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COMMUNICATIONS

STUDIES ON DEVELOPING RETINAL VESSELS

IX. REACTION OF ENDOTHELIAL CELLS TO OXYGEN*

BY

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IN reviewing the mechanisms which might be involved in the phenomenon of oxygen vaso-obliteration in the immature retina, it was recently suggested that swelling of the retinal tissue around the vessels could be the primary factor concerned. The detailed evidence upon which this suggestion was based has already been reported (Ashton, 1957; Ashton, Graymore, and Pedler, 1957) and further supporting data have since been published (Graymore, 1958; Pedler, 1959a, b). It was pointed out, however, that a more direct action of oxygen on the vessel wall could not be excluded by the negative evidence then obtained, because the techniques available at that time were not entirely satisfactory. With the development of the new digest method of Kuwabara and Cogan (1960), it has been possible to isolate the affected capillaries, and with the acquisition of an electron microscope at this Institute a detailed study of the changes in the internal structure of the endothelial cell has become feasible. The findings obtained are presented and discussed in this paper.

MATERIALS AND METHODS

The experiments were carried out on the same plan as in previous work, the mother cats and kittens of 1 to 14 days old being placed in 70 to 80 per cent. oxygen for varying periods of time. To determine the time of onset of the retinal changes, five kittens of 5 days old were subjected to oxygen for periods of 6, 9, 12, 18, and 24 hrs respectively. The experiments were terminated by intraperitoneal Nembutal and various procedures were then adopted; in one animal both retinae were digested, in seven animals one eye was removed for digestion while the other was injected with Indian ink *via* the left ventricle to demonstrate the degree of vaso-obliteration obtained, in another animal one eye was digested and the other examined by electron microscopy, and in three animals one eye was injected and the other examined by electron microscopy. The retinal vessels of an adult cat (50

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days old) subjected to 16 days' oxygen were also examined by digestion. In addition, normal controls of both cats and kittens were examined both by digestion and electron microscopy.

For electron microscopy the eyes were fixed in osmium tetroxide as described below; in all other cases the eyes were fixed in 10 per cent. formol saline. In one experiment meningeal and cerebral capillaries were isolated for examination by the "shake" method (Ashton, 1949).

Digest Methods.—The retinae were digested either by our own pepsin-trypsin method or by the trypsin technique of Kuwabara and Cogan (1960), and the isolated vessels were examined by phase contrast and dark-ground illumination, and were subsequently stained with haematoxylin and eosin, periodic acid Schiff and haematoxylin.

Electron Microscopic Methods.—The eye was removed from the animal and bisected equatorially in the coronal plane. The posterior half containing the retina was then placed under a dissecting microscope dry, the vitreous was removed, and the retina was pulled out in fragments which were immediately immersed in a cold solution of veronal-buffered osmium tetroxide for a maximum period of 3 hrs. After graded alcohol dehydration, the tissues were immersed in a 1 per cent. alcoholic solution of phosphotungstic acid for approximately 1 hr in order to enhance electron contrast. With the exception of Fig. 14, which shows a methacrylate embedded vessel, the specimens were finally embedded in Araldite and sections cut in a Huxley-pattern mechanical advance microtome at thicknesses within the range of 500–1000 Å. The microscope used is an A.E.I. E.M.6 model, and the photographs were taken using an objective aperture of 25 μ. Magnifications are given as a product of the primary figure achieved in the microscope and the secondary optical enlargement.

Findings in Digest Preparations

In all test-animals the degree of obliteration was exactly as to be expected from our previous studies; that is, its extent depended upon the time of exposure and was complete in all kittens exposed for over 36 hrs.

The earliest discernible changes in the vessels in digest preparations of the retina were evident as early as 6 hrs after exposure; they consisted of widespread patchy capillary obliteration with elongation of the endothelial nuclei which had come to lie in single file in the long axis of the capillary; mural cells were indefinite at this stage of development (Fig. 1*a*, *b*, *c*, opposite).

This appeared to be followed by a gradual withdrawal (or displacement) of the endothelial cells, some of which showed pyknosis, from the closed capillary towards less affected vessels (Fig. 2, overleaf), similar to the process of retraction in normal capillary growth (Fig. 3, overleaf) (Ashton, 1961). At a more advanced stage (6–24 hrs) endothelial cells had undergone degenerative changes, the cytoplasm was markedly eosinophilic and had

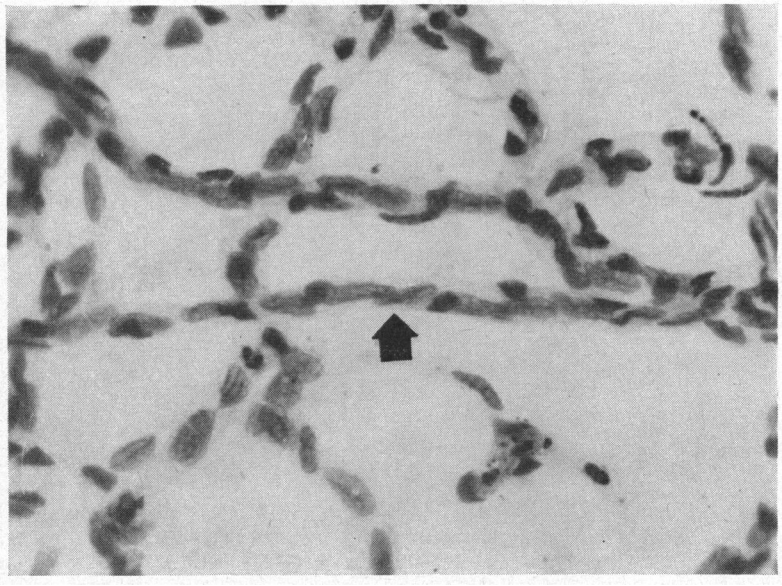


FIG. 1(*a*).—The first discernible change in the retinae of oxygen-exposed animals is capillary closure with elongation of endothelial nuclei which come to lie in single file in the long axis of the vessel (arrow). Digest preparation. Haematoxylin and eosin. ×500.

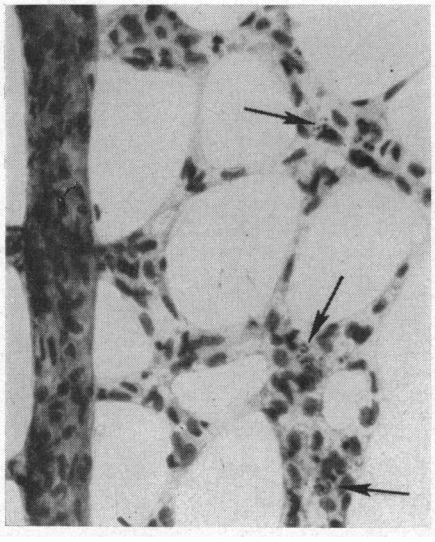


FIG. 1(*b*).—Capillary network (arteriole on the left) after 9 hrs' exposure to oxygen. There is already marked attenuation of some of the capillaries and degenerative pyknotic changes in the endothelial cells may be seen (arrows). Digest preparation. Periodic acid Schiff and haematoxylin. ×330.

