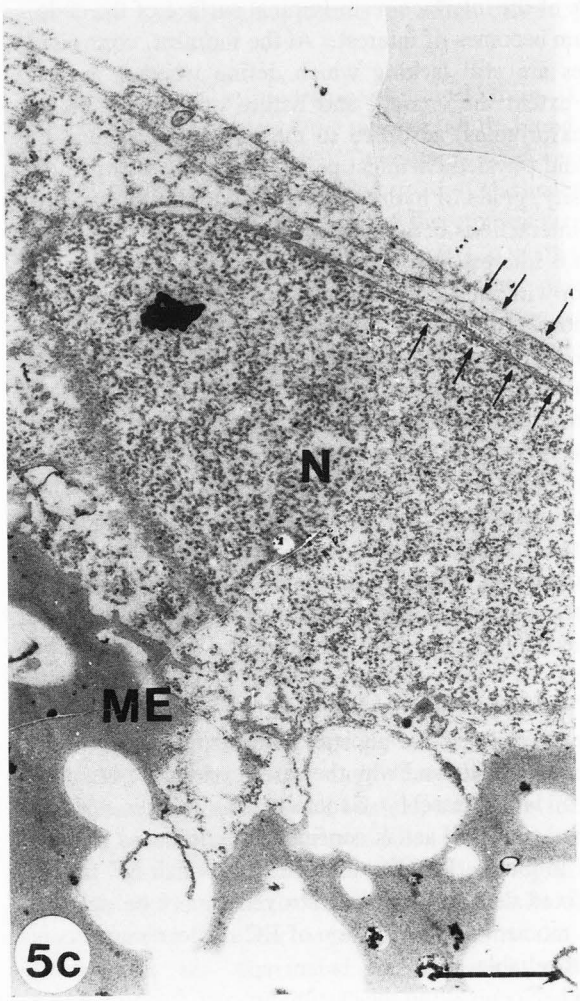
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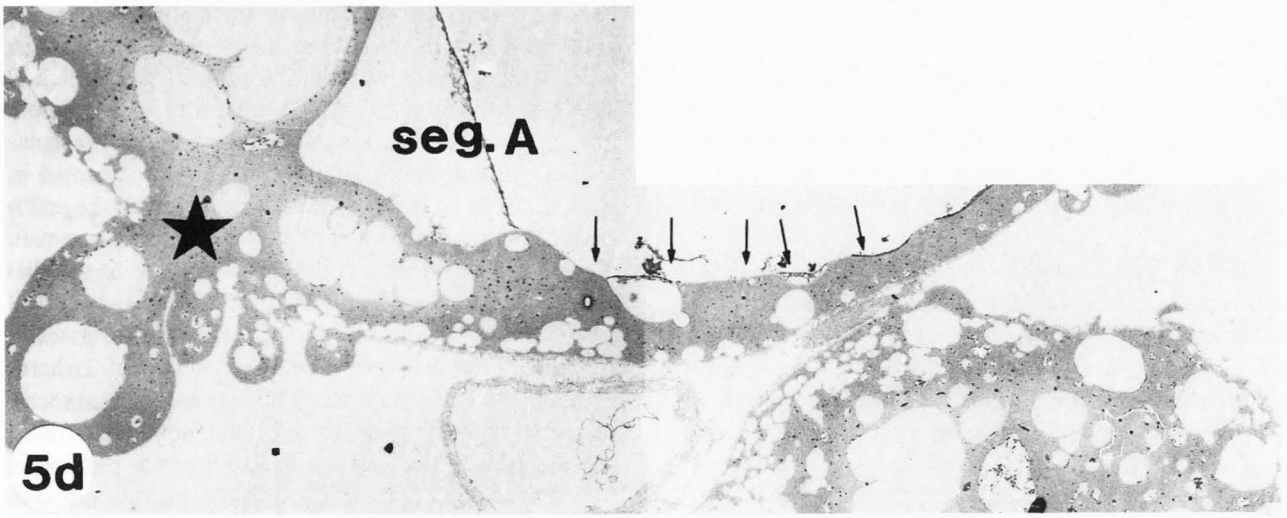

