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COMMUNICATIONS

STUDIES ON THE PERMEABILITY OF THE BLOOD-RETINAL BARRIER

III. BREAKDOWN OF THE BLOOD-RETINAL BARRIER BY CIRCULATORY DISTURBANCES*

A TOPOGRAPHICAL STUDY OF THE VASCULAR TREE

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AN alteration of vascular permeability has been proposed by numerous authors as the key factor underlying the development of most vascular retinopathies. Among others, Hanum (1939), Ballantyne (1945, 1946), Friedenwald (1950), and Ashton (1951, 1963) have stressed the probable importance of a change of vascular permeability in the formation of microaneurysms and development of diabetic retinopathy, and Hodge and Dollery (1964) clearly showed in hypertensive retinopathy a change in permeability of the retinal vessels to fluorescein.

The existence of a Blood-Retinal Barrier (B.R.B.) was shown by Ashton (1965), Ashton and Cunha-Vaz (1965), and Cunha-Vaz, Shakib, and Ashton (1966), and its site (at least as regards the behaviour of certain substances) was located in the vascular endothelial membrane. The existence in the retina of such a mechanism of defence and protection has some bearing on retinal pathology, and the fact that the endothelial membrane is an essential component points to the importance of its injury in the development of the vascular retinopathies. Recently, in the Bowman Lecture, Ashton (1965) accentuated the fact that "certain pathological lesions of the retina are shared by a variety of apparently unrelated diseases, which points to the likelihood that, although pathological stimuli may vary widely, there are in the retina special circumstances, be they of structure, function, or environment, which limit its scope of reaction". It is probable that at least one of these "special circumstances" could be the peculiarity in permeability of the retinal vessels.

The present studies were designed to examine the vascular patterns of permeability changes which follow breakdown in the B.R.B. from two circulatory disturbances, to which an important role in the development of vascular retinopathies has been attributed; namely, ischaemia (Ashton, 1963) and venous stasis (Ballantyne, 1946; Ashton, 1963).

For these studies the colloidal carbon technique of Majno and Palade (1961) was used because, from previous investigations (Ashton and Cunha-Vaz, 1965; Cunha-Vaz and others, 1966; Cunha-Vaz, 1966), it was known that the carbon particles can be used as an indicator of B.R.B. damage. With this technique, when the permeability is altered, the particles label the affected vessels and permit their visualization under the light microscope (Cunha-Vaz, 1966).

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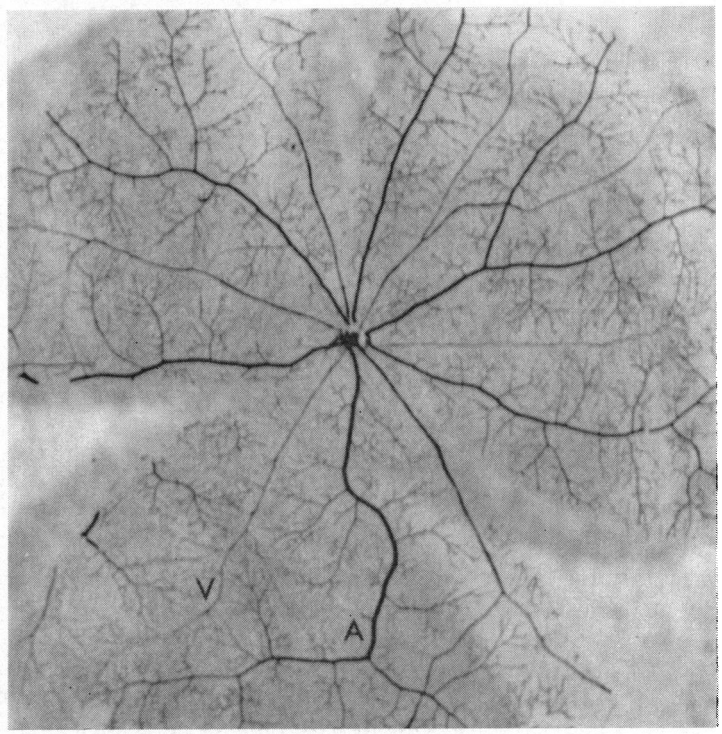


FIG. 1.—Flat preparation of adult rat retina injected with Indian ink. Note that the superficial net is almost entirely arterial, being formed by the tree-like ramifications of the large arterioles (A), which do not intercommunicate with the venules (V) at this level. The deep venous plexus can be seen faintly in the background. × 15.

Material

Male albino rats were used in these experiments because their retinal vascular architecture is particularly useful for topographical studies (Ashton and Blach, 1961; Cunha-Vaz, 1966) (Fig. 1). They were anaesthetized with subcutaneous sodium barbitone after a previous intraperitoneal injection of Largactil. When necessary ether was used to deepen the anaesthesia. In addition, similar experiments were carried out on a few cats of either sex to show that comparable results could also be obtained in other species.

