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PRESERVATION OF IN VIVO MORPHOLOGY OF BLOOD VESSELS FOR MORPHOMETRIC STUDIES

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Abstract

Preservation of in vivo morphology of the blood vessels is important for morphometric studies. With each fixation method (e.g., immersion fixation, perfusion fixation), there are inherent pitfalls which can easily distort vessel wall morphology, thereby creating artifacts. Alteration of the vessel wall cell volume due to tissue preparation is another common source for errors. Selection of proper morphometric protocols for comparative studies is crucial. Lack of attention in these areas is a cause of confusion and contradiction in the literature. In this review, the merits of various commonly used methods for vessel wall preparation and measurements are discussed.

KEY WORDS: Blood vessels, morphometry, smooth muscle, perfusion fixation.

Introduction

For a better understanding of the role of blood vessels in health and disease (e.g., hypertension), various morphometric methods have been used. However, the reliability of such morphometric studies depends not only on the morphometric protocols, but also on the methods used for the preparation of the blood vessels. The effect of the preparation method for microscopy on cell volume is one of the many factors which will distort the in vivo morphology of the blood vessels. It is well established that different fixation, dehydration and embedding procedures have different effects on the cell volume, in that a drastic volume reduction can occur in some procedures, especially when tissues are prepared for scanning electron microscopy [17]. Other factors include: method of fixation of the arteries (e.g., immersion versus perfusion fixation); physiological states (either relaxed or contracted) of the vessels at the time of fixation; and sampling of the arteries. Selection of parameters to be measured (e.g., cross-sectional areas versus wall thickness), and the morphometric protocols used (e.g., histometric method) are other compounding factors. Lack of attention on these issues is often the source of conflicting reports among different morphometric studies. In this review, the merits of various specimen preparation methods, and morphometric protocols for the study of blood vessel wall dimensions are discussed.

Specimen Preparation Methods

Immersion Fixation

This is the least satisfactory method for the study of vessel wall dimensions, because vessels prepared this way are either collapsed, compressed or contracted. The degree of collapse or contraction will influence measurements on the size of the lumen, and on the thickness of the vessel wall. This seems inevitable, especially in studies involving human vessels, as preparation of the vessels can

only take place at necropsy. To overcome this problem, some authors used a gelatin mixture to expand the vessel wall before fixation. For example, Short and Thomson [26] used a bismuth oxychloride-gelatin suspension at 80°C and injected this into the mesenteric arteries from humans at a pressure of 150 or 200 mm Hg. Russell [25] used a fine suspension of barium sulphate in gelatin instead, and injected it into the surface vessels of the brain at a pressure of 90-100 mm Hg at 55°C. In both instances, after hardening of the gelatin, the vessels were fixed by immersion in formalin, and later used for radiographic and light microscopic measurements and studies. In view of the need for using high temperature to maintain the fluidity of the gelatin, it is doubtful that such preparation is suitable for ultrastructural studies Leakage of the mixture from the arteries is another problem with this technique [25]. Another alternative is to fix the arteries in situ with the blood still inside by superfusion. In the mesenteric arteries of rats, the elegant studies of Rhodin [23] involving intravital and electron microscopic analyses have shown that during superfusion of glutaraldehye (GA), there was a 20-50 sec delay before the blood flow came to a complete stop. Ultrastructure of some endothelial cells was less well preserved, and the appearance of the vascular wall was slightly altered.

Perfusion Fixation

Vascular perfusion fixation is considered the method of choice, because it provides rapid and uniform exposure of the tissue to the fixatives, and preserves the natural spatial relations of various tissue components [23,29]. Ideally, a suitable procedure involving fixatives with different osmolarities and composition should be developed for each animal species. In practice, most researchers paid more attention to perfusion pressure than to the effects of the fixatives on the cell volume. Other factors, such as viscosity and colloid osmotic pressure of the fixatives; and the physiological states of the vessels (i.e., contracted or relaxed) at the time of fixation, can also have profound effects on vessel wall dimensions, as outlined below.