Figure 5 (a-c on facing page 730; d above). Fine structure of prefixed (**a** and **b**) and unfixed (**c** and **d**) tail fin vessels of tadpoles of *Xenopus laevis Daudin* cast with diluted Mercox (**b**, **c**, and **d**). (**a**). Capillary. Longitudinal section. E: endothelium; CF: collagen fibers in the perivascular space; LU: lumen; M: mitochondrium; N: nucleus; rER: rough endoplasmic reticulum. Bar = 1 μm. (**b**). Segmental arteriole (d = 50 μm). Transverse section. Note the electron dense content of the luminal vesicles of the ECs (arrows) and the electron lucent ("empty") spaces within the intraluminal Mercox (ME). Bar = 1 μm. (**c**). Segmental arteriole (d = 25 μm). Transverse section. The luminal plasma and nuclear membranes of the unfixed EC are destructed. The cytoplasm is mixed up with Mercox (ME). Abluminal plasma and nuclear membranes are still present (arrows). Bar = 1 μm. (**d**). Segmental arteriole (d = 65 μm). Transverse section. Note the focal extravasation of Mercox (asterisk). The extravasations are rounded, their interior reveals round, electron lucent structures. Mercox forms an electron dense rim along the vessel periphery (arrows). Bar = 3 μm.

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Discussion with Reviewers

M.A. Konerding: You have macerated your specimens with 15% KOH although you refer in the **Discussion** to the paper of Sims and Albrecht (1993). Why did you not use a lower concentration?
Authors: The conclusions of Sims and Albrecht (1993) that concentrations of KOH or NaOH higher than 5% inevitably lead to saponification and thus, prolong or even hinder maceration, are the results of experiments with fatty samples (0.3 grams of beef suet, chicken liver, bovine tracheal rings). In our experiments, we had to macerate a few layers of endothelial cells (only those, which are not fatty); in these samples, therefore, saponification is very unlikely to occur even with higher concentrations of KOH (see also Fig. 2b in Sims and Albrecht, 1993).

M.A. Konerding: Is there, in your mind, still a field where the vessels should be left unfixed prior to casting?
P.A. Sims: Given your results, do you recommend casting **only** fixed tissue? Is there an instance where you would not cast fixed tissue?
Authors: Fixation results in the loss of the elasticity of the vessel wall and increases the vascular resistance. Loss of elasticity makes the vessel prone to wall rupture, an increase in resistance impairs resin inflow and thus the degree of filling. Fixed fetal or larval vessels with a very delicate wall, but a large diameter, will be most prone to rupture when cast with a slightly elevated injection pressure. Future experiments have to show if casting of the larval or fetal vascular bed is more successful when prefixation is omitted.

S. Aharinejad: You state that endothelial cell imprints are found more often on the surface of cast large vessels because "Generally, the thicker wall of larger vessels will act as confinement and keeps the EC nuclei in place". We have found (Aharinejad and Böck, 1993a,b, 1994; Aharinejad *et al.*, 1993b) that Mercox penetrates into endothelial cells, no matter how thick the vascular wall is. Beside, both our data and those presented in your study show that Mercox destroys part of the endothelial lining, whereas other cellular components remain relatively unchanged. Is this probably due to the distance of these components from the endothelial lining?
Authors: Figures 4c and 5c show that there is actually a gradient of destruction of cellular components, whereby luminal areas, which face Mercox, are more severely destroyed than components in the abluminal areas. One might speculate that, as you have suggested previously (Aharinejad and Böck, 1993), a phase separation occurs within the Mercox and that a noxious phase, which remains confined to the luminal areas, causes the destruction of structures in these areas. On the other hand, as you have previously demonstrated (Aharinejad and Böck, 1993), Mercox infiltrates the luminal areas of endothelial cells and polymerizes (mummifies). One might therefore speculate that it is the heat (generated by the polymerization of Mercox within these luminal areas) which accounts for the destruction. The fact that heat decreases with the square of the distance could explain the great differences in structural destruction within a small distance. Until further experiments have analyzed precisely the impact of these factors, we can only describe the effects but their explanation(s) remain purely speculative.

S. Aharinejad: In your micrographs of cast endothelial cells, both fixed and unfixed, there is a gap between the cellular layer surface and Mercox. What do you think causes this gap?
Authors: For TEM studies, we used filter membranes which were held in position by a plastic holding device. For embedding the membrane-filter grown endothelial cells into Epon 812, the membrane had to be removed from the holder. Although this was done very carefully, some mechanical forces onto the specimen obviously could not be avoided and may have led to the observed gap between luminal surface of the endothelial cells and the overlying Mercox.