Colloidal Carbon.—An identical preparation to that described in previous papers (Ashton and Cunha-Vaz, 1965; Cunha-Vaz and others, 1966) was used. This contains 100 mg./ml. carbon with average particle size of 100–200 Å. The standard dose for intravenous injections was 0·1 ml./100 g. body weight.

Dextran.—A 3 per cent. solution of Dextran, grade A, 200,000 to 275,000, (British Drug Houses Ltd., Poole, England) was used.

Methods

(1) Induction of Retinal Ischaemia

Two methods were used: (*i*) elevation of the intra-ocular pressure above the retinal arterial pressure and (*ii*) clamping of the optic nerve.

(*i*) *By Elevation of the Intra-ocular Pressure.*—This technique was essentially the same as used by Smith and Baird (1952). The rat pupils were dilated with atropine. One eye always served as a control. A 5 litre aspirator containing 2·5 litres of saline was connected to an electric pressure pump, the saline being maintained at the required pressure by means of a regulating valve (Fig. 2, opposite).

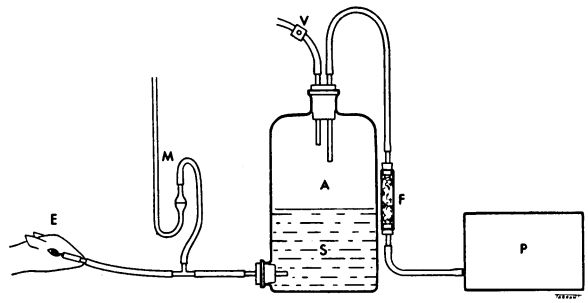


FIG. 2.—Schematic drawing of the apparatus used for raising the intra-ocular pressure.
A. Aspirator bottle. E. Eye. F. Air Filter. M. Manometer. P. Pump. S. Saline. V. Regulating Valve.

The saline in this reservoir was connected to the anterior chamber of the eye by a needle inserted through the periphery of the cornea. No aqueous fluid was lost in this procedure. The peripheral location of the needle permitted continuous observation of the retina during the experiment at a 10 x magnification with a Zeiss Opton microscope by applying a small coverslip to the cornea, the space between the coverslip and the cornea being filled with saline. The pressure delivered to the eye was continously monitored by a mercury manometer connected in line with the outflow to the eye. The intra-ocular pressure necessary to produce a static segmentation of the blood in the retinal arteries was about 110 mm. Hg in the rat, and somewhat higher in the cat. Constant stagnation of the blood flow was obtained when the intra-ocular pressure was maintained at 150–160 mm. Hg. The ischaemic pressure was maintained for varying periods in different animals and this was followed by injections of colloidal carbon.

(*ii*) *By Clamping the Optic Nerve.*—Lateral canthotomy and section of the conjunctiva with exposure of the optic nerve were first carried out. A specially-made adjustable forceps, its branches covered with rubber to offer the least possible traumatizing surface, was then applied to the optic nerve. Under direct observation of the retinal vessels with a Zeiss Opton microscope and a coverslip applied to the eye, the compression of the optic nerve and central retinal vessels was adjusted by a regulating screw to a level just sufficient to produce complete and stable stagnation of the blood in the retinal arteries.

(2) Induction of Venous Stasis

Two methods were used: (*i*) elevation of the intra-ocular pressure and (*ii*) repeated injections of high molecular weight Dextran.

(*i*) *By Elevation of the Intra-ocular Pressure.*—The technique was the same as that used in the production of retinal ischaemia. Here, however, the intra-ocular pressure was controlled between 80–90 mm. Hg in order to induce a definite sludging in the retinal veins, as seen by direct microscopical observation through a coverslip applied on the eye. In each animal one eye was submitted to a single episode of venous stasis and the other eye served as control. The stasis pressure was maintained for varying periods in different animals.

(*ii*) *By Injections of Dextran.*—A 3 per cent. solution of high molecular weight Dextran was injected intravenously in three doses of 2, 3, and 3 ml. (Thorsén and Hint, 1950). After the third injection there was sludging in the retinal veins, and colloidal carbon was injected intravenously.

Colloidal Carbon Injections

To demonstrate the leaking vessels the colloidal carbon suspension was injected into the femoral vein either immediately after the experimental circulatory disturbances were induced, or after intervals of 3 and 24 hours.

Injection Techniques.—In the first experiments the entire vascular system of the animal was irrigated *via* the left ventricle with heparinized saline to remove any carbon that might have remained in the lumen of the vessels. Later this procedure was discontinued as it was found that one hour after the colloidal carbon injection, when the animals were killed, there were constantly no carbon particles left in the retinal circulation of the control eyes, as expected from the studies of Halpern, Benacerraf, and Biozzi (1953), which showed that by this time all the carbon had been taken up by the reticulo-endothelial system. In some experiments, irrigation was followed by injection of dilute Berlin blue to provide a clearer picture of the labelled vessels, the black carbon contrasting with the light blue of the Berlin blue solution.

