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CASTING WITH MERCOX-METHYLMETHACRYLIC ACID MIXTURES CAUSES PLASTIC SHEETS ON ELASTIC ARTERIES. A SCANNING AND TRANSMISSION ELECTRON MICROSCOPIC STUDY

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Abstract

Lungs and systemic vessels of rats were cast with Mercox, both undiluted and diluted with monomeric methylmethycrylate (MMA; v:v/4:1), and studied by scanning electron microscopy (SEM). A sheet, less than 5 μm thick, surrounded the surface of cast pulmonary arteries and the aorta, when using diluted Mercox. This envelope was absent when casts were prepared with undiluted Mercox. Transmission electron microscopy (TEM) of unmacerated cast arteries showed all constituents of the vascular wall. Sections of thoroughly macerated vascular casts, however, still showed elastic lamellae in the vascular wall region, whereas muscular components and endothelial lining were completely digested. It is suggested that dilution of Mercox with MMA leads to phase separation during polymerization. An unidentified compound is released which penetrates the vascular wall, and makes preferentially the elastic lamellae resistant against tissue digestion. This assumption is supported by occurrence of such covering sheaths exclusively around elastic arteries.

Key Words: Scanning electron microscopy, transmission electron microscopy, corrosion casting, elastin, artifacts.

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Introduction

Methylmethacrylate compounds have been successfully used as casting media for scanning electron microscopy (SEM) of microvascular corrosion casts since the pioneering work of Murakami (1971). Methylmethacrylate enables us to cast the entire vascular bed including capillaries; observation of such casts in SEM provides a three-dimensional view of microvascular domains. Although soft tissue is corroded away, various adhering structures may be occasionally observed on the surface of cast preparations. Such structures may be extravasations of casting material or remnants of soft tissue due to incomplete maceration (Rosenbauer and Kegel, 1978). Moreover, it has been shown that endothelial cells can be infiltrated by methylmethacrylate compounds, resulting in mummified cells which resist maceration (Aharinejad and Böck, 1993).

When studying pulmonary corrosion casts prepared with Mercox diluted with methylmethacrylate, we observed wide-spread laminar structures around differently sized arteries, which often covered the entire cast surface. This constantly occurring phenomenon has not been described as yet. Hence, it was a challenge to examine the precise morphology of these lamellar structures. For this purpose, we combined SEM of vascular corrosion casts with transmission electron microscopy (TEM) of tissue sections.

Material and Methods

Scanning electron microscopy

Thirty Sprague Dawley rats of both sexes (weighing 200-220 grams) were used. The animals were anesthetized with sodium pentobarbital (intraperitoneally, 40 mg/kg body weight), the abdomen and thorax were opened by a median cut, and the thoracic and abdominal aorta, as well as the caudal vena cava were cannulated with a plastic catheter (Argyle 0.8 x 19 mm, Sherwood Medical, St. Louis, MO, USA). The cannulas were connected to a two-way connector (LS2, B. Braun-Melsungen, Germany), and the systemic (via the aortic arch) and pulmonary circulatory system (via the caudal vena

cava) were rinsed with 42°C heparinized Tyrode solution (5,000 IU/l) and/or physiologic saline solution mixed with dextran (Macrodex; v:v/4:1), until the efflux of the incised caudal vena cava at the level of renal arteries (as the systemic circulatory system was perfused) or the efflux of the abdominal aorta incised at the level of renal veins (as the lungs were perfused), was clear. Thereafter, we either injected Mercox (Dainippon Ink & Chemicals, Tokyo, Japan) diluted (v:v/4:1; final volume 20 ml; Hodde, 1981) with monomeric methylmethacrylic acid (0.75 g catalyst was mixed with 20 ml methylmethacrylic acid (MMA), i.e., 0.075 g catalyst in 10 ml Mercox/MMA mixture); or we injected Mercox mixed with catalyst (1 g catalyst, 55% benzoyl peroxide, per 10 ml Mercox; Ladd Research Industries, Burlington, VT), through the aortic arch or the caudal vena cava (Aharinejad *et al.*, 1993).

