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ARTERIAL ELASTIN AS SEEN WITH SCANNING ELECTRON MICROSCOPY: A REVIEW

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Abstract

All large arteries contain elastin, collagen, and muscle which can be seen with light microscopy and transmission electron microscopy. Elastin forms an internal elastic lamina (IEL) in all arteries, but also forms multiple fenestrated sheets in the media of the aorta and other large arteries. The fenestrations in the media are larger than those in the IEL. The adventitial elastin is more fibrous and often contains tubular elastin surrounding vasa vasorum when prepared by removing all non-elastin by placing the aorta in 0.1 N NaOH at 70-75°C for five hours. The fenestrations are larger near branches and in an experimentally created poststenotic dilatation. Atherosclerosis appears associated with both new elastin formation in early atherosclerosis and elastolysis in late disease.

KEY WORDS: Elastin, Artery, Scanning Electron Microscopy, Fenestrations, Elastolysis, Atherosclerosis, Aneurysms.

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Introduction

Elastin is a rubber-like protein which can be extended to almost twice its initial length and still recoil completely. Elastin is present in virtually all tissues and organs which must change in size physiologically (e.g. spleen, heart, arteries, lung, skin), but has been studied most extensively in the ligamentum nuchae of the cow and ox and in the large arteries of mammals.

Ross and Bornstein (1969) studied ligamentum nuchae from young cows and showed that elastin is made up of two components. Microfibrils appear first and are about 110 Å in diameter, and usually oriented in parallel arrays, often as beaded fibres. Later an amorphous component develops to imbed the fibrils and form fibres which were about 6 μm in diameter. Both components are quite inert chemically, but the microfibrils are digested by trypsin and chymotrypsin, while the amorphous component is removed with elastase. Both components are resistant to heat, and strong acid and alkali.

Elastin is very inert chemically and is usually purified by removing other tissues which are more soluble. We (Song and Roach, 1983, 1984) have used 0.1 N NaOH at 70-75°C for three to five hours, as have others (Lowry et al., 1941; Starcher and Galione, 1976; and Steven et al., 1974). Gosline (1978) autoclaved the tissue for many hours, while Ayer et al. (1958), Lansing et al. (1952) and several others used hot acid. Histological and chemical studies suggest that all of these methods preserve elastin while removing all non-elastin. Crissman and Pakulski (1984) found ultrasound accelerated the digestion process if used with 0.5 N NaOH and later autoclaving.

Elastin prepared with any of these methods can be studied chemically, mechanically, or morphologically. The completeness of digestion can be assessed chemically or microscopically.

Concepts of Arterial Elastin from Light Microscopy

Elastin is identified in arteries with a variety of stains including Weigert's and aldehyde fuchsin. Counterstains allow the elastin to be separated from collagen and muscle. Banga (1966) discusses these methods in detail.

The most detailed analysis of elastin in mammalian aortas is that by Wolinsky and Glagov (1964) who developed the concept of the medial lamellar unit or MLU. They described this as two adjacent layers of elastin (seen in cross-section) separated by muscle, fine elastin, and collagen. They studied these in a large variety of mammals, ranging from the mouse to the horse, by removing the aortas, stretching them to their in vivo length, and then fixing them at physiological pressure with

the lumen filled with a mixture of gelatin and barium which was radio-opaque and so could be used to determine the internal radius of the sections. They sectioned these in the middle of both the descending thoracic aorta and the abdominal aorta, and drew the following conclusions:

(i) The elastin became straighter as the vessels were distended with about 80 mm Hg pressure, and the distance between adjacent lamellae decreased uniformly throughout the wall as the pressure was increased, and the fine elastin fibers between them became oriented more circumferentially as did the muscle and collagen fibres (Wolinsky and Glagov, 1967a).

(ii) The number of MLU increased as the weight of the animal increased, so the tension/MLU in the thoracic aorta was approximately 2000 dyn/cm regardless of the species.

(iii) Vasa vasorum were seen only in aortas with over 29 MLU, so they presumed the thinner aortas were nourished entirely by diffusion from the lumen (Wolinsky and Glagov, 1967b).

(iv) The tension/MLU for the abdominal aorta was constant for all 10 species studied except for man where the tension/MLU was higher as the lamellar units were much thicker and had no vasa vasorum (Wolinsky and Glagov, 1969).

van Baardwijk and Roach (1983) studied the aortas of sheep and lambs which had been pressure-fixed with filling the lumen with barium and gelatin but they studied the differences along the length of the aorta, while Wolinsky and Glagov looked only at two regions, i.e. the middle of the thoracic aorta and the middle of the abdominal aorta. They showed that the number of MLU decreased linearly along the descending thoracic aorta at both ages, but was relatively constant along the length of the abdominal aorta. The tension/MLU increased linearly along the whole length of the lamb aorta, but increased abruptly at the diaphragm in the sheep aorta.

Roach (1983) used published data to show that the number of MLU in the middle of the abdominal aorta was linearly related to pulse pressure, while the number of MLU in both the thoracic and abdominal aorta appeared to increase exponentially with stroke volume.

The pathological literature is full of references to elastin, but most of these are mentioned in passing and contain little quantitative information. We will discuss these only with respect to the lesions such as aneurysms, atherosclerosis, and poststenotic dilatation which we have studied ourselves.

Transmission Electron Microscopy (TEM)

Wolinsky and Glagov (1964) supplemented their quantitative light microscopic studies with qualitative TEM studies. They compared the components of rabbit aortas at different distending pressures and concluded that the lamellae were tubular, and that elastin straightened out above diastolic pressure. At normal pressure they felt the fine interlamellar elastin fibres which went between elastin lamellae were arranged randomly at low pressures, but became oriented circumferentially at higher pressures. They did not see any connections between the elastin and collagen, and suggested that the elastin seemed to distribute the forces uniformly through the wall.

Clark and Glagov (1979, 1985) did a more detailed TEM study of the aorta, but based their conclusions on aortas distended at normal and elevated pressures in different ages of pigs and rabbits. They concluded the muscle played a more crucial role than elastin in the basic structure of the aorta. The muscle cells were aligned circumferentially and their ends tended to overlap as the pressure increased. The fine collagen fibrils seen between adjacent cells appeared to be in sheets one or two fibrils thick, and were usually very close to the basal lamina. In distended vessels, these fibrils tended to be approximately perpendicular to the long axes of the cells, and ran obliquely to form interlacing networks around the cells.

In young animals, they saw islands of elastin which appeared parallel to the long axes of the cells. As the animals became mature, these two components appeared to be attached to each other.

Figure 1 shows a TEM photograph of part of a cross-section of a rat aorta which was fixed at 100 mm Hg pressure with buffered 10% formaldehyde. The edges of the elastin appear irregular, and fibrils of collagen appear intimately related to both the edges, and at times the interior, of the elastin. While TEM pictures are best to show the interconnections between cellular and fibrous elements, they are less satisfactory than SEM studies to give a three-dimensional picture of the arrangement and connections between the elastin lamellar and the interlamellar elastin fibers.