Perfusion Pressure. Many authors are of the opinion that in order to preserve the in vivo dimensions of the blood vessels, it is desirable to perfusion-fix the vessels at physiological pressure. Thus, in rats [e.g., 30] and rabbits [e.g., 24,32], a perfusion pressure of 100 mm Hg (in rabbits) or higher (in rats) had been used, despite the fact that the normal blood pressure of the rabbits is usually about 80 mm Hg. Viscosity of GA fixative is usually below 1 cP at 37°C [29], as compared with 2.5 cP in the whole blood [7]. Consequently, a flow rate three times or higher than normal blood flow is needed to generate the in vivo pressure. In the rabbits, the flow rate varied between 90 and 200 ml/min between preparations in order to generate a pressure of 30 mm Hg in solutions containing 2.5% dextran [29]. At higher pressure, a marked edema developed within a few minutes [29]. In the mesenteric vascular beds of rats, we have found that at a flow rate of 7.6 ml/min, which generated only a perfusion pressure of 37 mm Hg, the lumina of the large and small muscular arteries were over-expanded [16]. A low flow rate (1 ml/min/100 g body weight) and a low perfusion pressure (approximately 16-24 mm Hg) are preferred because lumina of these arteries are not affected. High perfusion pressure also causes vascular and perivascular edema, which develops rapidly, being faster with higher pressure (unpublished data).

Preperfusion with Electrolyte Solutions before Fixation. When GA is perfused into the vasculature without prior clearance of the blood with buffers or electrolyte solutions, contraction of the arteries usually occurs. The mechanism through which GA causes contraction of the smooth muscle cells is unclear. In the large elastic and muscular arteries of rats, contraction of the smooth muscle cells still occurred despite the use of in vivo pressure, as indicated by the irregular shape of the cell outlines, wavy cell membrane, and crenated nuclei [30]. On the other hand, perfusion of the microvessels from the mesenteric bed of rats with GA without prior clearance of blood did not alter the outer diameter of any segment of the microvascular bed [23], and the ultrastructure of these vessels showed that the smooth muscle cells were not contracted. The use of a high perfusion pressure (80 mm Hg) might have prevented the contraction of these microvessels. It would therefore appear that with high perfusion pressure contraction of the muscle cells is prevented in small vessels, but not in large arteries. In the mesenteric beds of rats, we have found that perfusion of the vascular bed with oxygenated Krebs for about 15 min at low pressure (16-24 mm Hg, at a rate of 1 ml/min/100 g body weight) was sufficient to produce maximal relaxation of the arteries, because addition of vasodilator (sodium nitroprusside), or beta-agonist (isoproterenol) or calcium-chelating agent (EGTA) into the perfusate did not alter the perfusion pressure [15]. More importantly, contraction of the vessels due to the introduction of GA in the perfusate was also eliminated. Walmsley et al. [32] also observed that in rabbit ear arteries, intermittent perfusion with Krebs bicarbonate containing 10^{-3} M sodium nitrite for 15 min was sufficient to fully dilate the vasculature, and subsequent perfusion with 10% neutral buffered formalin did not cause contraction of the vasculature. However, the use of electrolyte solutions in the perfusate prior to fixation can also induce artifacts in the vessel wall, especially when a high perfusion pressure is used. Richardson et al. [24] have noted that in rabbit aorta at a perfusion pressure of 100 mm Hg, prolonged preperfusion with some oxygenated electrolyte solutions (pH 7.4) induced changes in the endothelium similar to those reported as the response to injury induced by other stimuli. They found that Ringer-Locke solution perfused for 5-15 min caused severe damage to the

endothelial cells, and microvillus formation, whereas Krebs-Henzleit solution or modified Eagles medium did not. Whether Ringer-Locke solution will cause similar damage in other arteries besides aorta is not known. It appears that Krebs solution is preferable as the pre-fixation perfusate because of its ability to maximally relax the arteries, as well as eliminating the contractile response of the vascular smooth muscle cells to the fixatives [15].