Cast specimens were left at room temperature for 2 hours, then put into a 60°C water bath overnight. Lungs and the aortae were removed and macerated in a 5% KOH solution at 40°C for 12 hours, before the maceration solution was renewed and maceration was allowed to continue for another 2 days. Afterwards, the specimens were repeatedly rinsed in a beaker containing tap water. The specimens were then cleaned in 5% formic acid for 30 minutes at room temperature and in several passages of distilled water, each for 2 minutes. Casts were frozen in a small volume of distilled water and then freeze-dried. The specimens were coated with evaporated carbon for 3 seconds, and then sputter-coated with gold for 600 seconds (Aharinejad *et al.*, 1989, 1990). The cast preparations were mounted onto copper foils with silver paste using the conductive bridge method of Lametschwandtner *et al.* (1980). Most of the lung cast preparations were frozen in distilled water and cut at -20°C with a specially adapted circular saw into 1-2 mm thick slices (Aharinejad *et al.*, 1991; Aharinejad and Lametschwandtner, 1992) and prepared for SEM as described above. Casts were observed in a Cambridge 90 M SEM operated at an accelerating voltage of 10 kV and working distance of 60 mm.

Transmission Electron Microscopy

After injecting Mercox and polymerization of the resin, small blocks were obtained from the lungs and the aortae. These specimens were immersion fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH = 7.4, for 2-4 hours, washed in the same buffer and postfixed in 1% osmium tetroxide for 4 hours. Dehydration and embedding in Spurr's medium was performed in a series of ethanol solutions with graded concentration of ethanol. In addition, small pieces of macerated cast preparations were immersed in 1% osmium tetroxide for 4 hours and embedded in Spurr's low viscosity medium as described above.

Sections were cut on a Reichert OmU2 ultramicrotome. Sections were stained with alkaline toluidine blue O for light microscopy, and with uranyl acetate and lead citrate for TEM. Specimens were observed with a Zeiss EM 10 TEM.

Figure 1. Vascular corrosion cast of a pulmonary artery made by MMA-diluted Mercox. Note the delicate enveloping sheath which extends toward the branches. This sheath is detached from the luminal cast surface (arrows) and is fractured in circumscribed areas (arrowhead).

Figure 2. Corrosion cast of a pulmonary artery made by MMA-diluted Mercox. Where the covering sheet is removed, the characteristic endothelial cell nuclear reliefs are exposed. Micrograph shows that this envelop is not firmly attached to the cast surface.

Figure 3. Higher magnification of part of the vascular cast shown in Figure 2. Surface of the periarterial sheet might be either smooth or it might show reticular and porous substructure.

Figure 4. Cast aorta prepared with MMA-diluted Mercox. The enveloping layer is ruptured into two pieces exposing the underlying luminal cast surface. Cast vasa vasorum of the aortic wall (arrows) are seen outside the covering layer, indicating the perivascular sheet as being located in the vascular wall region.

Figure 5. Cast aorta prepared with MMA-diluted Mercox, isolated and fractured. Note that the enveloping layer entirely surrounds the cast vessel but it is separated from the cast surface by a gap of varying width. The fractured surface shows fine granular substructure.