Scanning Electron Microscopy (SEM)

SEM is particularly useful for studying surfaces, but is much less satisfactory for comparing different types of fibers. All digestion methods, including ours with 0.1 N NaOH at 75°C for 5 hours leave elastin behind because the elastin is so inert chemically. As long as the digestion is complete, any material seen will be elastin. The completeness of the digestion can be assessed chemically or microscopically as described above.

The elastin that remains after digestion of the aorta or other tissues (spleen, kidney, lung, heart) is oyster-white in color and translucent, and is neutrally buoyant with water. Thus, if the specimen is placed in water it appears to have the same shape and probably size as the artery from which it was prepared (Fig. 2). The artery is frozen in water to maintain this shape, and then is freeze-dried, coated with Au/Pd, and then scanned.

This approach shows that the thick elastin lamellae are tubes or sheets which presumably are arranged like concentric cylinders since the arteries are cylindrical. The lamellae are attached to each other by fine elastin fibres (Fig. 3), although the number of connections has not been quantified.

The innermost layer is called the internal elastic lamina (IEL) and is present in all arteries. Higher powers (e.g. 800× or more) show that the sheet contains small holes which are quite uniformly distributed. The size of the fenestrations can be obtained readily with a digitizer and computer (Campbell and Roach, 1981). In the IEL most of the holes or fenestrations are almost circular as shown in Fig. 4, but we have found that the size varies considerably in different parts of the aorta and in different species. Data obtained in our laboratory is shown in Table 1. The fenestrations vary over a wide range, but the fenestrations tend to get larger the more peripheral the segment regardless of the species. Since the fenestrations are larger in the dog which is intermediate in size between the rabbit and the sheep, the variation at any one location does not appear related to animal size.

The media can be seen by looking at the cut edge of the artery, and is made up of multiple fenestrated sheets in the thoracic aorta, and to a lesser extent in the abdominal aorta. Fig. 5 shows both the IEL and the media of the abdominal aorta of a dog. We (Noss, Song, and Roach - unpublished) have developed a method to expose these medial layers so they can be studied en face (Fig. 6). After the specimen is freeze-dried it becomes glass-like

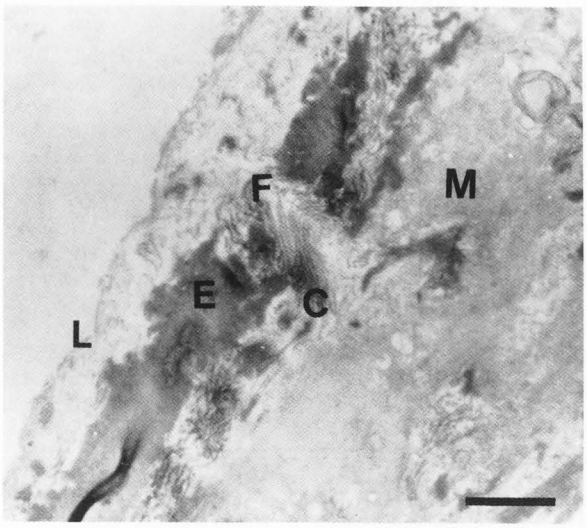

Fig. 1 - TEM photograph of a rat aorta pressure-fixed at 100 mm Hg. L = lumen, E = elastin, F = fenestration, C = collagen, and M = muscle. The bar is 1 μm.

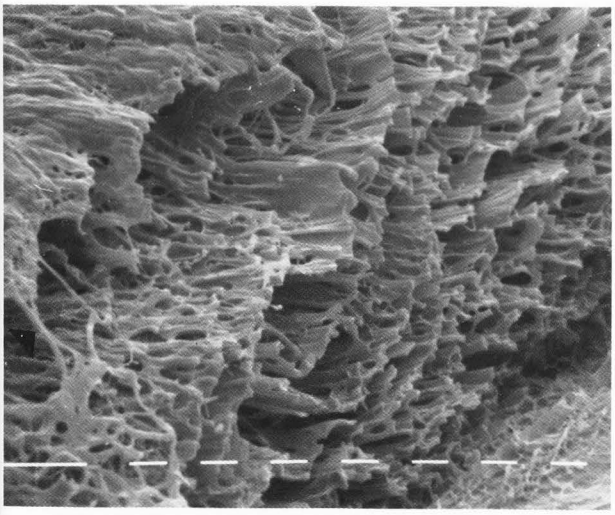

Fig. 3 - Cut edge of the wall of the digested thoracic aorta from a sheep. Note that there are multiple sheets, and often they are connected by fine fibers as seen near the arrow on the left. The small bars are 10 μm.

and brittle. With care it is possible to make a series of oblique cuts through the media to expose at least some of the layers. These can then be analyzed as above and scanned with zero tilt. Small tilt angles can be corrected for by multiplying by $1/\cos\theta$ where θ = angle of tilt, but this is rarely necessary as the layers are parallel to each other and so to the surface of the stub. Fig. 7 shows the data obtained from five mature pigs with an average

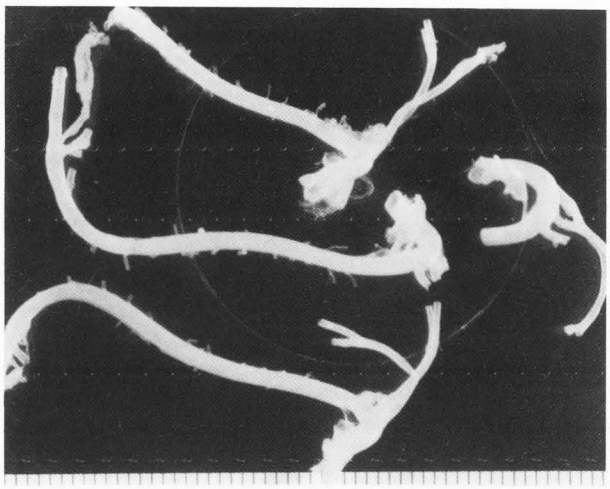

Fig. 2 - Rat aortas digested for five hours. The scale at the bottom is in mm. Note that the vessels are translucent and maintain their shape.

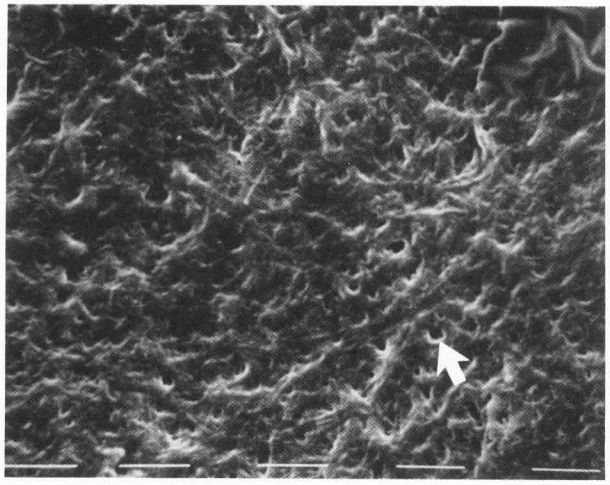

Fig. 4 - IEL of thoracic aorta of sheep. Note the rough surface and the small round holes (such as the one above - the white arrow) which are quite evenly distributed. Bars are 10 μm.