S. Aharinejad: Your Figure 4b shows that Mercox has a fine granular structure once it is diluted with MMA, while large drops of Mercox are seen when the resin is not diluted with MMA (Fig. 4c). How do you explain this?
Authors: Figure 4b shows polymerized (diluted) Mercox on prefixed endothelial cells with no destruction of cellular components. Figure 4c shows (undiluted) Mer-

cox polymerized on unfixed endothelial cells with heavy destructions of cellular components in the upper cell layers. From our experiments, we cannot conclude whether it is the different substrates (unfixed versus fixed endothelial cells) or the dilution with MMA which caused the differences in the internal Mercox structure (fine granules vs large drops) seen in Figures 4b and 4c.

S. Aharinejad: How do you know that the extravasation of Mercox in unfixed tadpole capillaries observed in intravital video microscopy is due to a "focal chemical destruction" and not (e.g.) due to (even) minimal contraction of endothelial cells and opening of intercellular gaps?
Authors: We cannot rule out that some of the extravasations are caused by opening of interendothelial junctions. However, in unfixed cast capillaries of the tadpole tail fin, we found many tiny extravasations scattered over an area of the size of an endothelial cell (unpublished observations). If interendothelial junctions were the preferred sites for extravasations, there should be an alignment of these structures reminiscent of endothelial cell borders.

R. Christofferson: You have shown that prefixation preserved the EC integrity even at the ultrastructural level after casting. Unfortunately, formaldehyde and glutaraldehyde are vasospastic (activate arterial sphincteric structures), and, in my experience, hence impair the completeness of vascular casts. Do you know how to overrule the vasospastic effects of fixatives.
Authors: No, we do not know. As long as we do not know positively how Mercox activates sphincters (via receptors?, chemical activation?, osmotic effects?) and how the shortening of the sphincter (contraction, swelling with subsequent shortening of the sphincter) leading to a narrowing of the vessel lumen is caused, we have to face vasospastic effects when fixation is done prior to casting.

R.Christofferson: Would it be a good idea to investigate the effect of microwave treatment on EC integrity? The animal (e.g., a tadpole) would then be anesthetized, cannulated, killed, immediately put in a water bath and microwave treated, and then subjected to vascular casting. Microwave treatment and casting of cultured cells would be simpler.
Authors: Your idea is great, particularly as experiments with cultured ECs are concerned! Provided that technical difficulties (e.g., safe placement of a small, cannulated animal into a water bath with the cannula securely held in place) are overcome, microwave treatment could be done right after blood washout. Moreover, fixation by microwave might not generate vasospasms as does chemical fixation with aldehydes. Murakami *et al.* (A modified method for vascular casting and scanning electron microscopy: Its utility in the demonstration of rat pancreatic insulo-acinar and other blood vascular systems. Arch. Histol. Cytol. **55**: 233-238, 1992) placed specimens (adult rats) injected with monomeric methyl methacrylate (supplemented with 1.0% benzoyl peroxide and 1.0% N,N-dimethylaniline) into a water bath and heated them with a microwave processor (2,000-2,200 Mhz and 500-550 W) to 60°C. This enabled them to polymerize the injection medium within 10 minutes. At room temperature (20°C-22°C), the medium needs 90 minutes or more to polymerize completely!

R. Christofferson: The exact composition of Mercox is not revealed by the manufacturer. Which agent do you think is responsible for the severe cellular damage observed. Would it be the plasticizer, the stabilizer, the pigment, or the heat generated during gelling (Mercox gels quickly, MMA slowly)?
R.M. Albrecht: Components, for example, the inhibitor, are found in Mercox and not in methylmethacrylate. Do you have any ideas about what it is in the Mercox that is causing the damage?
Authors: We observed severe destruction of unfixed ECs by clear, unpigmented, undiluted Mercox without catalyst MA (Fig. 4d). On the other hand, unfixed ECs revealed little destruction if cast with MMA with catalyst MA (Fig. 4f). Heat, pigment, or catalyst MA alone therefore cannot account for the destruction. We assume that unspecified compounds of Mercox (chemical compounds other than hydroxy-methylmethacrylate) and the physico-chemical properties of Mercox (e.g., hydrophilic, hydrophobic or lipophilic properties; osmotic properties) resulting from its composition are responsible for the observed severe cellular destructions. Future experiments with chemically well defined methylmethacrylate-mixtures (hydroxy-ethyl-; hydroxy-propyl-; hydroxy-methylmethacrylates) should enable us to pinpoint the destructive agents or processes.