Preparation of Specimens for Microscopical Examination.—The eyes were excised and placed in 10 per cent. formol saline. The retinae were removed after 24 hours' fixation, mounted flat, and cleared in glycerin. After examination and photography, some of the retinae were digested by the method of Kuwabara and Cogan (1960) as modified by Ashton (1963) and stained with periodic acid-Schiff and haematoxylin (PASH); others were embedded in paraffin wax and transverse and flat sections were stained with either haematoxylin and eosin (H.E.) or periodic acid-Schiff and haematoxylin (PASH).

Controls

The material described in previous papers (Ashton and Cunha-Vaz, 1965; Cunha-Vaz, 1966) served as additional controls for the present studies. Injections of trypan blue, using the techniques described previously (Cunha-Vaz and others, 1966), were performed to control the breakdown of the B.R.B., which was demonstrated in the retinae where vascular labelling was present.

Results

(1) Vascular Labelling after Retinal Ischaemia

(i) By Elevation of the Intra-ocular Pressure.—When carbon was injected after the retinae had been submitted to a single episode of ischaemia lasting up to one hour, no vascular labelling could be seen in the retinal vessels and the histological sections showed only a moderate swelling of the inner layers of the retina especially involving the inner plexiform layer.

(*a*) *Retinal Ischaemia lasting for 70 minutes.*—When *carbon was injected immediately after* the single ischaemic episode and the animals were killed one hour after the injection, some retinal vessels could be seen to be labelled. The carbon labelling was restricted to vessels of capillary size and involved only short segments of the vessel walls, giving a general appearance of a "spotty" blackening. The capillaries affected were almost exclusively located in the superficial vascular net of the retina, *i.e.* on the arterial side of the circulation (Fig. 3, opposite). Digest preparations of these retinae showed the nuclei of the endothelial cells and intramural pericytes to be pale and somewhat enlarged. The basement membrane showed a significant increased staining with PAS. The deposition of PAS-positive material in the vessel walls was specially accentuated where carbon deposits could be seen. On histological examination, the retinae showed moderate oedema of the innermost layers.

When *carbon was injected 3 hours after* the ischaemic episode and the animals were killed one hour after the injection, the vascular labelling was more widespread and involved larger

portions of the vessels, but there was still a definite preference of the labelling for the superficial net, although a few more vessels of the deep net were now involved. The alterations observed in the digest and histological preparations of these retinae, although more intense, were similar to those already referred to in the experiments where colloidal carbon was injected immediately after the ischaemic period.

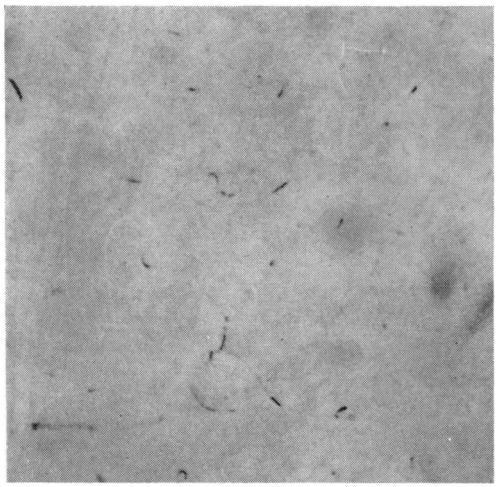


FIG. 3.—Flat preparation of a retina from a rat injected with carbon immediately after ischaemia lasting 70 minutes. Note labelling localized to short segments of vessels of capillary size belonging to the superficial net. × 65.

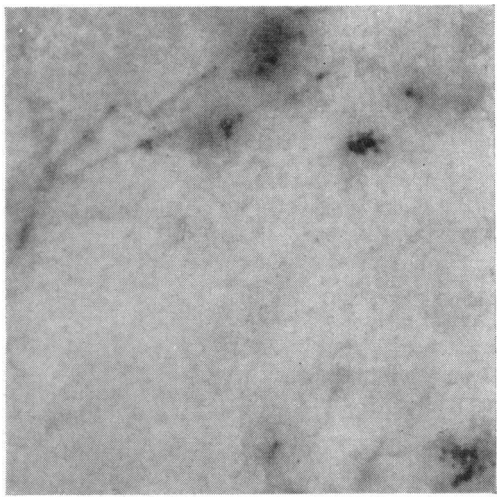


FIG. 4.—Cleared retina from a rat injected with carbon 24 hours after ischaemia lasting 70 minutes. Note carbon deposits in the walls of small vessels of the deep net and spreading into the surrounding tissue. × 65.

When *carbon was injected 24 hours after* the ischaemic episode and the animals were killed one hour after the injection, a striking change in the vascular labelling pattern was observed. The labelled vessels were still essentially of capillary size but were mainly located in the deep net, *i.e.* venous side of the circulation. Some venules also showed carbon deposits in the walls, and adjacently there were a few small round haemorrhages with carbon particles (Fig. 4). In some vessels the carbon deposits had spread into the surrounding tissue. Digest specimens showed enlargement of the nuclei of the endothelial cells and intramural pericytes, and early proliferation of the endothelial cells. A few intramural pericytes had lost their characteristic round shape and presented signs of migration. The vessel walls showed a marked increase in PAS-positive material. Histologically, the retinae showed generalized oedema, especially affecting the innermost layers which were loose and vacuolated. The ganglion cells showed signs of early degeneration and the retina at this level contained cells staining positively with PAS. The vessel walls were thickened and PAS-positive material had spread around the vessels of the superficial net. In the deep net there were signs of stasis, the capillaries being dilated and engorged.