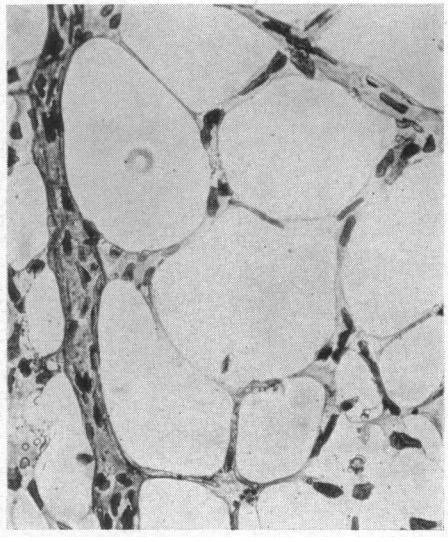


FIG. 1(*c*).—Capillary network (arteriole on the left) after 12 hrs' exposure to oxygen. Attenuation of the capillaries is now extreme, endothelial cells have withdrawn from the obliterating capillaries, and there is an overall loss of endothelial nuclei. Digest preparation. Periodic acid Schiff and haematoxylin. ×330.

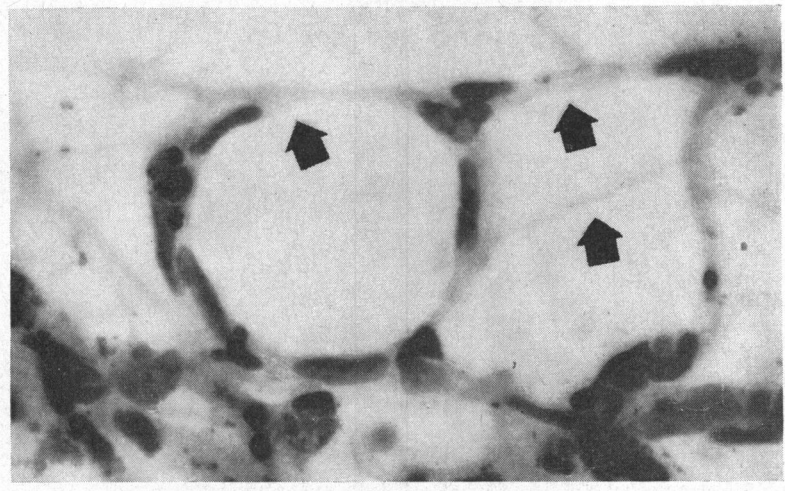


FIG. 2.—Capillary closure is followed by withdrawal of endothelial cells from affected vessels (arrows), as in the normal process of capillary retraction (see Fig. 3). Digest preparation. Haematoxylin and eosin. ×700.

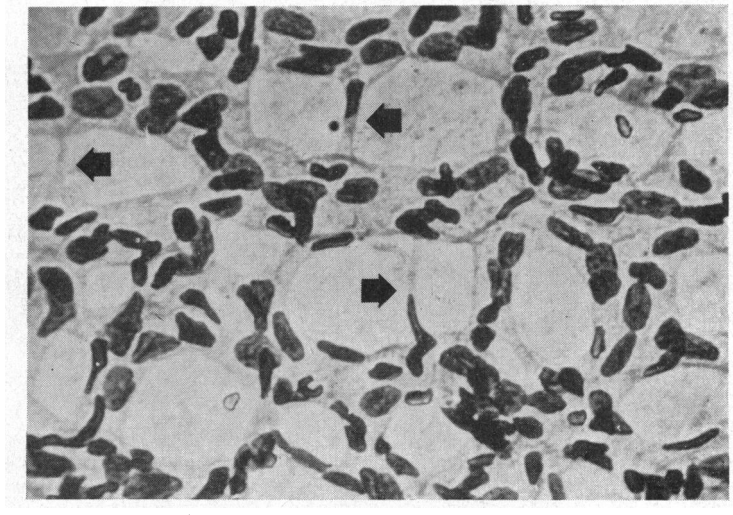


FIG. 3.—Normal developing kitten retina, showing numerous retracting vessels in some of which the endothelial cells can be seen withdrawing from the constricting capillaries (arrows). Digest preparation. Haematoxylin and eosin. Phase contrast. ×525.

broken down into irregular globules, and the nuclei showed karyorrhexis, pyknosis, and finally chromatolysis (Figs 4 and 5).

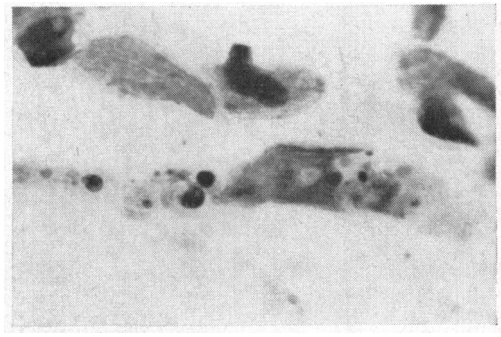


FIG. 4.—Endothelial cell undergoing degenerative changes. The cytoplasm becomes eosinophilic and breaks down into irregular globules, while the nucleus shows karyorrhexis, pyknosis, and finally chromatolysis. Haematoxylin and eosin. ×1,200.

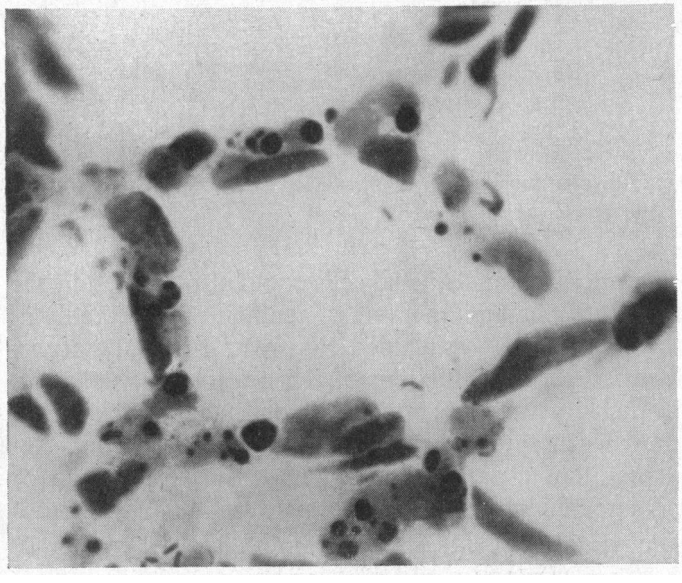


FIG. 5.—Degenerating endothelial cells in capillary network. Haematoxylin and eosin. ×1,200.

As this process extended, the degenerating cells which appeared in the walls and lumina of the capillaries (Fig. 6, overleaf) gradually converged upon isolated capillary islands from which they could escape no further, and here, together with eosinophilic and neutrophilic polymorphonuclears, they formed bizarre aggregations of degenerative cellular debris, while the original capillary network was represented only by delicate threadlike strands devoid of nuclei except for a few pyknotic fragments (Figs 7, 8, 9, 10, overleaf).

Finally the islands were absorbed and no trace of the endothelial cells, except for the persisting strands, remained.