Sampling of the Arteries. During sampling of the arteries for morphological studies and measurements, it is essential that a consistent sampling protocol is established, especially for the study of microvessels. Ideally, arteries from similar anatomical locations should be sampled. Some studies [e.g., 1,22] used the branching pattern of the vessels as the basis for the division of the vessels into various categories. Others [e.g., 28] used the percent distribution of the cross-sectional area of the vessel, or that of the vessel wall diameter as the basis for categorizing arteries. In the mesenteric bed of rats, branching pattern of the microvessels varied from rat to rat [15,22]. Nevertheless, before a better method can be devised, reliance on branching pattern is still the most logical method for the classification of the microvessels. Dividing the arteries on the basis of lumen size or vessel wall area is more hazardous, in view of the findings that the lumina of the microvessels are easily affected by the perfusion pressure used [16]. This shortcoming becomes more obvious when such sampling method is applied to the study of vascular changes of the arteries in hypertension. It is known that in various hypertensive animal models as well as in humans, the medial layer of the arteries is thickened. Depending on the vessel type, this thickening is due to either hyperplasia or hypertrophy of the vascular smooth muscle cells [19,21]. It is conceivable that at any given pressure, the arteries from the hypertensives will be more resistant to expansion than those from the normotensives, because of their thicker wall. Consequently, arteries from normotensives may be incorrectly perceived as having larger lumen than hypertensives. Studies by Cox [5] indeed found that at a perfusion pressure of 0-30 mm Hg, the external diameter of carotid arteries from normotensive rats was smaller than those of hypertensives. However, at higher pressure range (60 mm Hg and above), normotensive vessel diameter became larger than the hypertensives. Any attempts to normalize wall thickness or area measurements with lumen will inevitably result in errors, unless the arteries were fixed at low pressure in a maximally relaxed state [15,16].

Fixation Effect on Cell Volume. Most researchers who are engaged in quantitative morphological studies recognize that preparation procedures for light and electron microscopy can have profound effects on cell volume: some procedures causing swelling, and others causing shrinkage [17]. Probably because of the difficulties involved in finding a suitable method to best preserve cell volume, it is not surprising that few investigators have taken the appropriate actions on this important matter. For our study on the vascular changes in various animal models for human essential hypertension, we have tested several fixation procedures involving different concentrations of GA, and different buffers with a range of osmolarities [12-14]. One method was found to give an overall best preservation of cell volume and morphology for both scanning and transmission electron microscopy when applied to vascular smooth muscle cells. This method is now routinely used in our laboratory for morphometric studies of arteries [18]. Our fixation procedure has also been used in other laboratories [e.g., 32].

Morphometric Studies of the Arteries

There are basically two approaches for the study of vascular wall dimensions. Both utilize either planimetric or point and intersect counting methods to measure the area of the vessel wall components (e.g., lumen, media, adventitia). The difference is that in the "morphometric" method, the only mathematical compensation applied on the measured results, is correction for eccentricity of the sections due to oblique sectioning angles [15]. In contrast, the histometric method uses various mathematical methods to convert values obtained from collapsed or contracted arteries into hypothetical relaxed state of the vessels.

Morphometric Method

A standard point-counting system is usually used to determine the cross-sectional areas of the lumen, intima, media and adventitia in the relaxed vessels [e.g., 15,16]. Depending on the size of the arteries and magnification used, appropriate transparent morphometric test grids can be chosen for such purposes [33]. Alternatively, a digitizing pad can be used to determine the areas planimetrically [20]. In either case, it is essential that correction for eccentricity of the sections due to oblique sectioning angles should be applied. The basis is that a section through a cylindrical vessel at angle θ to the exact cross-section (circular profile) will result in an elliptical profile having a short (S) and a long (L) axial diameter (Figure 1). The ratio of S/L equals the cosine of θ, which can be used as a correction factor for eccentricity [15]. This correction should be applied to all the measured vessel wall components. Obviously, this type of correction is suitable only for maximally-relaxed arteries. In contracted arteries, because of the convoluted appearance of the lumen, an accurate measurement of the two axes is difficult.