(*b*) *Retinal Ischaemia lasting for 2 hours.*—The pattern of distribution of the vascular labelling, observed when colloidal carbon was injected immediately or 3 or 24 hours after an ischaemic episode lasting for 2 hours, evolved in a similar way to the pattern already described after an ischaemic period of 70 minutes, the only difference being one of degree,

with a marked increase in the intensity of the lesions and labelling in the 2-hour ischaemic specimens (Figs 5, 6, 7).

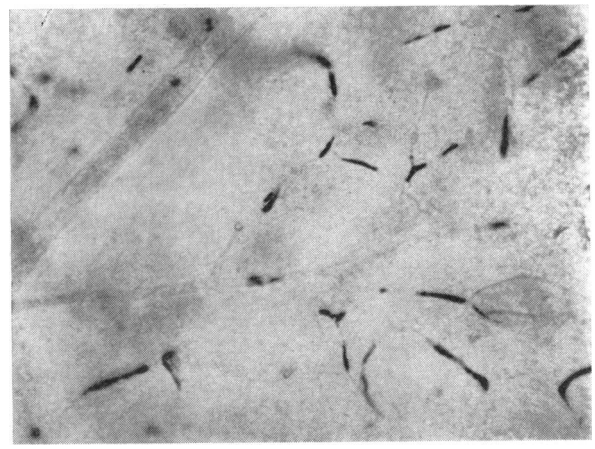
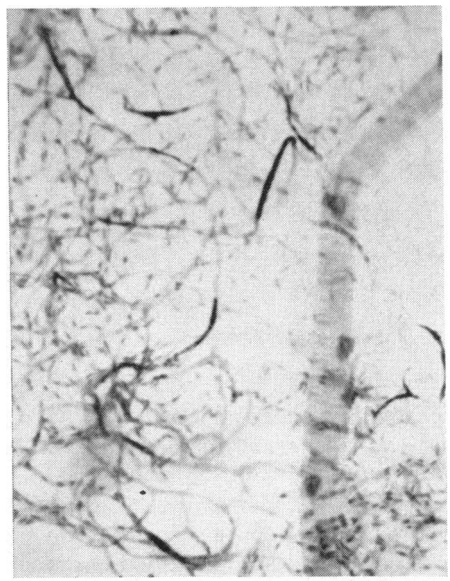


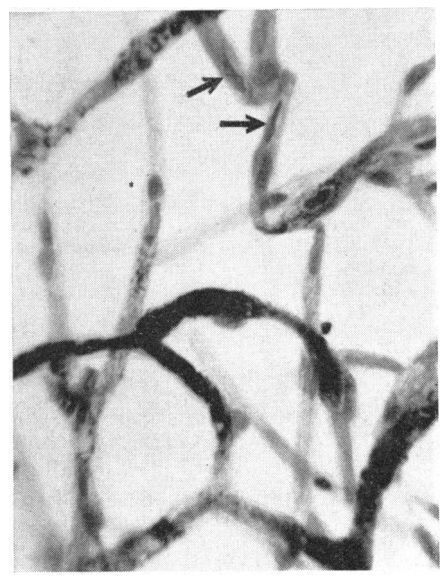
FIG. 5.—Cleared retina from a rat injected with carbon immediately after ischaemia lasting 2 hours. The vascular labelling is limited to the superficial net as in Fig. 3, but is now more generalized and involves also arterioles. × 75.

FIG. 6.—Digested retina from a rat injected with carbon immediately after ischaemia lasting 2 hours. Numerous labelled vessels can be seen near an arteriole. × 85.

FIG. 7.—Digested retina from a rat injected with carbon immediately after ischaemia lasting 2 hours. Note irregular distribution of carbon deposits in vessel walls. Some intramural pericytes have lost their characteristic round shape (arrows). × 440.

(6)





(7)

(*c*) *Retinal Ischaemia lasting for 6 hours.*—When the carbon was injected immediately after this longer ischaemic episode and the animals were killed one hour after the injection, the vascular labelling affected only the arterial side of the circulation as in the specimens subjected to shorter ischaemic periods, but this preference for the arterial side was here strikingly evident, and it could be seen that all arterioles of large and smaller size were involved, in contrast with the venous side of the circulation which was free of carbon deposits (Figs 8, 9, 10, opposite).

(ii) By Clamping the Optic Nerve.—Rats subjected to single episodes of experimental clamping of the optic nerve lasting for periods of 70 minutes and 2 hours were injected with carbon immediately afterwards. The pattern of labelling was similar to that observed in the experiments where retinal ischaemia was produced by elevation of the intra-ocular pressure.