R. Christofferson: Could the preservative effect of MMA be explained by a fixative action?
Authors: We do not know.

R. Christofferson: Perfusion and, indeed, vascular casting of small tadpoles with soft and jelly tissues is truly remarkable! I have tried to cast fetal mice through the heart, and I am fully aware of the immense technical difficulties. Could you please give me some practical hints on how you expose vessels, use the micromanipulator, what cannulas you use, etc. You infused the resin at 5-8 ml per hour, what is the "systemic arch output"

in a tadpole? How many tadpoles did you try to perfuse/cast in order to obtain the six used in this study?
Authors: The deeply anesthetized tadpole is placed in a supine position onto a wax-plate. Then, the thin epidermis overlying the systemic arch is incised with microscissors under the dissecting microscope (magnification: x20-x40) and two ligatures (surgical suture material 10-0 or 11-0) are placed 1-2 mm from each other around the systemic arch, and tied. Then the systemic arch between the two ligatures is ripped open along its long axis with a fine, sharpened insect needle (size:00). Thereafter the self-made thin-walled glass cannula (tip diameter: 100-200 μm) is introduced with a mechanical micromanipulator under microscopic control through the opening into the vessel and pushed forward close to the distal ligature. This ligature then is opened (for further details see Aharinejad and Lametschwandtner, 1992, pp 28-31), the cannula is put forward beyond the ligature which then is closed. This approach results in minimal bleeding, the opened vessel can be seen, and enables the insertion of the cannula even into very delicate vessels. Our glass cannulas are made from glass used by electrophysiologists for pulling their electrodes for intracellular recordings, patch clamp or voltage clamp experiments.

We do not know the blood flow (ml/minute) which passes one systemic arch in the tadpoles used (the tadpole has two systemic arches). The flow rate of 5-8 ml per hour for the injection of Mercox was chosen because pilot experiments showed that this flow re-established the systemic arch diameter to control levels.

Because we have great practical experience with micro-dissections, perfusions, and casting, casting of the tadpole vascular system is no more a problem to us.

R. Christofferson: I have looked through your transmission electron micrographs. All of them appear to be mounts, with zigzag lines across the surface. The cell membranes cannot always be resolved. Why did you not use 2.5% glutaraldehyde? Did you really use cacodylate as buffer? Why have you not used single micrographs?

Authors: Pilot experiments have shown that tissue preservation in the tadpole is best with buffered 2% glutaraldehyde (0.15 M sodium cacodylate). We did not use single micrographs because we were particularly interested in the structure of the endothelium **and** the Mercox within the lumen of the vessel.

P.A. Sims: Have you considered any alternatives to using Mercox like partially polymerized methylmethacrylate?
Authors: Yes, we have considered the use of partially prepolymerized methylmethacrylate (e.g., Gannon BJ Preparation of microvascular corrosion casting media: procedure for partial polymerization of methyl methacrylate using ultra violet light. Biomed. Res. **2**: 227-233, 1981) in those casting experiments where the preservation of the integrity of the endothelial lining is in the center of the study rather than the three-dimensional arrangement of the cast vessels.

R.M. Albrecht: Some previous studies (e.g., Christofersson, 1988) show relatively well preserved vascular endothelium even when using Mercox on in unfixed vessels. Do you have any comments on how the length of time in contact with the unhardened Mercox (i.e., time from initial contact to polymerization) and temperature during the polymerization stage may be affecting the structure of the Mercox exposed vessels or cells?
Authors: Phase contrast microscopy has shown that within seconds after exposure to diluted and undiluted Mercox (with or without catalyst MA), endothelial cells were destroyed. Because Mercox without catalyst MA does not polymerize, the destruction of the endothelial cells under this experimental condition cannot be caused by the heat of polymerization. To our knowledge, there are no controlled experiments done so far, which clearly enable to separate the effects exerted by the physicochemical properties of the resins used for vascular casting upon the vessel wall and their components from the effects which are due to the heat generated by the polymerization.