TABLE 1

Fenestration Diameter (μm) in IEL

	UTA	LTA	UAA	LAA
Dog	0.954	1.100	1.514	2.227
Sheep	0.883		1.040	1.283
Rabbit	0.819		1.088	1.480

UTA = upper thoracic aorta (between third and fourth intercostal)
LTA = lower thoracic aorta (between seventh and eighth intercostal)
UAA = upper abdominal aorta (between coeliac and renals)
LAA = lower abdominal aorta (below large branches and above iliacs)

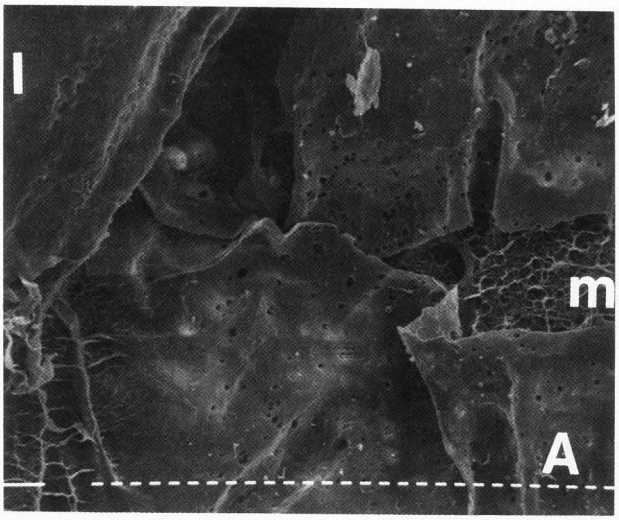

Fig. 5 - Digested dog abdominal aorta (A). The IEL is a smooth fenestrated sheet. The fenestrations are larger and less evenly distributed. The surface is cut on the right to show the larger holes in the media (m). The left side shows the iliac (I). The wrinkles are produced by trying to flatten a region which is normally curved. The small bars are 10 μm.

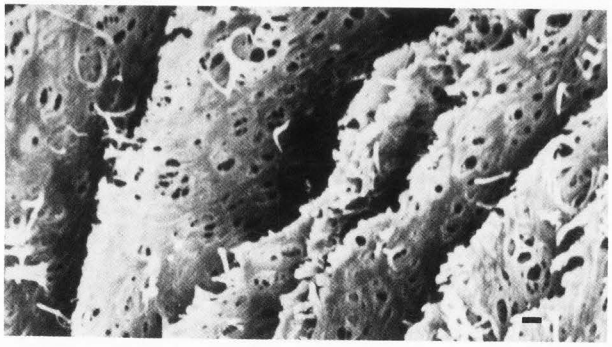

Fig. 6 - Exposure of medial layers of the ascending aorta of a pig. The IEL is on the left. Note the large holes. The bar is 10 μm.

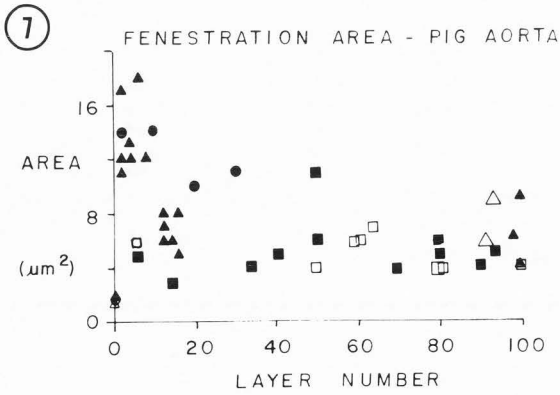

Fig. 7 - Area of fenestrations for the ascending aorta of five adult pigs. Note that the medial holes are much larger than the IEL ones (layer O). The different symbols are from different pigs. Note that virtually all of the IEL fenestrations are the same size so the data points are superimposed. (Unpublished data of Noss, Song, and Roach.)

weight of 150 kg. Note that the medial holes are much larger than those of the IEL, and that there is very little difference in size throughout the media. Figs. 3 and 6 show that there are many fine elastin fibers joining the lamellae. These appear qualitatively to be arranged as an interlacing network, but no quantitative studies of their size or orientation have yet been done. They persist even with longer digestion times and so are almost certainly elastin.

The adventitial (outside) elastin appears more fibrous, and is often penetrated by tube-like structures which we have shown are vasa vasorum (Song et al., 1985). However, there are many more fenestrations than there are vasa. Fig. 8 shows a typical picture from a dog aorta.

The continuity of the elastin network revealed by SEM provides strong support to the suggestion by Wolinsky and Glagov (1967a) that the elastin serves to distribute forces evenly throughout the wall of the aorta. Comparable studies should be done with collagen and muscle, but are much more difficult as the elastin is so hard to remove that non-elastin structures are usually damaged as well. Note how much easier it is to see the three-dimensional elastin network with SEM than with either light microscopy or TEM.

The Problem of Artifacts

(1) Digestion Damage - This has been discussed already in the Introduction. Very long digestion times probably damage the elastin, particularly the microfibrils which are slightly more soluble than the amorphous elastin. If the elastin were not interconnected with elastin, but rather with collagen, the arrangement of the network would be altered. We have found no evidence that this is the case.

(2) Shrinkage Artifacts - Campbell and Roach (1983c) developed a method using fluorescent particles to show that the elastin in digested human cerebral arteries shrank by 6.9 ± 0.2% (SE) during the freeze-drying. There are still no quantitative studies to show if the fenestrations increase or decrease in size or remain the same during the freeze-drying process. Calculations suggested that the shrinkage may be comparable to the thickness of the Au/Pd layer so that the two artifacts would cancel each other. However, this remains to be proved experimentally.

(3) Fixation Artifacts - Wolinsky and Glagov (1964) have stressed the importance of fixing arteries at physiological pressures. At low pressures the elastin is wavy, presumably because the muscle and collagen recoil as the luminal pressure is lowered. Since elastin is a rubber-like material, it can be stretched up to 200% of its initial length and still recoil to its original size, it seems likely that the unstretched length of elastin may be close to its diastolic length. This would support the observation of Wolinsky and Glagov (1964) that the elastin of rabbit aortas became straight at close to diastolic pressure. Note in Figs. 4, 5 and 8 that the elastin is flat rather than wavy, and so is probably close to its diastolic length.