The capillaries were the vessels most profoundly affected, particularly at the periphery of the growing vascular complexes, but we were not able to decide whether the arterial capillaries were more markedly affected than those

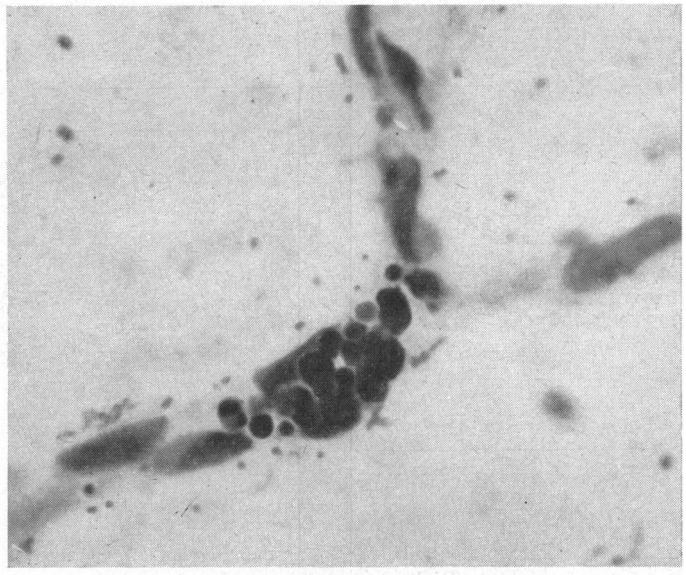


FIG. 6.—Pyknotic cellular debris lying in the lumen of a capillary. Haematoxylin and eosin. ×1,100.

on the venous side. Degenerative endothelial cells could also be seen in the larger vessels, both in the arteries and the veins—again more markedly at the periphery—and the impression was gained that pyknosis and karyorrhexis were more evident in the endothelium of the arterial walls than in those of the veins. In the larger vessels migration of the endothelial cells did not occur and they degenerated *in situ*. The changes, however, undoubtedly affected the capillaries predominantly, and also selectively, for other cells in the immediate vicinity of the capillaries were apparently completely unaffected. The retinal vessels of the cat submitted to oxygen for 16 days showed no endothelial changes in digest preparations. The cerebral and meningeal vessels examined in shake preparations showed no degenerative endothelial changes.

FINDINGS WITH ELECTRON MICROSCOPY

Normal Vessels.—Comparison between oxygen-treated and control eyes were made, as far as possible, between vessels of similar diameter. This precaution is perhaps not so important in the developing retina as it would be in more mature tissues, since it appears from our studies that, during vessel growth (when oxygen sensitivity is still present), vessels of a widely differing diameter show an exactly similar mural construction. This is further borne out by the fact that we observed similar pathological changes in vessels of many different sizes. An example of the morphological similarity between

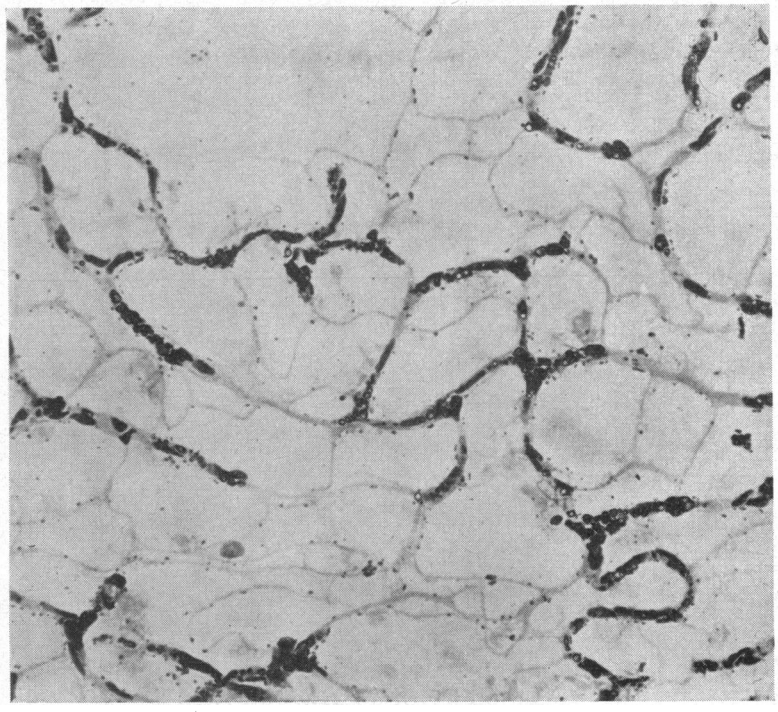


FIG. 7.—General picture of the process of vaso-obliteration as seen in a digest specimen. Degenerating endothelial cells have withdrawn into isolated capillary islands, while the original capillary network is represented only by delicate thread-like strands. Digest preparation. Haematoxylin and eosin. ×172.

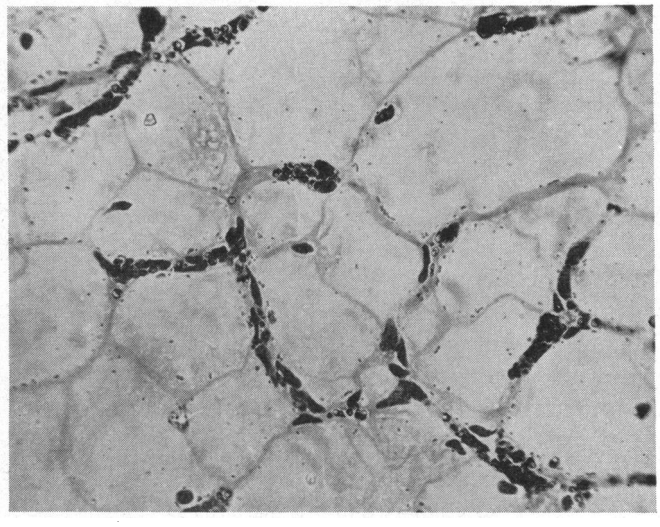


FIG. 8.—High-power view of Fig. 7, showing greater detail of obliterative process. ×330.

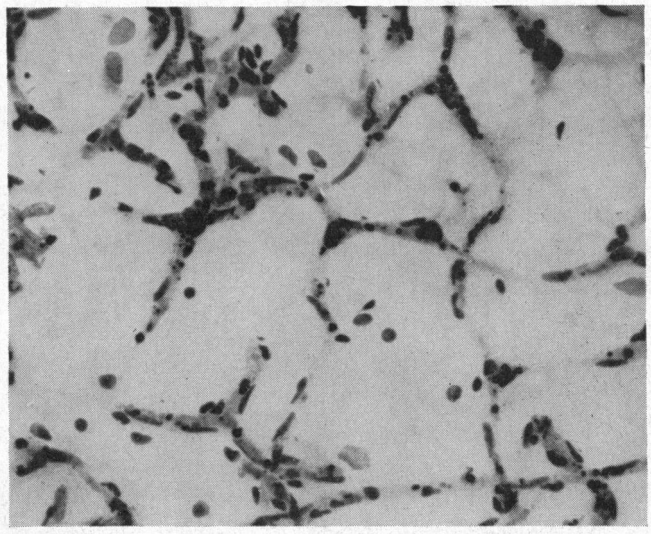


FIG. 9.—Extent of nuclear disintegration as endothelial cells withdraw from capillaries to form islands of cellular debris. Digest preparation. Haematoxylin and eosin. ×315.