Histometric Method

When arteries are sampled at necropsy, the arteries are either collapsed or contracted, therefore making the measurement of lumen size, and the dimensions of vessel wall components at a relaxed state impossible. Suwa and colleagues in 1961 (quoted from [9]) have devised a

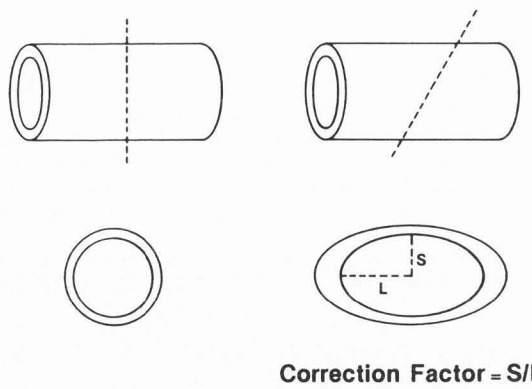

Figure 1. Illustration showing how an oblique sectioning angle affects the cross-sectional area of the vessel wall, and the correction factor which can be used.

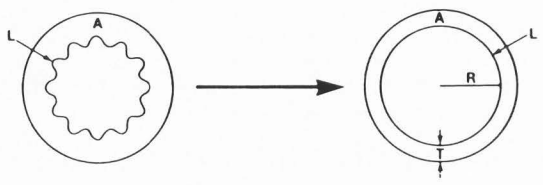

Figure 2. Principle involved in the histometric method to reconstruct a relaxed vessel from a contracted vessel. L = length of IEL, A = area of vessel wall, R = radius, T = wall thickness.

histometric method, in order that a hypothetical relaxed state of the contracted arteries can be constructed. Variations of this method have been introduced [e.g., 2], but the principle involved is the same. Basically, the length of the internal elastic lamina (IEL) of the contracted arteries is measured. The area of the vessel wall is determined either planimetrically, or by point counting method. Using the IEL length as the circular circumference of the lumen, mathematically idealized vessels can be reconstructed (Figure 2). This method presupposed that the length of the IEL, and the cross-sectional area of the vessel wall and its components remain unchanged during contraction of the vessels. The validity of these two assumptions will be examined here.

Short [27] suggested that the cross-sectional area of the vessel wall is the only reliable standard for comparison between hypertensive and normotensive arteries, but no reasons were given. Cook and Yates [2] assumed that on contraction, smooth muscle cells may change their shape, but not their volume, so that one might assume that the cross-sectional area remains constant. Our studies on the large mesenteric arteries from hypertensive and normotensive rats indeed showed that the cross-sectional area of the vessel wall remained constant when these arteries contract [16]. Similar observations were also made in the arterioles from hamster cheek pouch [31], arterioles from rabbit ear [10], and small muscular arteries from rats [8]. In the elastic arteries, muscular arteries and arterioles from the mesenteric arteries of rats, we found that expansion of the vessel wall by high perfusion flow did not alter the cross-sectional area. It is evident that cross-sectional area of the arteries is a reliable constant of the vessel wall under most physiological conditions.

Whether or not the length of the IEL is altered when the arteries contract or expand, seems to depend on the type of the arteries. In large elastic arteries such as human external iliac arteries, Cook et al. [4] have found that the IEL was resistant to stretching, because they did not find any difference between the length of IEL from collapsed and dilated arteries. Dobrin [6] noted that in large elastic vessels, the pressure at which expansion of the vessel wall becomes evident is between 150 and 250 mm Hg. In rat mesenteric arteries, doubling the perfusion pressure from 18.5 mm Hg to 37 mm Hg did not alter the lumen size of the superior mesenteric artery (another elastic artery), but the lumina of large mesenteric (a muscular artery) and small mesenteric (an arteriolar vessel) were significantly enlarged [16]. The presence of elastin laminae in these elastic arteries may be the reason why these arteries are more resistant to stretching than muscular and arteriolar vessels. When higher pressures are applied ($\geq$ 60 mm Hg in rat carotid arteries [5]), expansion of the elastic arteries can occur. Wolinsky and Glagov [34] have found that in rabbit aorta, the lumen became bigger with the increase in intramural pressure. The medial wall and also the elastin lamellae became thinner. When muscular arteries contract, IEL of arteries from normotensive rats were found to shorten, and the thickness of the IEL was also increased [16]. Therefore, it is obvious that IEL from the muscular arteries is easily stretchable as it is collapsible. This is important, because in hypertension, most of the studies from animal models to date suggest that primary changes occur in these muscular arteries [19-21].