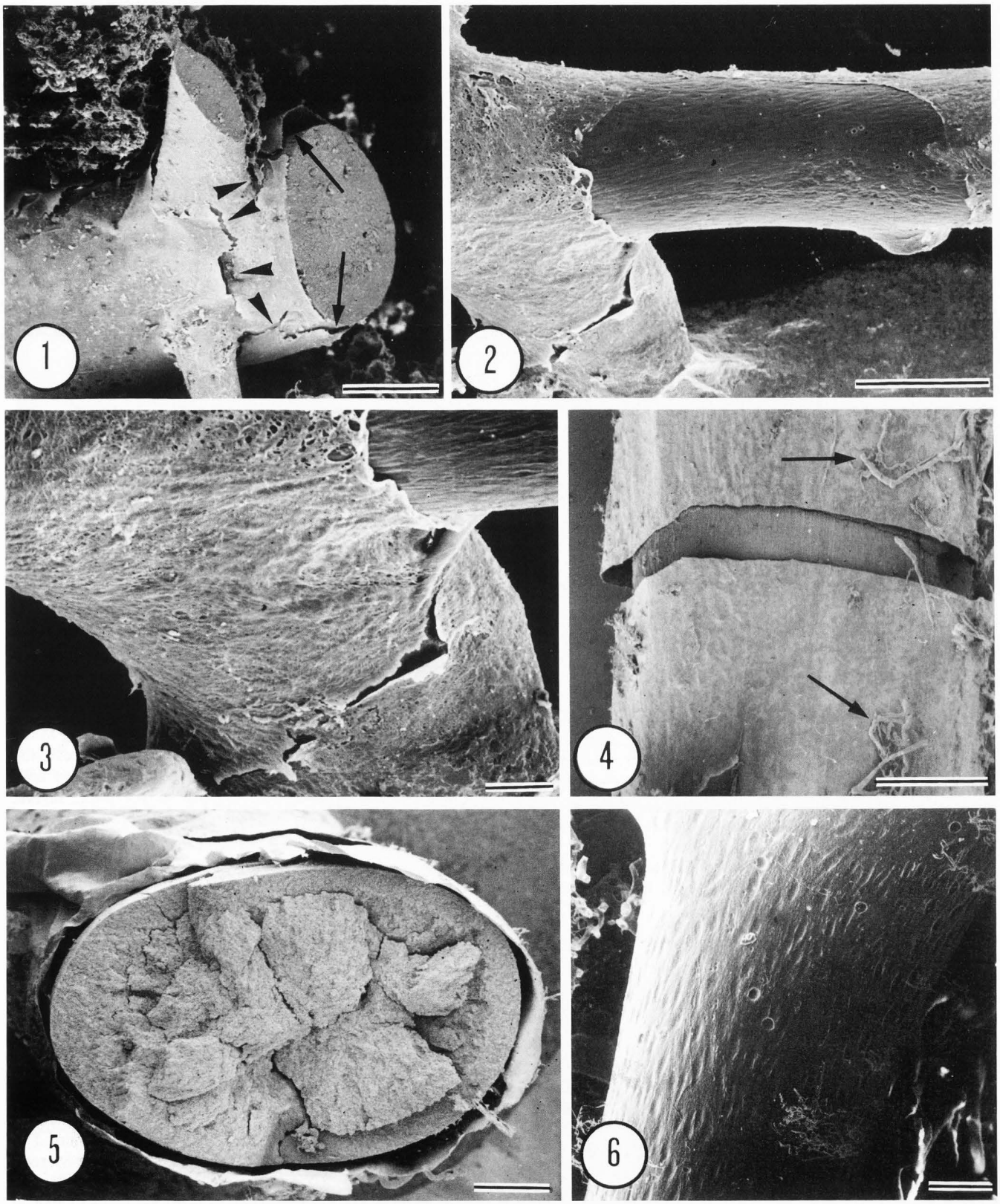
Figure 6. Cast pulmonary artery prepared with undiluted Mercox. Note the absence of an adhering lamellar layer. The endothelial cell nuclear imprints are replicated.