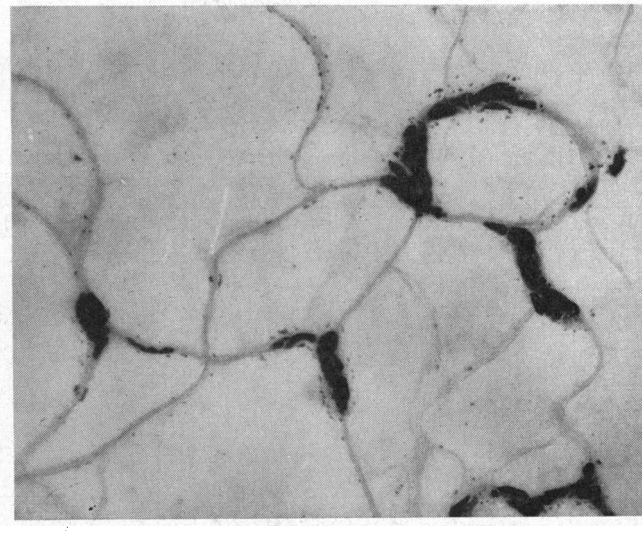
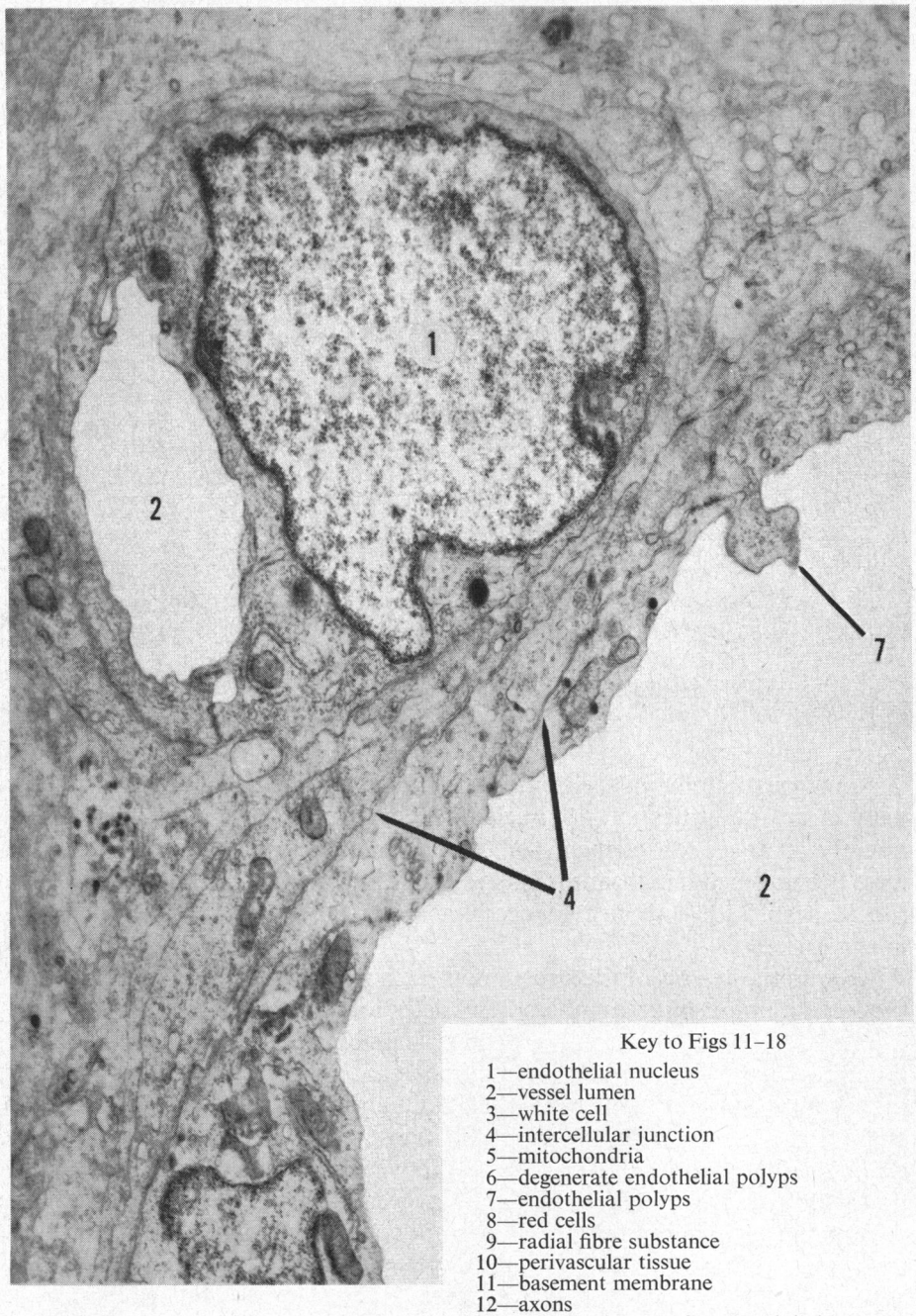


FIG. 10.—In the final stages of oxygen-obliteration the islands themselves disappear leaving only a skeleton of the original capillary plexus. Digest preparation. Haematoxylin and eosin. Phase contrast. ×330.

large and small vessels is shown in Fig. 11 (opposite), which is a transverse section of two vessels: at the top on the left is a single capillary composed of three cells; below on the right is the lumen of a larger vessel separated from the capillary by its endothelial lining. In both vessels, the endothelium is structurally similar and in the larger vessel there is no sign as yet of muscular, collagenous, or elastic components in the wall. Another feature which we have found to be characteristic of developing retinal vessels in the kitten is a number of endothelial protrusions contained within an intact plasma membrane projecting into the lumen (7), as in Figs 11 and 12. The size of these polypoid masses usually varies in direct proportion to the diameter of



Key to Figs 11–18

1—endothelial nucleus
2—vessel lumen
3—white cell
4—intercellular junction
5—mitochondria
6—degenerate endothelial polyps
7—endothelial polyps
8—red cells
9—radial fibre substance
10—perivascular tissue
11—basement membrane
12—axons
13—abnormal spaces found mainly in oxygen-treated retinae
14—swollen endothelium
15—thickened intercellular junctions
16—debris occluding lumen

FIG. 11.—Transverse section through two vessels of a control retina, showing similar mural construction of vessels of different size. ×18,000.

the vessel from which they take origin, although occasionally in the smaller vessels they appear almost large enough to occlude the lumen. Where the vessel wall is composed of more than one layer of endothelial cytoplasm, the protrusions always arise from the surface of the innermost cell (see Fig. 11).

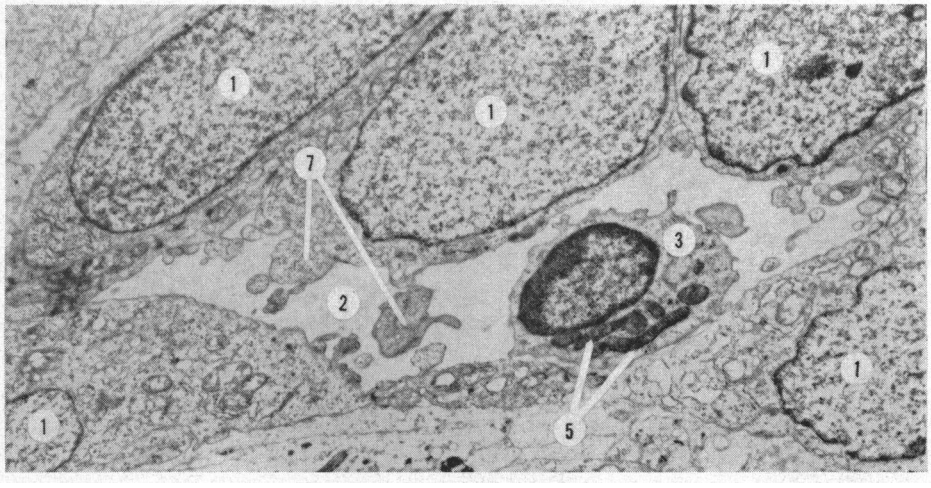


FIG. 12.—Transverse section through lumen of a control vessel, showing endothelial polyps protruding into the lumen. ×3,500.

Numerous mitochondria are present in the endothelial cytoplasm, together with large quantities of ribonucleoprotein granules, which are most frequently arranged on elements of the endoplasmic reticulum to form what have been termed membranes (Sjöstrand, 1956), and which have been thought (de Robertis, 1960) to be evidence of active protein synthesis—a likely event in this case.