Based on the information discussed above, it is obvious that one of the two major assumptions (i.e., IEL length is constant at contraction) crucial to the validity of the histometric method is incorrect, especially as it applies to muscular arteries. The consequence is that lumen size will be underestimated in some cases by this method.

Units of Comparison between Vessels

For comparative studies of arterial changes in some disease states such as hypertension and atherosclerosis, the choice of methods for comparison is an important factor.

Wall to Lumen, or Media to Lumen Ratio.

Wall to lumen ratio of arterioles was used by Kernohan et al as early as 1927 [11] to study vascular changes in human malignant hypertension. Many recent studies on hypertension still prefer to use wall to lumen ratio as the

unit for comparison [e.g., 3] probably because of the assumption that variation in vessel size can be eliminated by normalizing the values for vessel wall with vessel lumen. In view of the findings that values for lumen are generally not dependable, because they are affected by sampling, preparation method, and methods of measurements as discussed earlier, the sensitivity and reliability of wall to lumen ratio as the unit for comparison are generally of limited values. As illustrated in Figure 3, significant variation in wall to lumen ratio can occur either due to expansion of the lumen with high perfusion pressure, or contraction of the vessel wall. However, in cases where the arteries are fixed at maximal relaxation under low perfusion pressure, wall to lumen ratio provides an additional useful criterion for comparison.

Wall Thickness. This is the easiest parameter to measure, but is also the least dependable for comparative studies. For a given vessel, an increase in wall thickness can be caused as readily as by vasoconstriction, as a decrease in wall thickness by stretching the vessel wall due to high perfusion pressure.

Cross-Sectional Area of the Vessel Wall. To date, this is the most reliable parameter for comparative studies, because it is not affected by the physiological states of the vessels (i.e., whether relaxed or contracted) at the time of fixation, as discussed earlier.

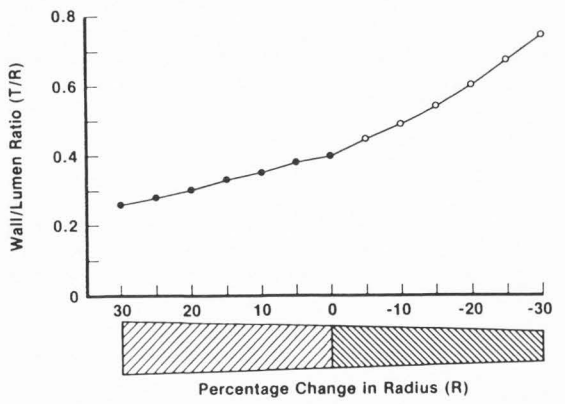

Figure 3. Variation in wall/lumen ratio in relation to either increase (due to expansion of lumen, towards left of 0) or decrease (due to contraction of vessel, towards right of 0) in radius of arteries.

Conclusion

The information generated from any morphometric studies of blood vessels is relevant only if the in vivo morphology of these vessels is preserved. In order to achieve this, careful attention has to be given to tissue preparation methods, as well as to the selection of proper morphometric protocols.

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References

1. Bohlen HG, Loback D (1978) In vivo study of microvascular wall characteristics and resting control in young and mature spontaneously hypertensive rats. Blood Vessels 15, 322-330.

2. Cook TA, Yates PO (1972) A critical survey of techniques for arterial mensuration. J. Path. 108, 119-127.

3. Cook TA, Yates PO (1972) A histometric study of cerebral and renal arteries in normotensives and chronic hypertensives. J. Path. 108, 129-135.