Results

Scanning Electron Microscopy

Casting with diluted Mercox Pulmonary corrosion casts showed all anatomical structures to be expected (Schraufnagel, 1990). However, in circumscribed areas, on the surface of pulmonary arteries of varying size, a tiny lamellar cover was seen. This sheath often enveloped the entire circumference of such arteries together with their branches (Fig. 1). These lamellar layers were sometimes fractured and detached from the underlying luminal cast surface (Fig. 1). The outer surface of these layers lacked any characteristic surface reliefs. In particular, endothelial cell nuclear imprints or endothelial cell border lines were missing. Occasionally, the covering sheet was accidentally discontinuous, exposing the underlining cast surface and its characteristic endothelial reliefs (Fig. 2). Where the cast surface was not covered by the described unidentified lamella, the axially oriented endothelial cell nuclear imprints and occasionally reliefs of trapped erythrocytes were observed (Fig. 2). Higher magnification of the covering sheets revealed a reticular relief which blended into porous areas near to the edge of the enveloping layer (Fig. 3).

The same phenomenon was regularly observed on the surface of aortae and their main branches. In addition, cast vasa vasorum were present atop the sheets but were never observed underneath (Fig. 4). As the



Bars = 500 μm (Fig. 1); 200 μm (Fig. 2); 50 μm (Fig. 3); 500 μm (Fig. 4); 250 μm (Fig. 5); and 100 μm (Fig. 6).

envelope's fracture lines ran circumferentially in circumscribed areas (Fig. 4), our assumption that the enveloping sheets embraced the entire circumference of casts, was supported. Observation of cross-sectioned isolated aortae proved this hypothesis (Fig. 5). It also showed the solid cast vascular lumen to be completely separated from the overlying sheet by a gap of varying width. The thickness of the enveloping layer was less than 5 μm. The interior of polymerized casting material showed a delicate granular appearance.

Casting with undiluted Mercox All cast preparations prepared with undiluted Mercox, both pulmonary and aortic casts, were devoid of overlying lamellar structures, therefore, they presented the almost perfectly replicated intimal surface details (Fig. 6).

Transmission electron microscopy

Cross-section of a rat aorta, fixed and embedded after casting with diluted Mercox and polymerization of the resin but prior to maceration, is shown in Figure 7. Elastic lamellae and smooth muscle cells are readily discerned, as well as the inner elastic lamina and adhering endothelial cell layer. The casting material itself was rather electron translucent and was only identified at higher magnification. The endothelial lining directly attached to the casting material (Fig. 8).

The situation was totally different as the sectioned cast specimens were viewed after maceration:

a. Elastic lamellae appeared condensed and their electron density was significantly increased;

b. Smooth muscle cells were no more present but holes within the aortic wall suggested their localization;

c. The thickness of the entire aortic wall region was shrunk;

d. The thickness of the inner elastic lamella, however, was rather unchanged, condensation of the inner elastic lamella was limited to its inner and outer surface;

e. The endothelial lining totally disappeared due to maceration, being indicated by a gap atop the casting material; and

f. The casting material showed increased electron density and its granular substructure became apparent (Fig. 9).

In the region of the aortic wall, an unidentified fine granular material was interspersed between elastic lamellae (Fig. 10).

Discussion

Mixtures of Mercox and monomeric methylmethacrylic acid (MMA) are widely used for microvascular corrosion casting studies (Aharinejad and Lametschwandtner, 1992). This method has been repeatedly modified by varying amounts of MMA to be mixed with Mercox (Lametschwandtner *et al.*, 1990; Aharinejad and Lametschwandtner, 1992). Application of these recipes results in adaptation of resin viscosity according to particular needs, allowing complete perfusion of various microvascular beds. We also used a mixture of Mercox and MMA (v:v/4:1; Hodde, 1981) in our studies on pulmonary microcirculation and observed a thin layer of indigestible material on large pulmonary arteries. As pulmonary arteries are of the elastic type and as it is known that maceration of elastin is problematic, we extended maceration time but this attempt was unproductive. In order to examine the hypothesis that most probably elastin causes this phenomenon, we studied aortic casts and found the same situation. The occurrence of vasa vasorum attached to the outer surface of the adhering layer indicates this layer to be located in the tunica media. Our previous studies (Aharinejad and Böck, 1993) have shown that phase separation occurs during polymerization of MMA-diluted Mercox resulting in mummification of vascular wall components. Therefore, we examined casts prepared with undiluted Mercox. Cast pulmonary arteries and aortae were found to be devoid of the enveloping sheets described above. Consequently, we conclude that MMA in the casting medium is responsible for the artifacts observed.