Around the vessels of the stratum opticum is an extremely complex mass of tissue containing large numbers of vesicles and small fibrils segregated into isolated polygonal masses by plasma membranes, which are themselves separated by an intercellular space of constant width. This is the immature radial-fibre complex which, in the adult animal, develops into the more characteristic and easily recognizable tissue surrounding all the neuronal elements of the retina (Pedler, 1961). Between the radial-fibre substance and the outer aspect of the endothelium, however, there is a narrow perivascular coat of fine granular material. This is not constantly found around all vessels and is probably related either to the developing radial fibres or to the feet of adjacent astroglia attached to the vessel wall; it is not yet possible to be certain which of these two alternatives is correct, although the difference between the granular material of the perivascular tissue and the vesicular components of the radial fibre complex makes a radial fibre origin less likely.

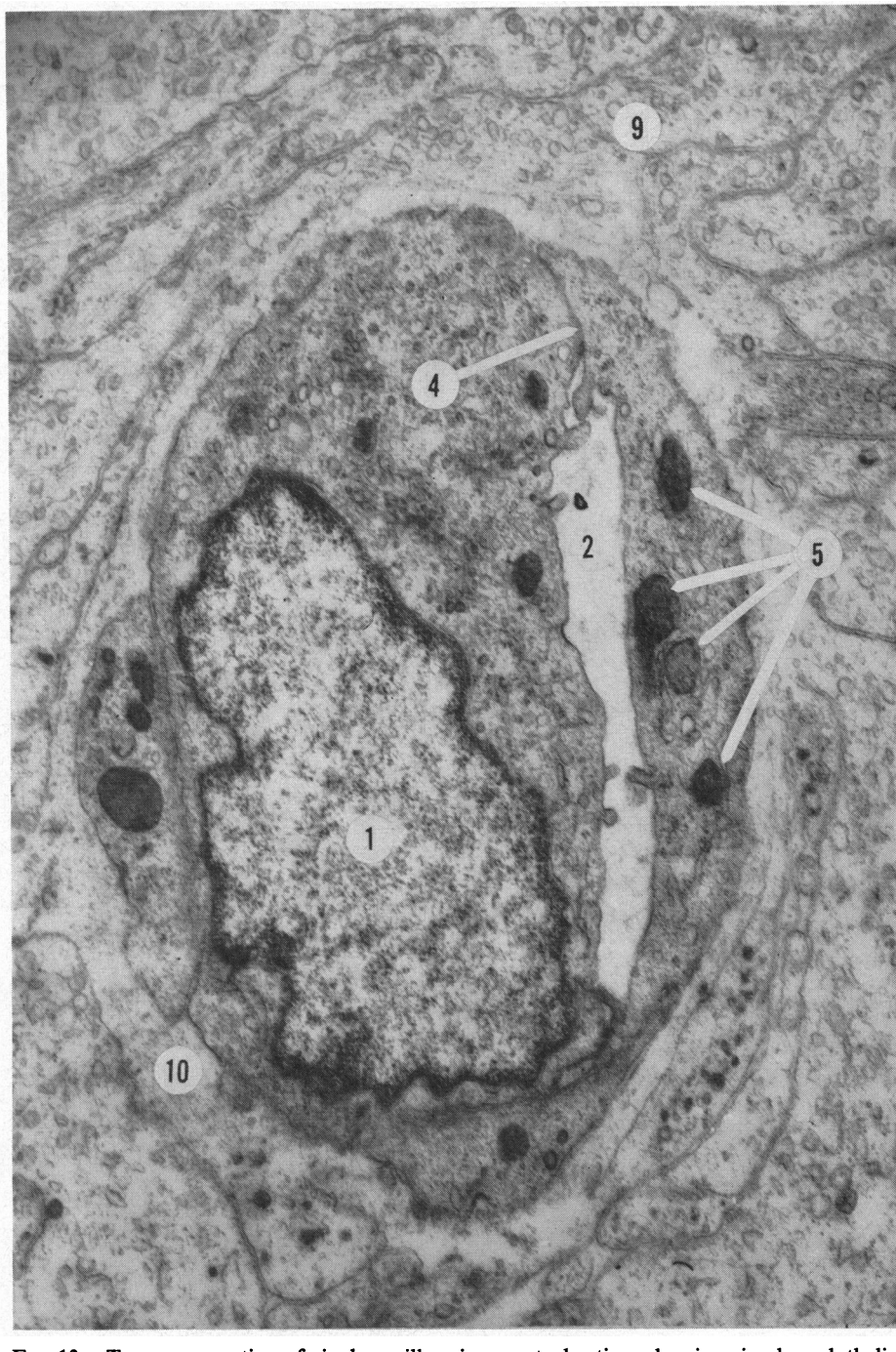


FIG. 13.—Transverse section of single capillary in a control retina, showing simple endothelial construction of the vessel and its close relationship with the surrounding retinal parenchyma. ×24,000.

Perhaps the most striking feature of the developing retinal vessels is the complete absence of the basement membrane. This is demonstrated by comparing Figs 13 and 14. Fig. 13 is a capillary from a control kitten of an oxygen experiment, and Fig. 14 shows a capillary from the nerve-fibre layer of the retina of a normal adult cat.