4. Cook TA, Salmo NAM, Yates PO (1975) The elasticity of the internal lamina. J. Path. 117, 253-258.

5. Cox RH (1981) Basis for the altered arterial wall mechanics in the spontaneously hypertensive rat. Hypertension 3, 485-495.

6. Dobrin PB (1978) Mechanical properties of arteries. Physiol. Rev. 58, 397-460.

7. Folkow B, Neil E (1971) Circulation. Oxford University Press, New York, 33-34.

8. Friedman SM, Nakashima M, Mar MA (1971) Morphological assessment of vasoconstriction and vascular hypertrophy in sustained hypertension in the rat. Microvasc. Res. 3, 416-425.

9. Furuyama M (1962) Histometrical investigations of arteries in reference to arterial hypertension. Tohoku J. Exper. Med. 76, 388-414.

10. Hashimoto H, Prewitt RL, Efaw CW, Chen IIH (1985) Calculated cross-sectional wall area of arterioles is a useful indicator of hypertrophy in conscious rabbits. Microvasc. Res. 30, 350-353.

11. Kernohan JW, Anderson EW, Keith NM (1929) The arterioles in cases of hypertension. Arch. Int. Med. 44, 395-423.

12. Lee RMKW, Garfield RE, Forrest JB, Daniel EE (1979) The effects of fixation, dehydration and critical point drying on the size of cultured smooth muscle cells. Scanning Electron Microsc. 1979; III: 439-448.

13. Lee RMKW, Garfield RE, Forrest JB, Daniel EE (1980) Dimensional changes of cultured smooth muscle cells due to preparatory processes for transmission electron microscopy. J. Microsc. 120, 85-91.

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

15. Lee RMKW, Garfield RE, Forrest JB, Daniel EE (1983) Morphometric study of

structural changes in the mesenteric blood vessels of spontaneously hypertensive rats. Blood Vessels 20, 57-71.

16. Lee RMKW, Forrest JB, Garfield RE, Daniel EE (1983) Comparison of blood vessel wall dimensions in normotensive and hypertensive rats by histometric and morphometric methods. Blood Vessels 20, 245-254.

17. Lee RMKW (1984) A critical appraisal of the effects of fixation, dehydration and embedding on cell volume. In: Science of Biological Specimen Preparation for Microscopy and Microanalysis, Revel J-P, Barnard T, Haggis GH (eds), SEM Inc., AMF O'Hare IL 60666, 61-70.

18. Lee RMKW (1985) Preparation methods and morphometric measurements of blood vessels in hypertension. Prog. Appl. Microcirc. 8, 129-134.

19. LEE RMKW, Smeda JS (1985) Primary versus secondary structural changes of the blood vessels in hypertension. Can. J. Physiol. Pharmacol. 63, 392-401.

20. LEE RMKW, Triggle CR (1986) Morphometric study of mesenteric arteries from Dahl strain of genetic hypertensive rats. Blood Vessels 23, 199-224.

21. Lee RMKW (1987) Structural alterations of blood vessels in hypertensive rats. Can. J. Physiol. Pharmacol. In press.

22. Mulvany MJ, Hansen PK, Aalkjaer C (1978) Direct evidence that the greater contractility of resistance vessels in spontaneously hypertensive rats is associated with a narrowed lumen, a thickened media, and an increased number of smooth muscle cell layers. Circ. Res. 43, 854-864.

23. Rhodin JAG (1986) Perfusion and superfusion fixation effects on rat mesentery microvascular beds. Intravital and electron microscope analyses. J. Submicrosc. Cytol. 18, 453-470.

24. Richardon M, Hatton MWC, Buchanan MR, Moore S (1985) Scanning electron microscopy of normal rabbit aorta: Injury or artifact? J. Ultrastruct. Res. 91, 159-173.

25. Russell RWR (1963) Observations on intracerebral aneurysms. Brain 86, 425-442.

26. Short DS, Thomson AD (1959) The arteries of the small intestine in systemic hypertension. J. Path. Bact. 78, 321-334.