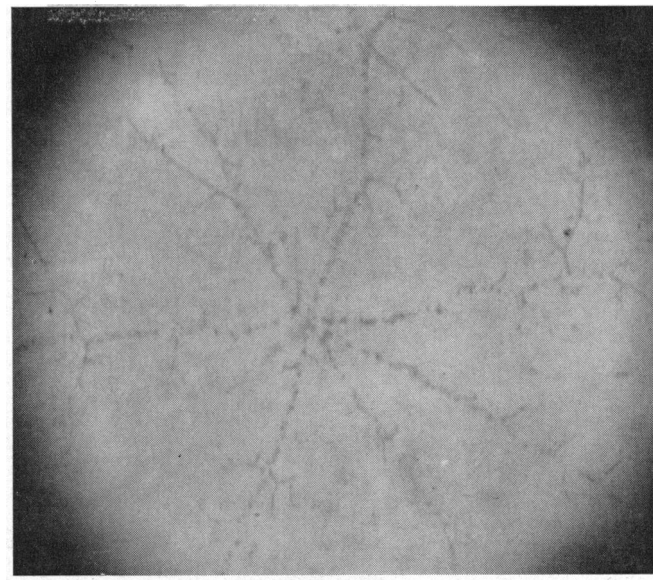


FIG. 8.—Cleared retina from a rat injected with carbon immediately after ischaemia lasting 6 hours. Vascular labelling can be seen in all arterioles of large and smaller size, in sharp contrast with the venous side of the circulation which is free of carbon deposits. × 15.

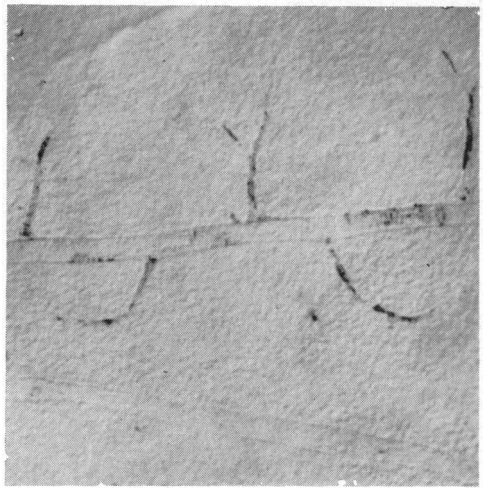


FIG. 9.—High-power view of Fig. 8, showing labelling of an arteriole of large size and its branches and an unaffected venule. × 110.

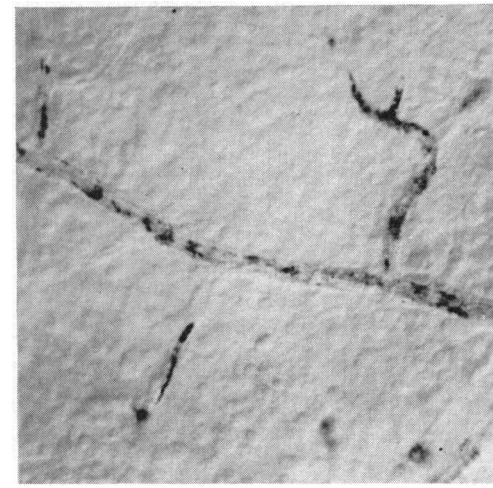
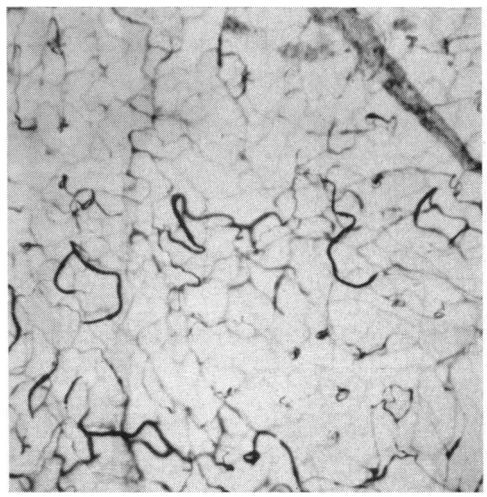


FIG. 10.—High-power view of Fig. 8, showing labelling in the arterioles. × 280.

(2) VASCULAR LABELLING AFTER VENOUS STASIS

(i) By Elevation of the Intra-ocular Pressure.—When carbon was injected after the retinae had been submitted to a single episode of venous stasis lasting up to 90 minutes and the animals were killed one hour after the injection, no vascular labelling could be seen in the retinal vessels. When the venous stasis was maintained for 90 minutes, a discrete labelling was observed in a few small vessels of the deep net, *i.e.* venous side of the circulation. The digest preparations of these retinae showed only moderate swelling of the nuclei of the capillary cells, and in the histological sections there was a moderate swelling of the inner plexiform and inner nuclear layers.