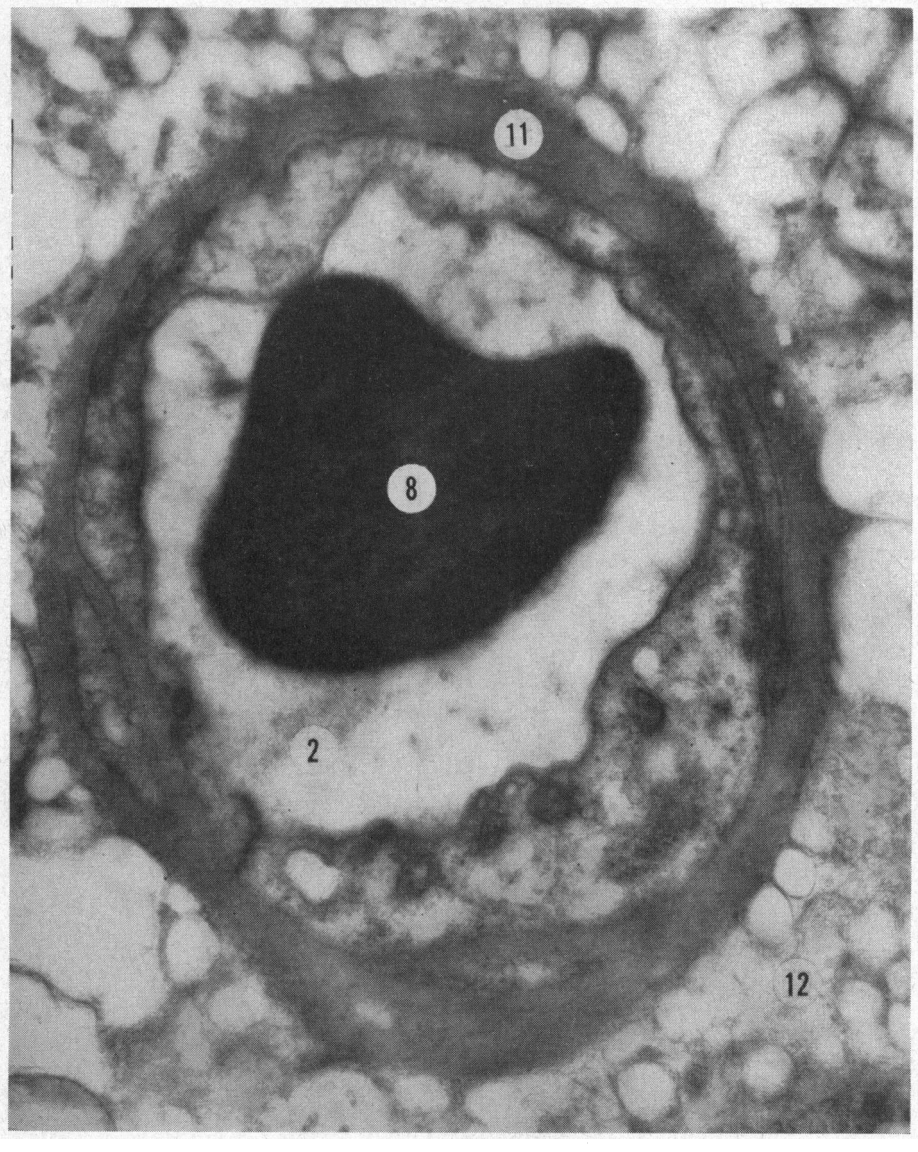


FIG. 14.—Transverse section of single capillary from nerve-fibre layer of an adult normal cat, showing presence of basement membrane. ×26,000.

It is at once obvious that the capillary of the kitten is entirely composed of endothelial cytoplasm, whereas the capillary of the cat is surrounded by a well-marked basement membrane enclosing and separating elements of endothelium. In general, the shape of the basement membrane in transverse section is very similar to the lines of intercellular junction present in analogous sections of immature vessels, suggesting that it is formed by structures somewhere in the region of the endothelial plasma membranes.

Changes following Exposure to Hyperoxia.—Figs 15, 16, 17, and 18 are examples of the different stages of oxygen-induced vessel damage in increasing severity.

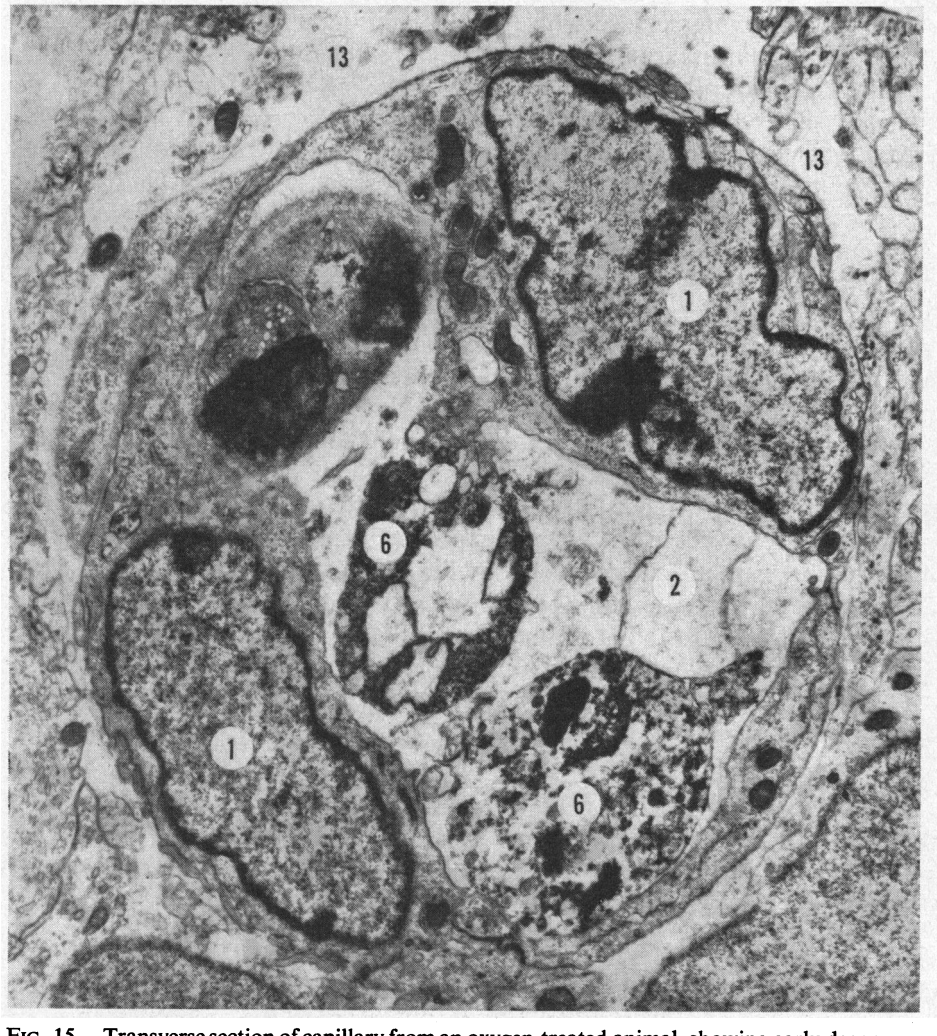


FIG. 15.—Transverse section of capillary from an oxygen-treated animal, showing early degenerative changes in an endothelial polyp. ×10,000.

The earliest vascular changes seen with the electron microscope were those in the endothelial polyps projecting into the lumen. An example is seen in Fig. 15, which shows a transverse section of an oxygen-treated capillary. The polyp is vacuolated and the cytoplasmic matrix has coalesced, leading to a marked increase in electron density, which may, since this is a preparation treated with osmium tetroxide, represent a form of lipoid degeneration. Part of the lumen is occluded by a further mass of degenerate endothelium, which, in this particular specimen, probably arose from a point *distant* to the plane of section, since it is partially enclosed by a surface membrane separating it from the internal aspect of the vessel wall. At the top on the left there is part of a degenerate leucocyte, and throughout the remaining lumen there is finely granular debris interspersed with fragments of disrupted cell. At this early stage of oxygen-induced damage, the endothelial cytoplasm shows only minimal changes other than in the polyps; the mitochondria are normal, no obvious cell swelling has occurred, and the plasma–membrane outlines remain intact and clear, particularly if due allowance is made for the probability that tissue outlines as seen in the electron microscope tend to be indistinct in developing tissues (Pease, 1960). Fig. 16 (opposite) shows a similar vessel also containing fragments of degenerate endothelium and an almost amorphous body of uncertain origin which may be part of a pyknotic nucleus or a partially absorbed red cell. The remaining lumen is again filled by finely granular material which is presumably plasma. Surrounding the vessel are groups of unmyelinated axons from the developing nerve-fibre layer, and radial-fibre substance split up by a number of irregular spaces bounded by ruptured tissue planes. At first it was thought that the spaces were an artefact of preparation, but after the examination of a number of specimens from different experiments, it was found that they occurred more frequently and more extensively in the oxygen-treated tissues. Any spaces seen in control retinae were all considerably smaller and often related to actual dehiscences in the Araldite resin and were not predominantly perivascular in distribution. Against this observation must be set the obvious fact that errors of sampling due to the small proportion of any one tissue examined in the electron microscope are very large.