27. Short D (1966) The vascular fault in chronic hypertension. With particular reference to the role of medial hypertrophy. Lancet I, 1302-1304.

28. Short D (1966) Morphology of the intestinal arterioles in chronic human hypertension. Brit. Heart J. 28, 184-192.

29. Thorball N, Tranum-Jensen J (1983) Vascular reactions to perfusion fixation. J. Microsc. 129, 123-139.

30. Todd ME, Laye CG, Osborne DN (1983) The dimensional characteristics of smooth muscle in rat blood vessel. A computer-assisted analysis. Circ. Res. 53, 319-331.

31. Walmsley JG, Gore RW, Dacey Jr RG, Damon DN, Duling BR (1982) Quantitative morphology of arterioles from the hamster cheek pouch related to mechanical analysis. Microvasc. Res. 24, 249-271.

32. Walmsley JG, Owen MP, Bevan JA (1983) Medial morphometry and mechanics of sequential rabbit ear arteries and myograph ring segments. Am. J. Physiol. 245, H840-H848.

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

34. Wolinsky H, Glagov S (1967) Nature of species differences in the medial distribution of aortic vasa vasorum in mammals. Circ. Res. 20, 409-421.

Discussion with Reviewers

R. Bolender: We have recently reported for liver that the small blocks prepared for transmission microscopy shrink or swell quite substantially [Bertram JF, Bolender RP 1983 Acta Stereol. 2 (Suppl), 281-284].

If the same is true for blood vessels, how can you account for different responses of the tissue compartments to fixation in health and disease? How, for example, will such volume artifacts of fixation influence estimates for volume, surface, and numerical densities?

Author: This is an important question for which we have no ready answer. For the blood vessels, our main focus was on the preservation of *in vivo* smooth muscle cell volume. It is conceivable that preservation of the volume of other vessel wall components (e.g., endothelium, fibroblasts, nerves) might not follow the same trend as that of the smooth muscle cells, so that estimates of morphometric parameters will be affected.

N. Thorball: Was any colloid substance added to the fixative solution used by you? What was the concentration of GA? In other words, is the viscosity of the fixation fluid known? That is a prerequisite when comparing flow and pressure in different experimental setups.

Author: No colloid substance was added to the fixative solution. The concentration of GA was 2.5%. We do not know the viscosity of our fixative. I agree that this is an important aspect.

N. Thorball: Concerning the development of edema, do you have any experience with employment of fixatives with varying concentrations of high molecular weight colloids?

Author: We have not yet tried to add colloidal substances in the fixatives to avoid edema formation. As pointed out by you, others [Bohman SO, Maunsbach AB 1970 J. Ultrastruct. Res. 30, 195-208; Rostgaard J, Behnke O 1965 J. Ultrastruct. Res. 12, 579-591] have achieved some success with the use of colloids.

N. Thorball: You did not comment on why the arteries relax when perfused with oxygenated Krebs for 15 min. Have you measured the Ca^{++}

content in the effluent fluid? Do you have any explanation of or any experience concerning the different effects of different electrolyte solutions on the endothelium? Is it simply a matter of electrolyte composition? Or might it be explained by the differences in the abilities of these electrolyte solutions to be oxygenated?
Author: We do not have any explanation as to why perfusion with oxygenated Krebs for 15 min caused the arteries to relax. We did not carry out any of the experiments you have suggested. In view of the recent findings that endothelium does play an important role in mediating the relaxation response of the smooth muscle cells to various substances, it will be interesting to investigate whether the relaxation response of the arteries to oxygenated Krebs is mediated by the endothelial cells.

N. Thorball: How was the data underlying Figure 3 obtained?
Author: It was based on a mathematical model. By varying the lumen diameter, wall to lumen ratio value for each specific diameter was calculated.

N. Thorball: Finally, can you please summarize which methods - step-by-step - that, in your opinion, will give the best results.
Author: For the fixation of vascular smooth muscle cells, we believe that our current method gives the best result for the presentation of cell morphology and volume. Details on the composition of the various fixatives and wash buffers, and the fixation pressure and times, are given in reference [18].