In order to analyze the structural details of these perivascular layers, we studied tissue sections of cast material, both before and after maceration. We found that smooth muscle cells and endothelial cells are completely digested away after maceration but elastic material persisted. The perivascular sheet, as observed in TEM, represents only part of the vascular wall with no cellular elements inside. The gap between luminal cast and overlying sheet is obviously due to digestion of the endothelial layer.

Applying identical maceration procedure to casts prepared with either diluted or undiluted Mercox, digests the entire aortic wall when using pure Mercox, while elastin persists and only cellular elements are macerated when using MMA-diluted Mercox. In conclusion, MMA may have altered the nature of vascular elastin resulting in higher maceration resistance. As mummified cells were not found in/or near to the perivascular layers, MMA has apparently a particular affinity to elastic fibers and lamellae. This assumption is supported by the higher frequency of perivascular sheaths in elastic arteries (this study). It is further supported by the occurrence of elastin remnants in the vocal cord as seen in laryngeal casts prepared with MMA-Mercox mixtures (our unpublished data).

Another factor which might contribute to the occurrence of the described periarterial sheet is the concentration of catalyst used. When using diluted Mercox, the final concentration of catalyst is 0.075 g per 10 ml Mercox/MMA mixture. But when using undiluted Mercox, the concentration of catalyst is 1 g per 10 ml Mercox. Hence, the concentration of catalyst in the former protocol is far less than in the latter. Although the curing of the resin seems to occur in an identical period of time when both protocols are used [for diluted Mercox about 5 minutes (Lametschwandtner *et al.*, 1990); for undiluted Mercox about 4 to 6 minutes (Aharinejad *et al.*, 1993)], the possibility cannot be excluded that monomers, which have been suggested to infiltrate vascular