Venous Stasis lasting for 3 hours.—When *carbon was injected immediately after* the single 3-hour episode of venous stasis and the animals were killed one hour later, the cleared specimens of the retinal vessels showed labelling of the small vessels of the deep net (Fig. 11), only a few vessels of the superficial net being affected. A preference of the labelling for the venous side of the circulation was evident. The carbon deposits were limited to the vessel walls without spreading into the tissue and presented an even distribution (Fig. 12).



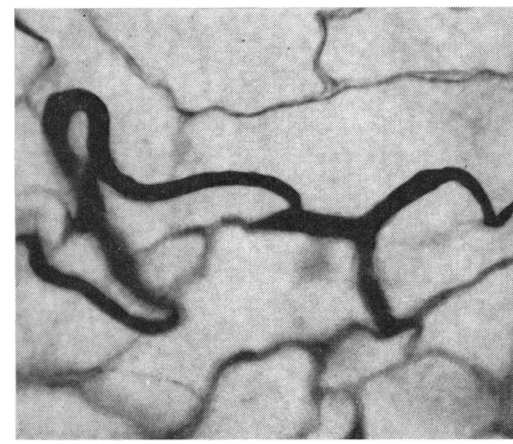


FIG. 12.—High-power view of Fig. 11, showing total blackening of a capillary of the deep net. × 333.

FIG. 11.—Cleared retina from a rat injected with carbon immediately after venous stasis lasting 3 hours. The rat had been perfused with dilute Berlin blue one hour after the carbon injection. The focus is on the deep net. Note widespread labelling of small vessels of deep net. There is no labelling in the large vessels. × 48.

A flag-stone pattern, indicative of preferential leakage through the intercellular junctions, was never observed. Digest preparations of these retinae showed an increased deposition of PAS-positive material in the vessel walls, especially in the labelled vessels. The nuclei of the endothelial cells and intramural pericytes were enlarged and pale, and some nuclei of the latter cells lost their round shape, and became irregular and elongated. Histologically, only moderate swelling of the inner layers of the retina could be seen.

When *carbon was injected 3 hours after* the 3-hour stasis period and the animals were killed one hour after the injection, a similar distribution of the vascular labelling was observed, but here there was an increased involvement of the vessels of the deep net with the labelling even extending to some large venules. The alterations observed in the digest and histological preparations of these retinae, although more intense, were similar to those already referred to in the experiments in which carbon had been injected immediately after the episode of stasis. The swelling of the retina in this group of animals, however, was more prominent in the inner nuclear layer.

When *carbon was injected 24 hours after* the 3-hour episode of stasis and the animals were killed one hour after the injection, a most striking distribution of the vascular labelling was observed. Most venules were intensely labelled, whereas the large and small arterioles were totally free of carbon deposits (Fig. 13); colloidal carbon had spread out from the walls of the labelled vessels giving the picture of "smudging" (Figs 14 and 15) and a few deep, round haemorrhages were apparent. Digest preparations of the retinae showed intense deposition of PAS-positive material in the permeable vessels. The endothelial nuclei were enlarged and had proliferated preferentially on the venous side of the retinal

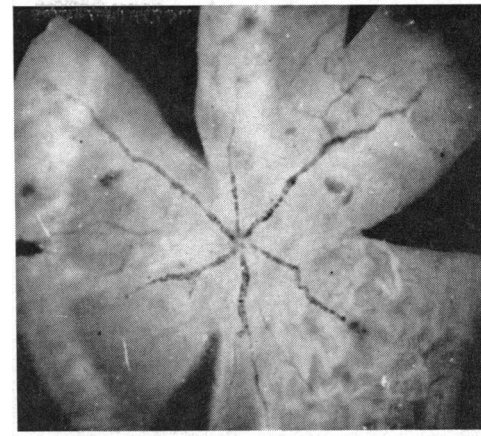
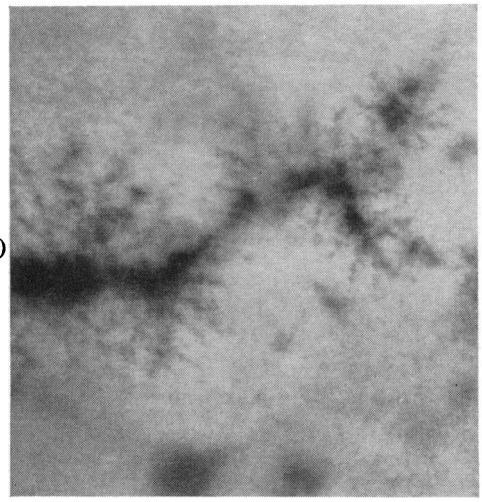


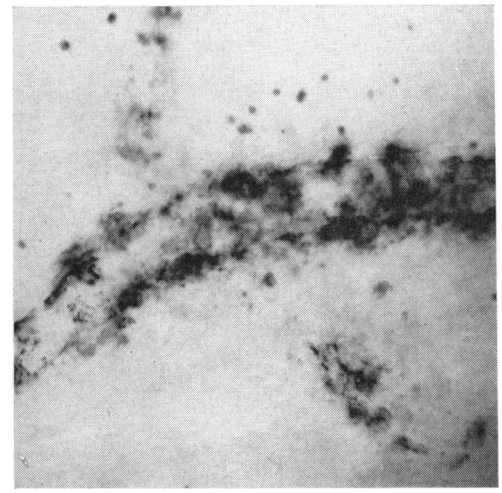
FIG. 13.—Cleared retina from a rat injected with carbon 24 hours after venous stasis lasting 3 hours. Vascular labelling is now confined to the large venules. A few small haemorrhages can also be seen. The arterial side is completely free of carbon deposits. × 12.