We have already noted that elastin is very inert chemically. Fung and Sobin (1981) have shown that it is very difficult, if not impossible, to fix elastin with any of the aldehydes such as formaldehyde and glutaraldehyde

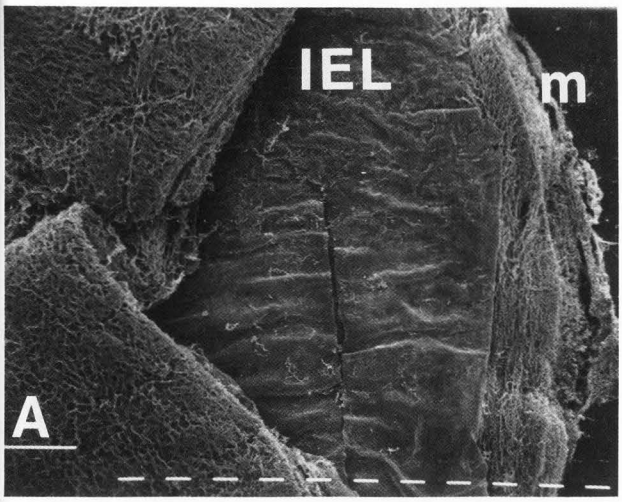

Fig. 8 - SEM of dog aorto-iliac junction. The flat IEL is shown in the center, the cut medial edge (m) on the right, and the adventitia (A) on the left. At this magnification the holes are not apparent in the IEL but are in the adventitia. The bars are 100 μm.

which quickly fix both muscle and collagen. When the elastin is interwoven with the muscle and collagen, the artery (or other elastin-containing structure) will remain at its fixed length. However, once the collagen and muscle are removed, the elastin will return to its unstretched length.

An alternative method of maintaining a certain length of elastin is to use the fact that Gosline and French (1979) have shown that elastin undergoes its glass transition at 97% relative humidity. Thus, dehydrated elastin will behave as a glass and should not recoil. The brittle nature of glass also makes it possible to crack off layers of elastin to study the underlying layers. Thus, it seems likely that if arterial elastin is put in distilled water, with which it is neutrally buoyant, the specimen will return to the unstretched length of the elastin, but the elastin is still very stretchable. Once it is dried, this shape and size will be retained as the elastin becomes brittle. This method appears more reliable than chemical methods of fixing elastin.

(4) Stretching Artifacts - Much of the published information on arteries talks of 'elastin fibers' and yet our data (Song and Roach, 1983, 1984), and that of Crissman (1984) show that the elastin is probably in sheets, at least in the lamellae. There are fine fibers between the sheet-like layers of elastin. To determine if sheets of elastin could be changed into fibers, we stretched the elastin by 10, 25, and 50% of its length and then froze it at this length. Fig. 9a and b show a typical result with 25% strain. Note that the edges have become fibrous, while the central region is still like a sheet. Since similar changes occurred with 10% strain, it is obviously important to avoid stretching the samples during the preparation process. This may partially explain why adventitial elastin appears more fibrous as it is often tethered to the surrounding tissue, and so might be stretched as the artery is removed. No studies have been done yet to determine if stretching the whole artery has the same effect as stretching the purified elastin. The question of why the central region remains sheet-like may be due to local regions of stress concentration because of the interlamellar fibers.

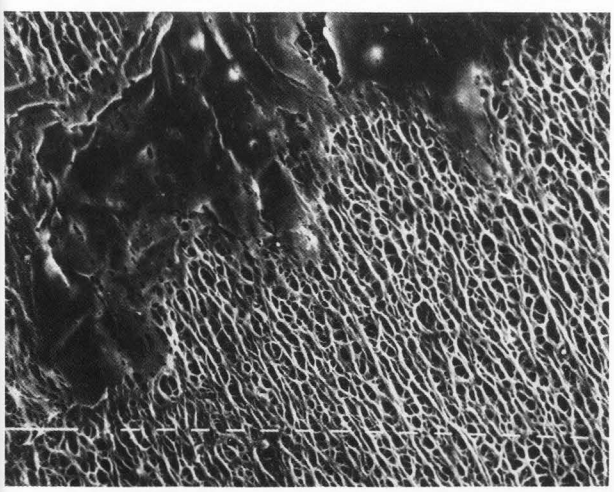

Fig. 9a - SEM of a digested dog thoracic aorta that has been stretched by 25% and then held at that length as it was dried. The edges near the grips are similar to the lower right corner and become fibrous instead of remaining as sheets. The bars are 10 μm.

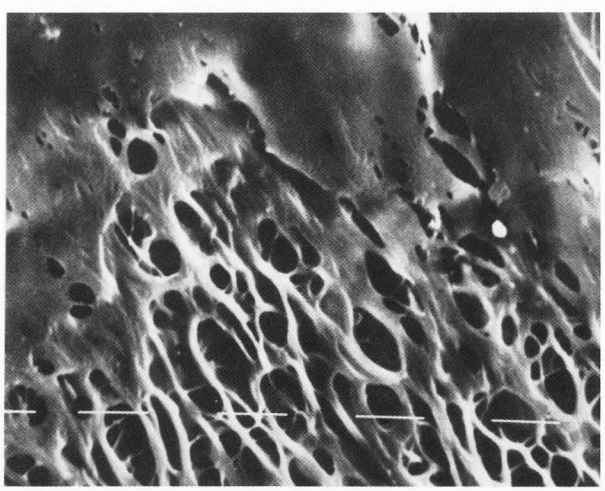

Fig. 9b - A higher magnification of the junctional region from Fig. 9a. Note that the junction between the sheet-like and fibrous elastin is irregular. The bars are 10 μm.

Alterations in Elastin with Location and Disease

(1) Cerebral Aneurysms. Campbell and Roach (1981) showed in human **cerebral arteries** that the fenestrations were much larger (5.9 μm) at the apex of bifurcations than in the straight sections (1.5 μm). **Aneurysms** often develop at the apex of these bifurcations.' Latex strips were marked with fenestrations identical in size and distribution to those in the arteries and then tested with uniaxial strains in an Instron tensile testing machine with and without the holes punched out. These showed that the concept of ligament efficiency could be used to describe the effects that the strips with large holes stretched more than those with small holes. Ligament efficiency is an engineering concept which describes the effect of holes in a thin sheet on the elastic properties of the sheet. Ligament efficiency equals the length of the solid band between holes divided by the distance between the centers of the holes (Campbell and Roach, 1983a,b). If the wall of the cerebral artery contained only elastin, the difference in size of the holes would predict that aneurysms would develop where the holes are larger (Campbell and Roach, 1984). This type of modelling cannot be done on the aorta until data is available on the medial layers and on the interlamellar fibers. Cerebral arteries contain only a single IEL and virtually no medial elastin.

Nyström (1963) showed with light microscopy that elastin appeared absent in cerebral aneurysms and granular with TEM. No SEM studies have been done to our knowledge, although we plan to do them in the near future.

(2) Poststenotic Dilatation (PSD). Poststenotic dilatation is a hemodynamic paradox as the artery dilates in the low pressure region distal to a stenosis. Roach (1979) showed that the PSD developed only if the stenosis created turbulence, and proposed that, since the artery was more distensible than normal in this region, the vibration of the wall induced by the turbulence caused structural fatigue of the elastin. She showed that the low pressure or elastin part of the distensibility curve was altered significantly, while the high pressure collagen region appeared unaltered.

Potter and Roach (1983) showed in a small group of rabbits that had developed PSD distal to a coarctation of the descending aorta that the fenestrations were larger in the PSD than in non-dilated controls. The average diameter of the fenestrations was 2.30 ± 0.53 (SE) μm distal to the stenosis but only 1.33 ± 0.16 (SE) μm proximally. These values were significantly different at $p<0.005$. Studies are in progress to determine if the size of the PSD is correlated with the size or the density of the fenestrations, but our present data (Fig. 10) are inadequate to predict how the two are related mathematically.