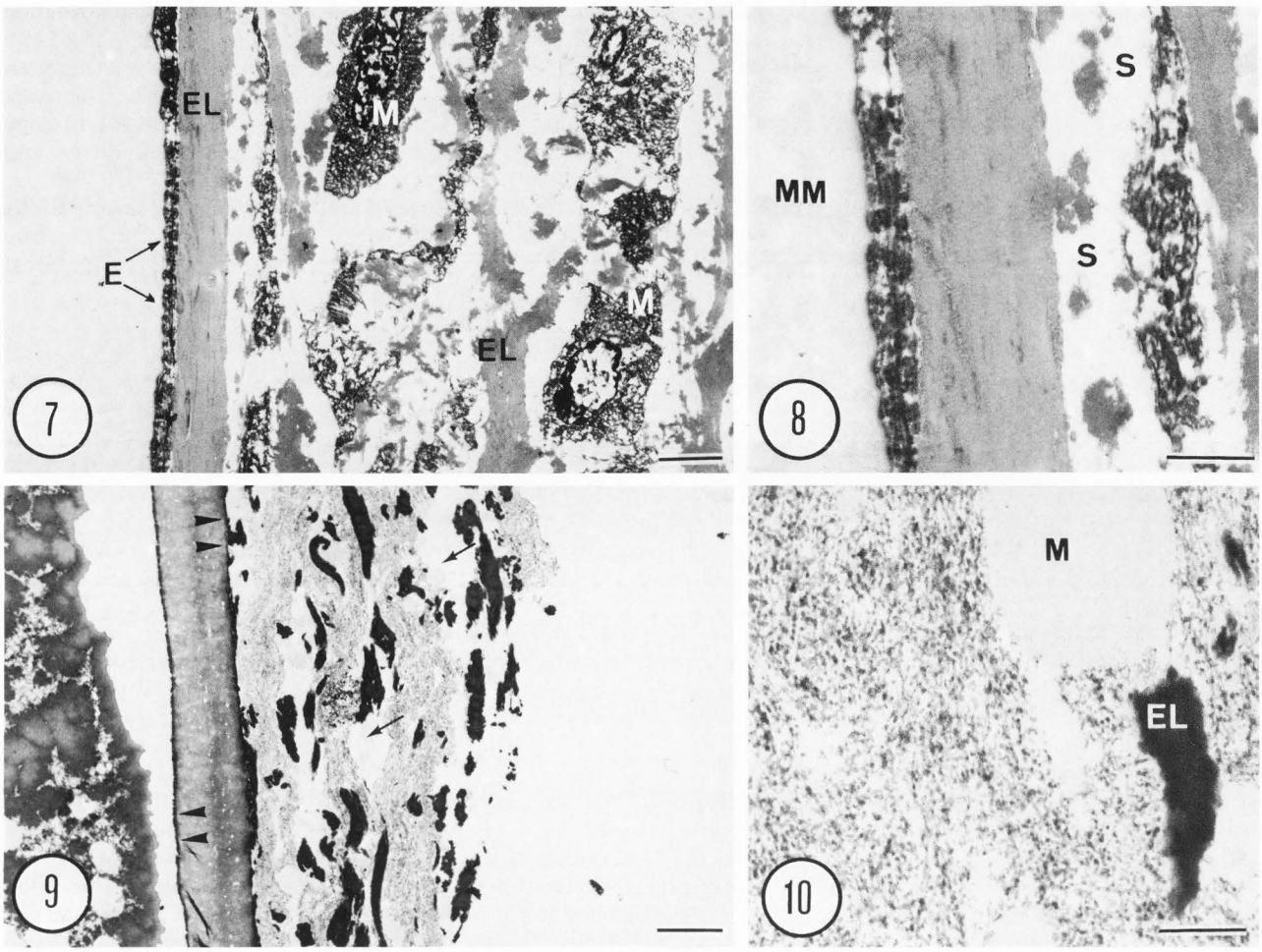


Figure 7. Rat aorta cast with MMA-diluted Mercox, fixed in glutaraldehyde/osmium tetroxide, and embedded after polymerization of the casting medium. Vascular wall components can be identified: Smooth muscle cells (M), elastic laminae (EL), and endothelium (E). The casting medium inside the lumen is not identified at this magnification (confer. Fig. 8). Bar = 2 μm.

Figure 8. Higher magnification of Figure 7. In the luminal region, dispersed polymerized casting material is now visible as droplets of low electron density. However, electron density of casting material (MMA-Mercox, MM) is slightly higher than that of the embedding medium (Spurr, S) seen in the vascular wall between its cellular and elastic elements. Bar = 1 μm.

Figure 9. Rat aorta cast with MMA-diluted Mercox, macerated, osmium tetroxide fixed, and embedded. The wall thickness is shrunk due to maceration; cellular elements disappeared, the electron density of elastin lamellae and of casting medium is increased. Note that the inner elastic lamella shows increased electron density only on its luminal and abluminal surface (arrowheads). Holes in the vascular wall region (arrows) correspond to digested smooth muscle cells, the gap between the inner elastic lamina and casting material corresponds to digested endothelial lining. Consequently, the material atop the casting medium corresponds to the enveloping layer seen on the surface of corrosion casts. Bar = 2 μm.

Figure 10. Higher magnification of Figure 9. In the region of the digested aortic wall, unidentified particles remain homogeneously intermingled with persistent elastin fibers (EL) and holes caused by digested smooth muscle cells (M). Bar = 1 μm.

walls, are still available. This phenomenon would be potentially important when using less catalyst and adding additional MMA and deserves further examination.