Fig. 17 (overleaf) shows a transverse section of a more severely affected vessel. The endothelial cell outlines, particularly internally, have now largely disappeared and those that remain are continuous with heterogeneous masses of debris almost filling the lumen. At this stage it is no longer possible to be certain about the nature of the material in the lumen; it is most probably a mixture, composed of degenerate endothelial remnants, red cells, white cells, and plasma. The fine structure of the endothelial cytoplasm is now also severely affected; the various organelles are swollen, misshapen, and partially disrupted, and in places the finely granular hyaloplasm has become fibrillar in nature, presenting an appearance similar to the tonofibrils of

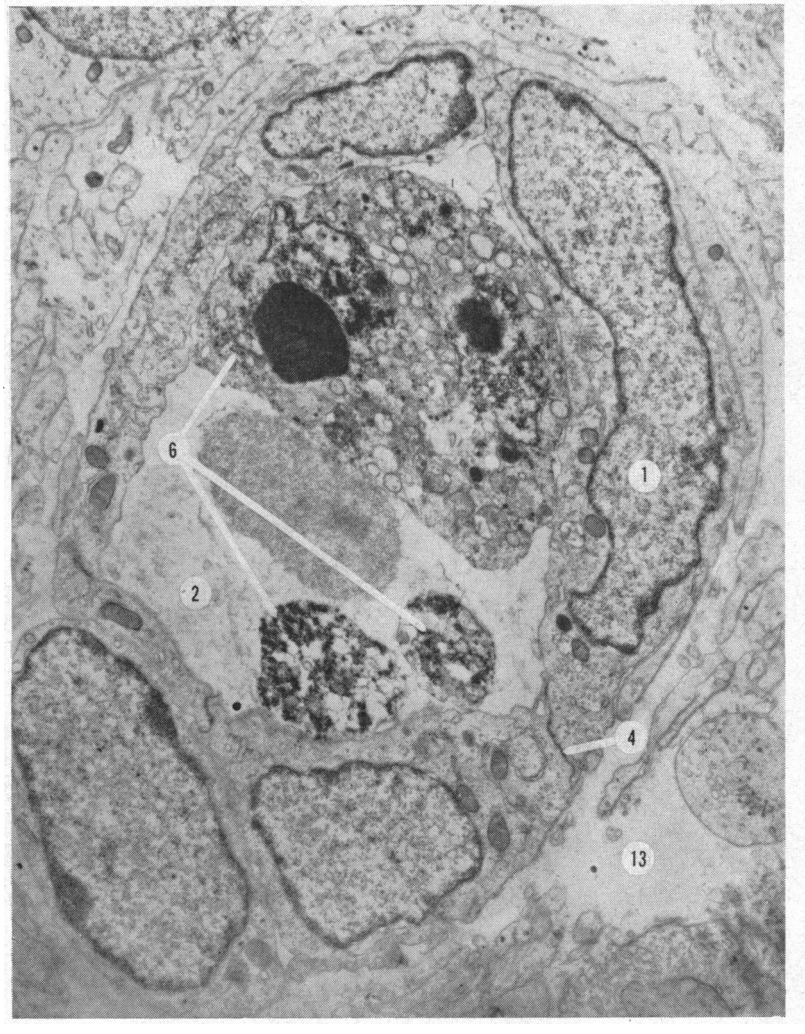


FIG. 16.—Transverse section of capillary from an oxygen-treated kitten. The lumen is partially occluded by endothelial debris and the perivascular spaces are now prominent. ×7,200.

desquamating epithelial cells. Cytoplasmic vacuoles are frequent and the perivascular spaces described above are prominent.

The vessel shown in Fig. 18 (overleaf) is an example of the most advanced changes seen in these experiments with the electron microscope: occlusion is now complete, the junction between the endothelium and the debris in the lumen is even less distinguishable and the intercellular junctions are thickened, dense, and uneven. In addition, the fibrillar changes in the cytoplasm are more widespread.

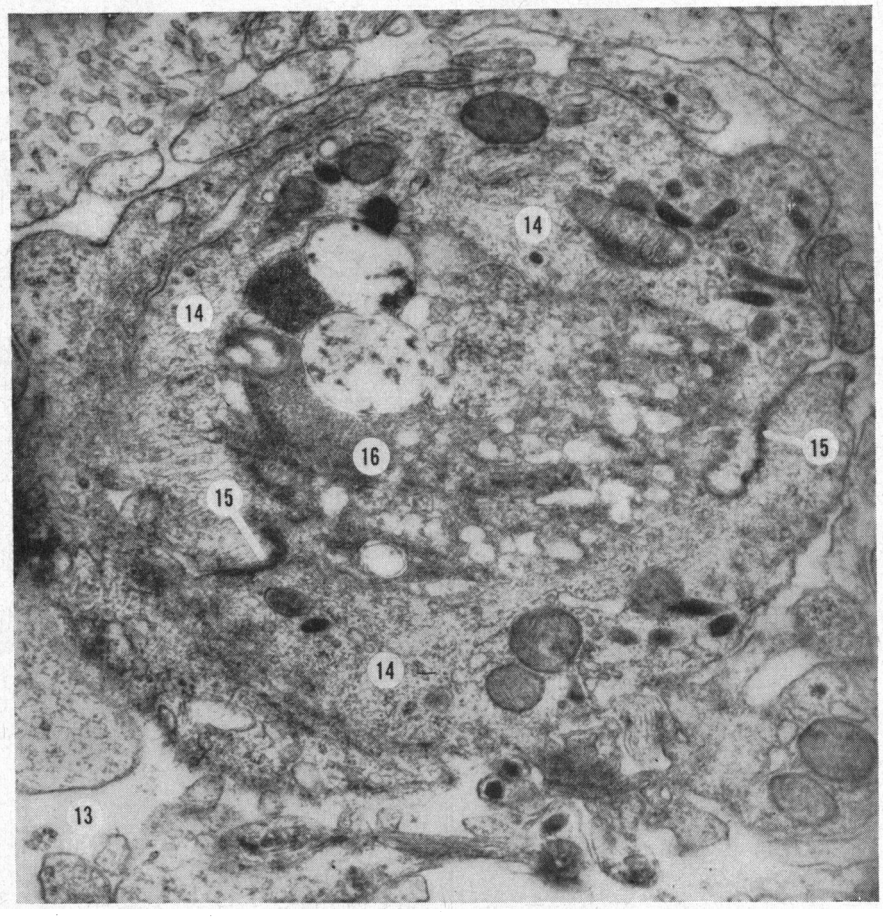


FIG. 17.—Transverse section of oxygen-treated vessel. Endothelial damage is now marked and the lumen is now almost completely occluded by debris. ×9,000.

In all the specimens of oxygen-treated retinae examined, the pathological tissue changes were confined to the endothelial material, and apart from the perivascular changes referred to above, all other retinal components appeared normal. Within the limits of the techniques used, tissue planes, cell contents, and plasma-membrane interrelationships were well preserved. Araldite-embedded material is particularly reliable in this respect and can be depended upon to produce minimal distortion (in contrast to the methacrylate polymers).