(3) Atherosclerosis. Atherosclerosis is a patchy disease that tends to occur particularly at bends and bifurcations, presumably because of altered hemodynamic forces in these regions (Roach and Smith, 1983). Roach and Fletcher (1976) showed that the location of lesions in cholesterol-fed rabbits was altered if flow into the branch was altered (e.g. near a blind stump of renal artery).

The pathology literature for over a century has argued whether elastin is fragmented or reduplicated in regions of atherosclerosis. Despite this controversy, there is general agreement that elastin is different from normal in the presence of atherosclerosis.

We have started a study on human aortic elastin in the regions of plaques, ulcers, and fatty streaks. Fig. 11 shows regions of damaged elastin under fibrous plaques. The edges are so ragged that it seems likely elastolysis has occurred. In ulcerated regions, the medial elastin is also damaged (Fig. 12), probably also from elastolysis, although local areas of stress concentration adjacent to calcified regions might create tearing stresses locally.

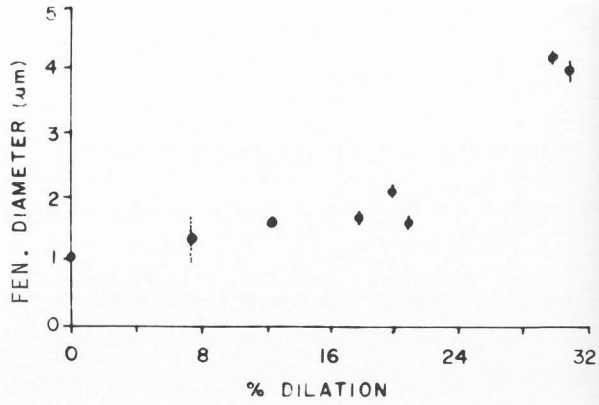

Fig. 10 - Preliminary (unpublished) data from Potter and Roach to show that the fenestrations in the IEL of rabbit thoracic aorta are largest with large PSD's. Each data point shows the mean and standard error of the mean from one rabbit. More studies need to be done between 20-30% dilation to determine the relationship between the two variables.

Nakatake et al. (1985) have recently shown that early atherosclerotic lesions in rat aortas are associated with an increase in dome-like elastic laminae with few fenestrations. They treated the aortas with 88% formic acid at 45°C for 2,3,4,5, or 6 days and used TEM to determine that all non-elastin was removed. Their TEM studies showed two types of change in the elastin. The first was reduplication of the IEL which was seen at multiple sites throughout the entire intima of the thoracic aorta. This new elastin appeared as a thin continuous sheet which branched from the original IEL and protruded a small amount toward the luminal surface to form crescent-shaped lesions which were always attached to the IEL and usually separated from it by a small number of irregularly shaped smooth muscle cells. The second change was an increase in the fibrous elastin which was often associated with microfibrils and seemed to run mainly longitudinally. The medial elastin was less affected but frequently appeared to have rough bristles on its surface rather than the usual smooth edges. SEM showed the new IEL protrusions to be 'belt-like' and oriented longitudinally. The fenestration pattern appeared altered. They felt there were less fenestrations, but we would suggest from their pictures that the fenestrations are less uniformly distributed than normal. They always found this new elastin attached to the original IEL with fibrous elastin. We have seen these under fatty streaks and have seen similar raised protrusions, coming from the IEL of the aorta of newborn lambs (Fig. 13). The elastin is probably produced by the smooth muscle cells (Giro et al., 1984), and we suspect these protrusions reflect the contour of the muscle cells from which they arise. These authors did not see any evidence of the elastolysis we have seen under advanced human lesions near the early lesions seen in rats.

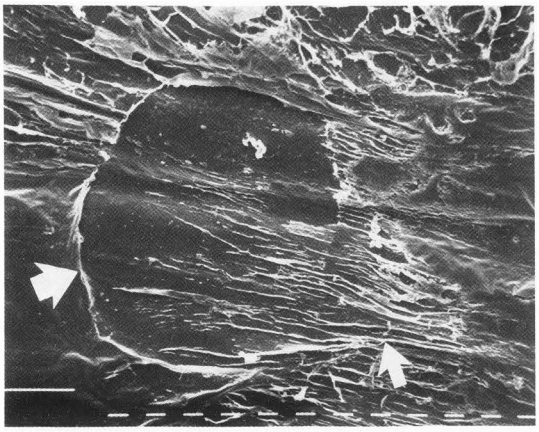

Fig. 11 - SEM of the IEL of a digested human aorta under a fibrous plaque. At the large arrow on the left are regions with rough edges where elastolysis has probably occurred. Regions by the small arrows on the right are probably regions with new elastin. The small bars are 100 μm.

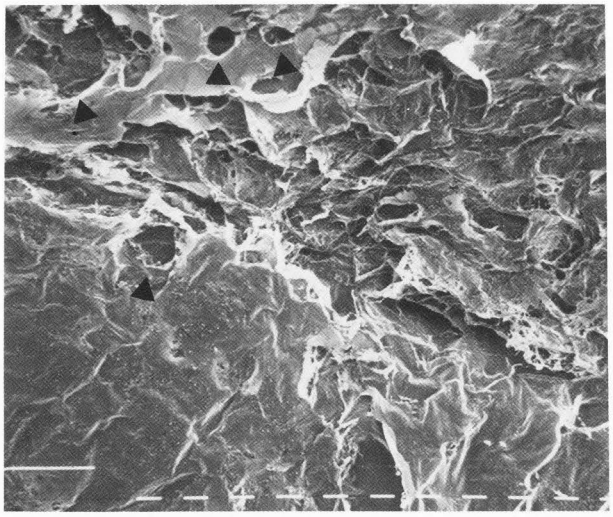

Fig. 12 - Digested human aorta with small ulcers at the arrows. Note the apparent elastolysis which at times includes the media. The small bars are 100 μm.

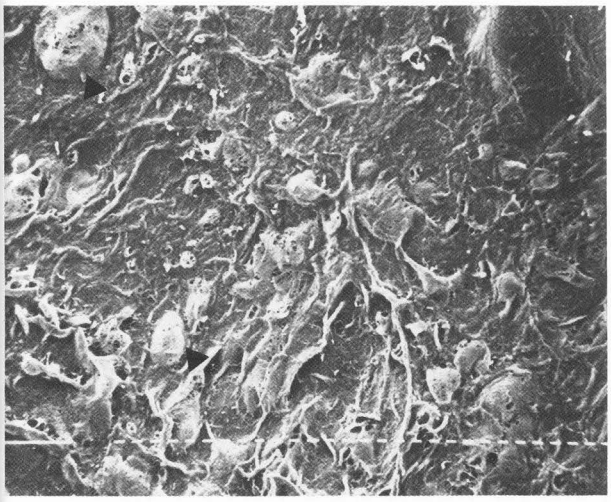

Fig. 13a - Low power view of the IEL of the digested thoracic aorta of a newborn lamb. Note the protrusions from the surface. Two of these are shown by arrows. The small bars are 10 μm.