Following conclusions might be drawn from this study. Dilution of Mercox with MMA components may cause a thin perivascular sheet. When casts without such artifacts are desired, dilution of Mercox should be omitted. However, as dilution of Mercox is sometimes necessary, e.g., in casting delicate cavities (tracheal system of insects; Meyer, 1989), casting embryonic material (Ditrich and Lametschwandtner, 1992; Lametschwandtner, 1990; Aharinejad and Lametschwandtner, 1992), such artifacts should be anticipated.

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Discussion with Reviewers

Y. Ohta: What color does this unidentified sheet have? We have seen a similar, unidentified material in our specimens when using a viscous, colloidal, oligomeric methylmethacrylate compound prepared from MMA according to our protocols [Ohta Y, Okuda H, Suwa F, Okada S, Toda I (1990) Plastic injection method for preparing microvascular corrosion casts for SEM and its practical application. Okajimas Folia Anat. Jpn. **66**, 301-312). Moreover, a similar phenomenon was seen in following cases: **a**) when diluting higher viscous casting resin with MMA; **b**) when using low viscosity oligomer; **c**) when using low temperature during maceration (below 13°C); and **d**) when using undiluted Mercox.

Authors: The color of the sheet observed under the dissection microscope in our cast preparations was gray with a shade of silver. Points **a**) and **b**) just substantiate our hypothesis that MMA is responsible for causing periarterial laminar sheets. Concerning point **c**), we think that low maceration temperature reduces maceration potency, requiring elongation of maceration time. As to point **d**), we never noted perivascular sheets when using undiluted Mercox.

Y. Ohta: Comparing the space in Figure 9 with that in Figure 5, why is the space width of the former narrower than that of the latter?

Authors: The sheet around corrosion casts is not firmly attached to the cast surface, as the interposed endothelial lining is digested away. As both figures show, the gap between perivascular sheet and cast surface varies. Hence, the width of the observed gap in TEM depends on the incidentally selected area of observation.

Y. Ohta: The authors report that this sheet is formed on vessels of 0.2-0.5 mm in diameter. Was this phenomenon also observed on arterioles, venules and capillaries?

Authors: No. Obviously, a larger amount of elastin than that in small arteries or veins is required to produce sheets which can withstand maceration.

A. Miodonski: The relative proportion of elastin and collagen fibers and matrix fibers can change along the vessels course as they run toward the periphery. Elastic bundles or lamellae and collagen fibers can be focally interrupted in form of small clefts. Do you think that

the occurrence of the described artifacts could follow these features in the same manner within proximal, mid, and peripheral segments of arteries of elastic type?
Authors: This hypothesis might be correct. However, as periarterial layers may not represent the entire elastic material of arterial wall (see above), discontinuity of the periarterial sheet not necessarily corresponds to discontinuity of elastic lamellae.

D.E. Schraufnagel: I wonder if these spaces are casts of tissue plans that could be connected with pulmonary lymphatics and, therefore, might be called prelymphatics. I have found sheets on the pleural surface of the lung that have a characteristic appearance [Schraufnagel DE (1992) Forms of lung lymphatics: a scanning electron microscopic study of casts. Anat. Rec. **233**: 547-554]. Did you find any lymphatics arising from these structures?
Authors: No, we did not observe any lymphatic channels arising from these sheets; the cast surfaces did not reveal any surface relief characteristic for initial lymphatics.

R.M. Albrecht: We routinely use a 1:1 mixture of MMA and Mercox for the casting of large elastin-containing vessels. Using the same maceration procedure you describe, we have not observed these "enveloping sheaths" around the vessels. Have you tried any other mixtures of MMA and Mercox to see if the same phenomenon occurs?
Authors: We have been using the 4:1 mixture of Mercox and MMA according to Hodde (1981). Undoubtedly, a 1:1 mixture of the mentioned components would affect the polymerization of the resin. We have no experience with other mixtures (e.g., such as yours) concerning the occurrence of this enveloping sheet. But considering your experience with 1:1 mixture, we may assume that the concentration of catalyst would significantly affect the occurrence of this sheath. This topic requires further investigation.