DISCUSSION

The evidence provided by the digest preparations and the electron microscope has thus shown quite clearly that the endothelium of the retinal vessels

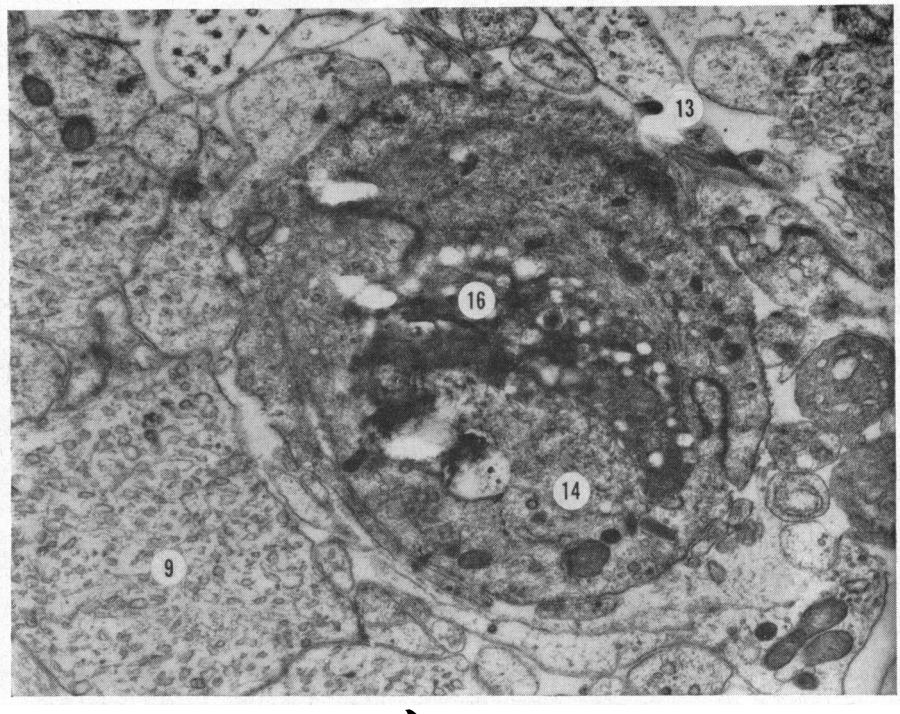


FIG. 18.—Final stages of oxygen-induced vessel damage. There is almost complete continuity between the endothelium and the debris filling the lumen. ×15,000.

of the kitten undergoes selective degenerative changes following exposure to ambient hyperoxia. That no endothelial degeneration could be found in "shake" preparations of the cerebral and meningeal vessels of the kitten, or in digest preparations of the retinal vessels of the cat subjected to hyperoxia, is in accord with our previous conclusion that the phenomenon of oxygen vaso-obliteration in this animal is confined to the growing vessels of the immature retina.

The present study shows that vaso-obliteration begins after about 6 hrs' exposure with capillary closure, together with, or soon followed by, degenerative changes in the endothelial cytoplasm, which first appear in the cytoplasmic polyps projecting into the lumen, but which progress to more severe changes of vacuolation and cellular disruption with pronounced pyknosis and occlusion of any remaining lumen by cellular debris. The next stage is most surprising, for the degenerating endothelial cells now move, or are displaced, from the affected capillaries towards less affected vessels, leaving only a thin strand of tissue behind. Finally, the endothelial cells undergo disintegration within islands where they become entrapped, and eventually disappear leaving only a skeleton of the original network.

Oxygen vaso-obliteration could therefore be regarded as a greatly exaggerated form of retraction, which is a recognized component of normal capillary growth (Sandison, 1928; Ashton, 1961), wherein the endothelial cytoplasm and nucleus of redundant capillaries retract into the parent capillary. Hence oxygen vaso-obliteration could be brought into line with a normal process and the implication that the ultimate pattern of normal capillary growth is also in some way related to oxygen tissue-tension would be interesting. Furthermore, the problem of oxygen vaso-obliteration in the immature retina would be largely solved (although many points would remain to be explained), since it could be attributed to a specific toxic action of oxygen upon growing endothelial cells.

Unfortunately it is not possible to say with certainty whether the endothelial disintegration in our animals results directly from the hyperoxia, for it might equally well be secondary to the failure of the circulation within the collapsed capillaries, and the movement of the endothelial cells may not be due to retraction, for it could be explained by the squeezing of the capillaries from external compression. It should not, however, be difficult to elucidate the question of endothelial injury, as, for instance, by observing the effect of circulatory failure induced by other methods, or by studying the influence of oxygen on endothelial cells in tissue culture.

With regard to the concept of retinal swelling as the primary cause of the initial capillary closure, the digest preparations add little beyond the fact of endothelial displacement. On the other hand, the electron microscope has shown no evidence of swelling, either in the endothelial cells or in the adjacent tissue, apart from the late development of irregular spaces in the perivascular radial-fibre substance, which, if significant, may well be a secondary effect. This study also emphasizes a selective effect on the endothelial cells in the degenerative process, all other cells in the immediate vicinity being entirely healthy; this again suggests a specific toxic action of oxygen rather than a circulatory failure.

Moreover, other work carried out in this laboratory since the hypothesis of retinal swelling was first suggested has failed to support it as a causative mechanism. These findings will be briefly reported here:

(1) Although chemical inhibition of metabolism *in vitro* by fluoride or iodoacetate is known to promote the uptake of water by the retina of the cat or rat, determinations of the water content of the retinae of kittens after treatment of the living animals with oxygen failed to reveal any significant difference from the normal (Graymore, 1957–8, 1958, 1959).

The details of these experiments were as follows:

Kittens aged 8 days were maintained in 80 per cent. oxygen for 3 days, at the end of which time the animals were anaesthetized and the eyes removed and dropped into liquid nitrogen (whilst still in an atmosphere of high oxygen). The frozen retinae were removed and their wet-weight: dry-weight ratios were determined in pre-weighed nickel crucibles. The mean

value for the oxygen-treated animals was 8·8 (±0·5) as opposed to 8·2 (±0·9) for a similar control group.

(2) By applying the same histological methods used to demonstrate retinal swelling resulting from intravitreal injections of sodium fluoride in the kitten's eye, no evidence of swelling in the retinae of oxygen-treated kittens as compared with normal controls could be demonstrated (Pedler, 1959; 1960).

(3) It has been found that the developing retinal vessels of young rabbits react to ambient hyperoxia and to intravitreal fluoride in a manner closely similar to those of the kitten. Since these vessels at this early stage of development appear to be entirely situated on the surface of the retina, this finding seriously questions the hypothesis that oxygen vaso-obliteration results from swelling of the retinal tissue (Ashton, 1960).

It is clear, therefore, that there is still much conflicting evidence to be resolved before the mechanism of oxygen vaso-obliteration can be fully understood, but all these new findings are more in favour of our original concept, namely that the vessels are directly affected by oxygen. Nevertheless, many of our subsequent observations point to retinal swelling as a factor of major importance and our immediate objective is to determine whether this does or does not participate in the phenomenon.

SUMMARY

(1) The effect of ambient hyperoxia on the endothelial cells of developing retinal vessels in the kitten was studied by a new digest method and by electron microscopy.

(2) It was found that oxygen vaso-obliteration begins after 6 hrs' exposure with capillary closure, together with or soon followed by, degenerative changes in the endothelial cytoplasm. These changes first appear in the cytoplasmic polyps projecting into the lumen, but progress to vacuolation and cellular disruption with pronounced pyknosis, and to the occlusion of any remaining lumen by cellular debris.

(3) The degenerating cells then move, or are displaced, from the affected capillaries and travel towards less affected vessels, leaving only a thin strand of tissue behind. In this way islands of cells are formed and these finally disintegrate leaving only a skeleton of the original vascular network. This reaction could be regarded as an exaggeration of the normal process of retraction.

(4) No such changes were found in other retinal cells, even if adjacent to the endothelium, in either the cerebral or meningeal vessels of these animals, or the retinal vessels of the adult cat exposed to hyperoxia.

(5) It is apparent that the endothelium of the retinal vessels of the kitten undergoes selective degeneration following exposure to hyperoxia and these new findings are discussed in relation to the role of retinal swelling in vaso-obliteration. It is pointed out that these observations, together with other

experimental results recently obtained, are more in favour of a direct injury to the retinal endothelium than of a perivascular compression, but the present evidence does not provide a conclusive answer to the problem of the mechanism of oxygen vaso-obliteration.

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