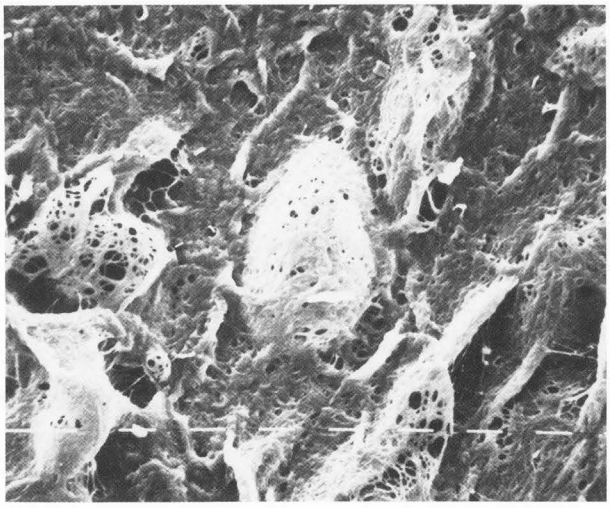

Fig. 13b - Large power view of the bumps from Fig. 13a. Note that these are produced by fenestrated elastin, often with quite large holes. They are probably regions of new elastin. The small bars are 10 μm.

TABLE 2

Statistical Parameters of the Holes in IEL of Sheep and Rabbits

	Upper Thoracic Aorta		Upper Abdominal Aorta		Lower Abdominal Aorta	
	Sheep	Rabbit	Sheep	Rabbit	Sheep	Rabbit
Mean Diam. (μm)	0.88	0.82	1.04	1.09	1.28	1.48
S.E.	0.02	0.02	0.02	0.02	0.03	0.02
Skewness	1.61	0.75	1.36	0.78	1.06	1.29
Kurtosis	7.24	3.96	5.83	3.51	4.45	5.21
Range (μm)	2.37	1.38	2.32	1.78	2.85	3.46
Median (μm)	0.82	0.79	0.98	1.06	1.25	1.36
Mode (μm)	0.78	0.77	0.90	1.00	1.15	1.27

This suggests that early atherosclerotic lesions or fatty streaks are associated with elastogenesis while the late lesions are associated with elastolysis.

Discussion

SEM is not useful to study arterial elastin if the arterial wall is intact as it is impossible to identify which surface features are due to elastin. However, since elastin is so inert chemically, the elastin appears to remain intact after all other wall components are removed by autoclaving or treatment with hot acid or alkali.

The first striking feature of arterial elastin seen grossly is that it retains the shape, and probably the size, of the aorta from which it was obtained. This suggests that the elastin is laid down in a 'formed' fashion and so retains its shape in the same way that the fingers of rubber gloves retain their shape even if they are split.

Elastin is hydrophobic and so will tend to squeeze water out and the fine fibers will collapse under the weight of the elastic laminae in air. Gosline and French (1979) have shown that elastin goes through a glass transition at a relative humidity of 97%, so it is important to keep the preparation hydrated until it is in its correct shape.

TEM studies often show fine microfibrils around the thick layers of amorphous elastin. These microfibrils are more soluble than the amorphous elastin, but probably survive hot alkali for the five hours used here. These microfibrils probably produce the irregular edges of elastin seen with TEM, but are too small to be visible with the 2500× magnification used as a maximum for our SEM studies.

We find, as have Crissman (1984), and Wasano and Yamamoto (1983) that SEM provides the best three-dimensional picture of elastin. The fact that the elastin is interconnected supports, strongly, the suggestion that it is the strutwork for the other wall components. The other components may also form a network within the wall, but they are harder to isolate by removing elastin as commercial elastase always seems to have some associated proteolytic activity.

The next factor which must be considered in this type of analysis is whether or not the fenestrations have a normal or gaussian type of distribution. Figs. 14 and 15 show data for three locations of rabbit and sheep aortas. The five rabbits weighed 1.5-2.1 kg and the five sheep 50-65 kg and Table 2 shows the shape analysis of these. None of the distributions are Poisson in type. Indeed, since the kurtosis in all of the populations from both species is greater than three, the populations are platykurtotic, but the significance of this is not obvious.

The final parameter of interest is the pattern of distribution of the fenestrations. In general the surface of the upper thoracic aorta appears almost felt-like in most species we have studied and the fenestrations are small and quite uniformly distributed (Fig. 4). By contrast, the surface of the abdominal aorta appears smoother (Fig. 8), the fenestrations are larger, and have a less uniform distribution. We have still not developed a useful way of mapping these patterns, but one would predict that they might cause more anisotropy in the elastic response than do the uniformly distributed holes. Some of the patterns

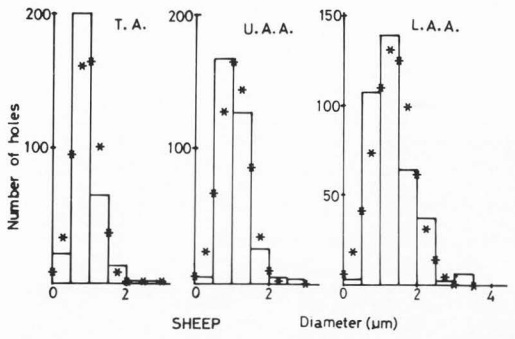

Fig. 14 - Distribution of fenestration diameters in the IEL of digested sheep aortas from the thoracic aorta (TA), upper abdominal aorta (UAA) and lower abdominal aorta (LAA). The asterisks show the medians and the bars the means.

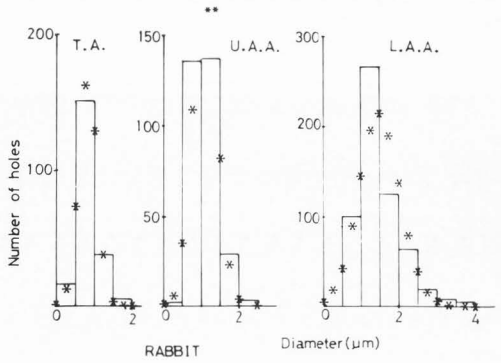

Fig. 15 - Distribution of fenestration diameters in the IEL of digested rabbit aortas from the thoracic aorta (TA), upper abdominal aorta (UAA) and lower abdominal aorta (LAA).

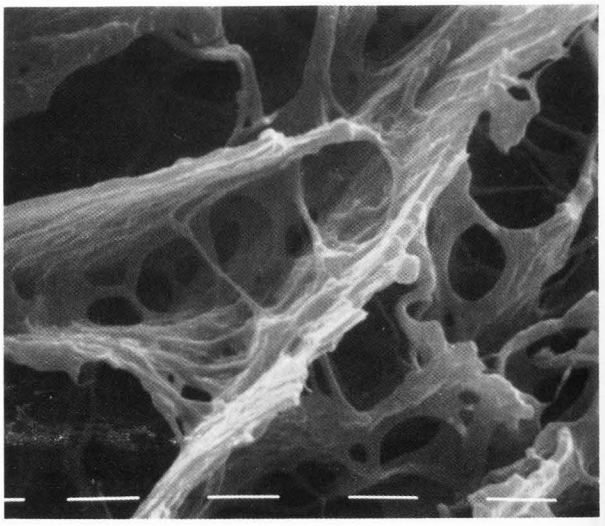

Fig. 16 - High power view of fenestrations in the media of a pig aorta. Note the complex nature of the holes and fibers. The bars are 1 μm.

are very complex as shown in Fig. 16, presumably due to indentations of other materials such as collagen (Fig. 1) and also possibly to vasa vasorum.