FIG. 14.—High-power view of Fig. 13, showing carbon spreading out from venular wall. × 65.

FIG. 15.—High-power view of Fig. 13, showing irregular deposition of carbon deposits in walls of a large venule. × 110.

(14)





(15)

circulation. The nuclei of the intramural pericytes showed evidence of migration. In histological preparations, the innermost layers showed swelling and cells containing PAS-positive material, and the inner nuclear layer presented dilated vessels with thickened walls, and a few haemorrhages. The carbon deposits could be seen extending beyond the vessel walls. The central retinal vein showed carbon labelling in contrast to the central retinal artery which was completely free of carbon.

(ii) By Injection of Dextran.—When carbon was injected immediately after the last injection of Dextran and the animals were killed one hour later, vascular labelling could be seen in a few large venules and small vessels of the deep net.

Discussion

The colloidal carbon technique used in the present investigation has revealed that the two types of circulatory disturbances studied, ischaemia and venous stasis, cause a breakdown of the B.R.B. to carbon particles. Furthermore, it was found that the

patterns of altered permeability were consistently characteristic for each type of circulatory disturbance studied (Figs 16 and 17).

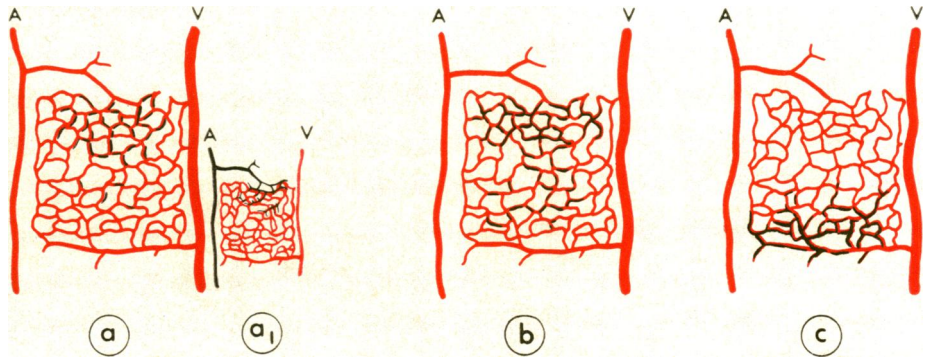


FIG. 16.—Vascular labelling patterns after retinal ischaemia: (*a*) Carbon injected immediately after 70 min.' retinal ischaemia. (a_1) Carbon injected immediately after 6 hrs' retinal ischaemia. (*b*) Carbon injected 3 hrs after 70 min.' retinal ischaemia. (*c*) Carbon injected 24 hrs after 70 min.' retinal ischaemia.

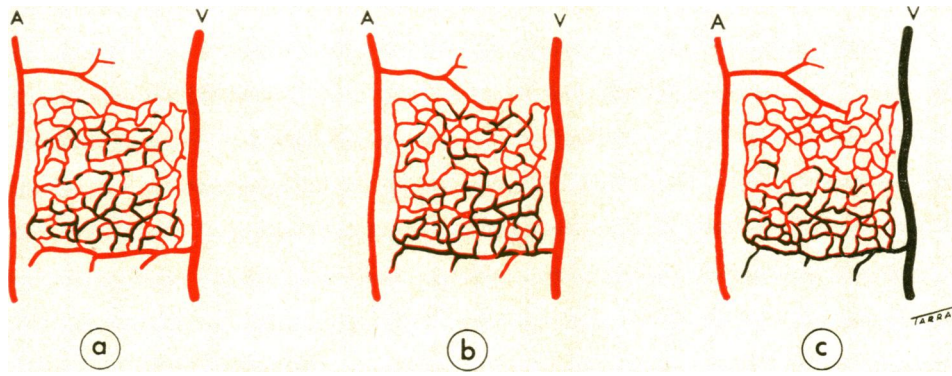


FIG. 17.—Vascular labelling patterns after retinal venous stasis: (*a*) Carbon injected immediately after 3 hrs' venous stasis. (*b*) Carbon injected 3 hrs after 3 hrs' venous stasis. (*c*) Carbon injected 24 hrs after 3 hrs' venous stasis.