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Discussion with Reviewers

T.F. Robinson: The authors point out the utility of the

selective extraction of all components but elastin and the difficulty of selective extraction of elastin in a complimentary type of experiment. In lieu of extraction of elastin, have they considered the possibility of selective staining of the collagen with some type of specialized stain; for example, silver stain samples viewed with electron microscopy or back-scattered scanning electron microscopy?
Authors: No. So far we have assessed only the elastin.

B.F. Miller: Different studies all fixed the vessels of interest at various pressures, but apparently some studies had first extracted the vessels from the animal before performing any fixation procedures (Wolinsky and Glagov, 1964). Would the authors comment on how the in vitro perfusion technique compared to the results obtained with an in vivo perfusion technique? How might the different methodologies affect the apparent interactions between the various vessel wall components?
Authors: Obviously perfusion fixation in vivo without any pressure drop will provide the best geometry, and this is essential for studying the endothelial surface. Our unpublished results on in situ perfusion fixation at physiological pressure vs fixation of isolated rabbit aortas restored to their in vivo length and fixed at pressure show few differences. However the aorto-branch junctions are distorted in isolated vessels. Our studies on digested aortas were done without any fixation for the reasons discussed in the text. At the present time there is still too little understanding of the exact geometry of the various wall components to devise a detailed model which also includes how the components are interconnected.

B.F. Miller: Some studies have described the fenestrations of the IEL as being arranged in a distinct pattern that is associated with the presence of anchoring filaments (Greensmith and Duling, Am. J. Physiol. 247:H687-H698, 1984). How did you determine that the fenestrations were distributed uniformly within the IEL?
Authors: We have not yet measured the distribution quantitatively. In the thoracic aorta the fenestrations are roughly uniformly spaced as determined by marking them on a clear sheet and then moving the sheet. They probably are not uniformly spaced elsewhere, and we still need to develop a computer program to analyze this. Campbell and Roach (1983a) tested the effect of different spaces and hole sizes on the elastic behaviour of sheets of latex rubber with holes removed, and found that the mean ligament efficiency (interedge distance/intercenter distance between holes) appeared to be the important variable for determining the effect of the holes on the elastic properties of the sheet.

B.F. Miller: What is the significance, if any, of the interlacing network of elastin fibrils that are on the luminal surface of the IEL (lower left corner of Fig. 5)? Why might such a network be evident on this surface?
Authors: Fig. 5 is at the aorto-iliac junction, and the wrinkles are produced by trying to flatten it out. The bigger fenestrations in the center are the junction while those on the right are on the aorta. The IEL of the aorta was cut to show the underlying medial elastin which has much larger holes. The fine 'fibrils' in the lower left are probably a combination of folding and stretching artifacts.

B.F. Miller: Fig. 3 suggested that the thick elastic lamellae are tubes or sheets which are arranged like "concentric cylinders". Later, "medial layers" of fenestrated sheets of elastin are described as being "parallel to each other and also to the surface of the stub" (Fig. 6). Would the authors describe how the "lamellae" are concentric and parallel to the stub at the same time? Are the tubes of elastin flattened prior to freeze drying, cutting, and measuring the size of medial fenestrae (eg. Fig. 8)? If so, how were the tubes of elastin flattened?
Authors: With the light microscope, cross-sections of the aorta show rings of elastin which encircle the lumen as concentric rings. If these are expanded into cylinders of artery, it has been assumed that there are cylinders of elastin. There is no doubt from our work and that of many others that the internal elastic lamina is continuous (except for the fenestrations), so it must be a cylindrical shell. The medial elastin is harder to expose, so we are guessing that these layers too are cylindrical shells.

We digest small aortas intact (fig. 2) and then cut them. Larger ones are digested as long rings and then cut longitudinally, usually into two strips which are semicylinders. In large aortas, the radius of curvature is large enough that the region glued on the stub is parallel to the surface. The medial layers are exposed by making oblique cuts in the freeze-dried specimens as described in the text. We have found that as long as the surface is within 10° of the horizontal (i.e. within 10° of being perpendicular to the beam) no correction for tilt is needed. It is easier to count the layers on the cut edge when it is almost perpendicular to the stub, but the details of hole size have to be measured from surfaces which are perpendicular to the beam.

B.F. Miller: What are the means ± standard deviation or standard error for the data in fig. 7? What criteria was used to determine "that there is very little difference in size throughout the media"? Does the adventitial elastin also have an organization that suggested the presence of lamellae?
Authors: This study was done by Mark Noss who worked with us as a summer student. You will note that we do not have data for all layers for any one pig - e.g. there are no solid circles beyond layer 30, and no open triangles under layer 90 except in the IEL. Technically it is very difficult to expose all of the layers in a single aorta. We tested this data in two ways. First, means were obtained for all aortas in 5 layer increments pooling data from all pigs. Here the data from the first few layers seemed to be in two groups (solid triangles and circles) and (open and closed squares). The first group was significantly different, but none of the other groups were. Since the IEL is hardest to cut, we are concerned this difference in two samples may be incorrect. We also calculated regression lines from layer 1-100 for all of the data and the slope was not significantly different from zero. We plan to do a more thorough study on more pigs and will do a statistical analysis on it.

With regard to the second question, we have calculated the area of the fenestrations using a digitizing board and trapezoidal rules. If the fenestrations are circular, as they are in the IEL and cerebral arteries, we have calculated the diameters. Many of the medial holes appear ellipsoidal and so these have been left as areas. The area is probably a more logical parameter.

B.F. Miller: In the section titled "Shrinkage Artifacts" reference was made to the fact that freeze-drying induced some shrinkage of elastin. How might the presence of other wall components within the specimens above influence the results? How did the authors determine what constituted a standard length of elastin? How did the authors identify an "unstretched length of elastin"

versus the other physical conditions of stretch and compression? Do the authors have any physical evidence that the process of dehydration does not affect or alter the morphology of elastin?
Authors: Water expands as it freezes (i.e. from 4-0°C), but shrinks as it cools. Most biological tissues contain a lot of water, but to our knowledge there have been no studies done to show how their size changes with freezing. Elastin becomes glass-like at 97% humidity and so should not change size once it reaches this level. Campbell and Roach (1983c) photographed specimens before and after digestion and then as the tissue was dried. They used the photographs, and also fluorescent beads on the surface, to measure shrinkage. They were not able to assess if the fenestrations changed size.

In terms of "unstretched length" used to normalize the length changes during measurement of elastic properties, this is considered to be the value where no stress (stress = force/unit area) is measured, and is usually obtained photographically.

We have no evidence that dehydration does not alter the morphology of elastin. Critical point drying tends to make it more fibrous. The elastin appears sheet-like in the wet state with phase contrast, but can be made visibly fibrous under these conditions if it is stretched.

B.F. Miller: Under "fixation artifacts" the authors note again that aldehyde fixation does not denature elastin and that, once the collagen and vascular smooth muscle are removed, hydrated elastin returns to its unstretched length. I would concur with the authors that the IEL in figs. 4, 5 and 8 were not "wavy" in the manner of immersion fixed vessels. However, I would be reticent to describe the IEL in figs. 5 and 8 as "flat". Would the authors please discuss why they seem to emphasize this improvement in shape more than called for?
Authors: If digested aorta is removed from water, surface forces fatten it and folds are common. Once this specimen dries, it has many strange shapes. If drying is not uniform, the dry elastin is brittle and the wet elastin very extensible and so edge effects can produce large artifacts. In virtually all cases if the elastin was freeze-dried in a container with water rather than alone, the shape of the vessel was maintained and the elastin then appeared smooth (it was flat locally if the radius of curvature was high).

Digestion of fixed vessels tended to cause irregular removal of non-elastin, and the edges of the elastin often appeared ragged. Data now being prepared for publication (Dunmore, Song and Roach) showed no difference in fenestration size if the vessels were digested fresh, immersion fixed, or pressure-fixed. While this works to study elastin, it is not a feasible method to study other parts of the arterial wall, or to determine how the elastin is related to the other components.

M. Richardson: How do the authors relate the structural inter-relationship of elastin to the proteoglycans of the arterial wall?
Authors: This a very good question. Proteoglycans almost certainly go through the fenestrations, but we have not assessed them directly. This question can probably be answered only with a good TEM study.

T.F. Robinson: What is the function of the fenestrations?
Authors: We believe they allow material such as collagen, proteoglycans and sometimes muscle to pass between the layers. Thus they strengthen the wall, but may also cause local changes in permeability. To our knowledge, this has not been studied.

T.F. Robinson: What is the significance of the change in configuration of the fenestrated lamellae and the fiber-like appearance in stretched samples? Does this change have relevance in vivo?
Authors: We are not sure. Arteries normally stretch more than this, but Wolinsky and Glagov (1964) have shown in rabbit aortas that the elastin is wavy below 60-80 mm Hg pressure so the waves are straightened rather than the elastin stretched below this. The observation might suggest that severe hypertension could cause elastin damage, but we have not assessed this experimentally yet. We have seen a similar change with critical point drying in a few cases, so this may be only an artifact. It does point out the importance of careful handling of the elastin.

M. Richardson: How does the observation of van Baardwijk and Roach (1983) that there is a different distribution of MLU along the length of the aorta relate to that of Wolinsky et al., specifically does the tension/MLU vary with anatomic position, but not with vessel size in species? Is the difference observed in the abdominal aorta of man compared to mice or horses, also seen in other bipeds?
Authors: Wolinsky and Glagov confined their studies to two regions - one the middle of the thoracic aorta, and the other the middle of the abdominal aorta, but studied them in many species. van Baardwijk and Roach studied only the lamb and the sheep, but did the analysis along the whole length of the aorta. From the latter study, we believe that the tension/MLU is probably constant along the length of the abdominal aorta, but changes significantly along the length of the thoracic aorta. It seems likely that this difference occurs in other species but this has not been studied to our knowledge. It is interesting that the gradient is continuous across the diaphragm in the lamb, but not the sheep. This difference in the sheep is sufficient to cause a discontinuity in the pressure wave, and so could set up reflections. It would be interesting to know whether species such as man that develop abdominal aortic disease have this abrupt change while others do not.

M. Richardson: Why does elastin change its morphology when subjected to 25% strain if it is capable of 100% stretch?
Authors: We are not sure. Many of the extensibility studies of elastin have been done on ligamentum nuchae. Ross and Bornstein (1969) have shown that elastin is made up of microfibrils and amorphous material, but the relative roles played by each are unknown. It is strange that the whole sheet does not become fibrous, although stress is known to be almost tripled at the edge of holes. This observation may be only an artifact, or could have physiological significance. We plan to do more studies to try to answer this question.

M.W.C. Hatton: Dilation of the arteries will increase the surface area of the vessel wall and, as a consequence, may distort the fenestrations in the IEL from a circular to an oval shape. Alternatively, during dilation, the fenestrations may not change shape significantly. The latter situation could arise if the elastin 'sheets' were sliding over each other, a movement which may either involve stretching of the fine fibers between the sheets or lead to a decreased distance between the sheets. From your analysis of stretched elastin, e.g. Fig. 9b, are the fenestrations noticeably elongated, are the inter-sheet fibres stretched or

is the inter-sheet distance decreased?
Authors: This is a very good question. Richard Potter is analyzing the changes in a poststenotic dilatation in rabbits as his Ph.D. project. Fig. 10 shows his analysis of the IEL fenestrations so far, but he plans to do a larger series to obtain a wider range of sizes of dilatation to determine if there is a correlation between the size of the fenestrations and the size of the distribution. Since the aorta has multiple layers of elastin, the changes, if any, in the medial layers must also be assessed. Campbell and Roach (1983a,b; 1984) showed that larger holes in latex make the sheet more distensible, and Fig. 7 shows that the medial holes are larger than the IEL ones in the ascending aorta of the pig. Since the IEL and the medial elastin are in parallel rather than in series, the stiffer layer (i.e. the one with the smaller holes if both sheets are the same) should play the predominant role. Our SEM and light microscopic studies both suggest that the IEL is thicker than the medial elastin, but we have no experimental information about their relative elastic properties. Potter is also assessing the width of the interelastin space and doing stereological studies on the muscle as seen with TEM. Hopefully when his study is completed we can answer your question. We are not confident that the spacing between layers seen in digested specimens is the same as that which would be present if the other wall components were present. To answer this question, it would be necessary to quantify all the artefacts with each technique and then compare them. Since we do not have our own TEM facilities we are unlikely to be able to answer the general question in the near future.

B.F. Miller: Reference was made to the work by Clark and Glagov (1979, 1985) which described the attachment of elastin fibrils to the vascular smooth muscle cells in maturing pigs and rabbits. Would the authors expand on how "these two components appear to be attached to each other"?
Authors: The TEM photographs show them adjacent to each other, but there was no detailed analysis of where or how they were attached. Since the wall remains compactly together after removal (except in some old and/or diseased human arteries), it seems likely adjacent layers are attached to each other, but this could be with an interlacing weave or with biological glue.

B.F. Miller: Have you been able to determine the average size of the fenestrations in the adventitial elastin? If so, how do they compare with those of the media? Does the adventitial elastin also have an organization that suggests the presence of lamella.
Authors: In our first studies on sheep aortas we found the adventitial fenestrations were much larger. The adventitia is the layer most apt to be damaged as the vessel is removed, and after we found that even 10% strain of pure elastin changed the sheets to fibers, we stopped measuring the adventitial fenestrations until we can resolve if the adventitia is or is not fibrous. At junctions, the light microscope shows a very complex pattern in the adventitia. Muscular arteries, which we have not studied, show an external elastic membrane made up primarily of sheets of elastin while the aorta does not have this 'membrane'. Since large vasa vasora have some elastin in the walls, it is important to identify these as distinct from adventitial elastin.

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