The overall survey of the ischaemic experiments showed, immediately after ischaemia, involvement of the terminal vessels of the arterial side (Fig. 16*a*), which progressed with longer periods of ischaemia to involve all the arterial side, including the large arterioles (Fig. 16a_1); 3 hours after ischaemia this preference for the arterial side was maintained and even increased (Fig. 16*b*), but after 24 hours the pattern changed to involve preferentially the venous side (Fig. 16*c*). The initial preferential labelling on the arterial side of the retinal circulation would appear to indicate that the endothelial cells of these vessels have a special sensitivity to anoxia. 24 hours after ischaemia the previously permeable vessels on the arterial side showed no evidence of labelling, although their endothelial cells did not appear to have recovered. The most probable explanation for this behaviour is the development of a very deficient circulation in these vessels due to stasis which would impede the access of carbon particles to the affected vessels and consequently prevent their labelling. Another important factor to consider in the development of deficient circulation in these vessels is the compressing effect of the tissue oedema, resulting from the initial

alteration of permeability and anoxic injury to the retina. The late involvement of the venous side, with engorgement, alterations in permeability, and haemorrhages, can be explained by the increased anoxia on this side resulting from the deficient circulation established at this stage on the arterial side.

The retinal venous stasis experiments showed, immediately after a single episode of stasis, a change in vascular permeability affecting the smaller vessels with a preference for the venous side of the circulation (Fig. 17*a*). In the experiments with longer survival time the labelling maintained its preference for the venous side (Fig. 17*b*) and at 24 hours had spread to involve all the large venules (Fig. 17*c*). This venous pattern of altered permeability can be easily explained on the basis of embarrassment of the venous outflow and the labelling of the small vessels is probably due to slowing of the circulation and increase in hydrostatic pressure. After this initial change in permeability, increased stasis develops with haemoconcentration and further slowing of the circulation, leading to hypoxaemia and increased permeability changes, ending with complete involvement of all the venous side of the circulation.

When venous stasis was of longer duration, slight involvement of the arterial side could be seen, but this was probably due to the fact that it was impossible to avoid the complication of some insufficiency of the arterial supply when stasis was induced by elevation of the intra-ocular pressure. That this could be the true explanation is shown by the results obtained when stasis was produced by increasing the viscosity of the blood with Dextran, where there was no insufficiency of the arterial supply and no labelling on the arterial side of the circulation.

In summary, the permeability changes in the retinal vessels resulting from temporary ischaemia are initially located on the arterial side, leading later to stasis and involvement of the venous side. The permeability changes in the retinal vessels where a temporary venous stasis is induced, either mechanically or by increasing the viscosity of the blood, begin in the small vessels on the venous side and extend later, with the evolution of the process, to the larger venules.

This involvement of the venous side with engorgement, stasis, and changes in permeability 24 hours after either an ischaemic episode or a temporary embarrassment of the venous outlet, shows the similarity of the pattern of permeability response of the retinal vessels to two distinct types of circulatory disturbances and indicates a limited spectrum of reaction. It is relevant to recall here some recent experiments on the effect of various types of injury to the retinal vessels, in which it was shown that the subsequent abnormal permeability resulted only from direct vascular injury (Cunha-Vaz, 1966). In these experiments, the pattern of permeability response of the retinal vessels was always the same, in contrast to most parts of the body where different patterns of permeability response resulting from liberation of endogenous mediators have been described (Wells and Miles, 1963; Majno, 1964; Sevitt, 1964), thus again emphasizing the limited scope of reaction of the retinal vessels in a breakdown of the B.R.B. whatever the pathological stimuli involved.

Regarding the B.R.B. and its anatomical location which has been shown to be in the vascular endothelial membrane, at least for carbon particles and trypan blue (Ashton, 1965; Cunha-Vaz and others, 1966), a few confirmatory observations were made. When shorter episodes of ischaemia and venous stasis were induced, there

was swelling of the retinal glia but no change in permeability of the B.R.B. to carbon particles. It has been suggested by the same authors that the existence of elaborate junctional complexes between the endothelial cells of the retinal vessels could play an important role in the characteristic impermeability of the retinal vessels. That the leakage of carbon through the retinal vessels in these experiments was not preferentially *via* this intercellular pathway was shown by the even distribution of the carbon deposits in the vessel walls. Finally, the experiments reported here show that retinal ischaemia on the one hand, and retinal venous stasis on the other, can both give rise to selective permeability changes in the vascular bed which are reminiscent of the vascular pathological patterns seen in the retinopathies occurring in man, particularly in diabetes.

Summary

The patterns of permeability changes which develop after breakdown of the Blood-Retinal Barrier (B.R.B.) in the retina after two different types of circulatory disturbance, namely ischaemia and venous stasis, were followed after *in vivo* injection of colloidal carbon; this technique leads to the labelling of vessels in a state of increased permeability to circulating particles.

When temporary *ischaemia* was induced, the resulting breakdown of the B.R.B. was initially located on the arterial side leading later, at 24 hours, to preferential involvement of the venous side.

When temporary *venous stasis* was induced, the permeability changes involved almost exclusively the venous side of the retinal circulation, beginning in the small vessels and extending later to the larger venules.

Some aspects of the significance of these findings in the basic understanding of the retinal response to disease are discussed.

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