COMMUNICATIONS

STUDIES ON THE PERMEABILITY OF THE BLOOD-RETINAL BARRIER

I. ON THE EXISTENCE, DEVELOPMENT, AND SITE OF A BLOOD-RETINAL BARRIER*

BY

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THE concept of a blood-brain barrier has gradually evolved from a large number of different observations concerning the peculiar impermeability of the central nervous system to a wide range of substances. The original observations (Ehrlich, 1885) and much of the subsequent work (Goldmann, 1913; Spatz, 1933; Broman, 1949; Jeppsson, 1962; Bakay and Haque, 1964; and many others) dealt with the behaviour of acid dyes, notably trypan blue which has been extensively used to demonstrate the presence or absence of this barrier.

Two problems of the blood-brain barrier, its development and its site, have been the subject of considerable discussion. If the first of these seems to have been settled by the works of Gröntoft (1954) and Grazer and Clemente (1957), who demonstrated complete impermeability to trypan blue in brains at various stages of development, the second, and most controversial problem, is still unsolved. Numerous hypotheses for the site of the barrier have been proposed, and to-day there are two main schools of thought: those who believe that the blood-brain barrier is located in the endothelium of the vessels of the central nervous system (Spatz, 1933; Krogh, 1946; Broman, 1949; Gröntoft, 1954; Rodriguez, 1955; Crone, 1960; and others) and those who believe it is located in the glial membrane facing the basement membrane of the cerebral capillaries (Gärtner, 1927; Walter, 1933; De Robertis and Gerschenfeld, 1961; and others).

In the retina of the rabbit, a similar barrier to trypan blue has been described by Schnaudigel (1913), Palm (1947), and Ashton (1965), and to the fluorescent dyes—diaminoacridines—by Rodriguez-Peralta (1962), but surprisingly there is otherwise little information in the literature regarding the permeability of the retinal vessels. Adler (1959), in his descriptive textbook of the physiology of the eye, refers only briefly to a possible difference in permeability between the choriocapillaris and the retinal capillaries.

Recently Ashton and Cunha-Vaz (1965), studying the effect of histamine on the permeability of the ocular vessels, found that the retinal and cerebral vessels shared the common property of being resistant to histamine, in sharp contrast to the other vessels of the eye which behave similarly to vessels elsewhere in the body (Majno and

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Palade, 1961). This and previous studies of the permeability of the retinal vessels have been carried out in adult animals and no attempt to study the development and site of a probable blood-retinal barrier has yet been reported.

The purpose of this study is to examine these last two aspects and to present new evidence which, together with our electron microscopical findings, points to the endothelial membrane of the retinal vessels as the barrier involved in the permeability of trypan blue.

Materials and Methods

Animals.—Adult cats, rabbits, and rats, and young rabbits, kittens, and ratlings were used. They were divided into three groups; the first group was submitted to one or two of the Broman's trypan blue tests, the second group was injected with colloidal carbon, and the third group included the animals studied by electron microscopy. All the experiments were carried out under general anaesthesia, the rats being anaesthetized with ether and the rabbits and cats with intraperitoneal pentobarbital (Nembutal).

Trypan Blue Test Techniques.—Two techniques described by Broman (1949) were used:

(1) 0.2 mg./kg. body weight of freshly prepared trypan blue in physiological saline was injected intravenously. The animals were allowed to survive for periods of 15 minutes to one hour; their entire vascular system was then irrigated *via* the left ventricle with physiological saline to wash all traces of dye from the circulation.

(2) While the animals were under deep anaesthesia, the thoracic cavity was opened, the descending aorta clamped and the pericardium incised; a hypodermic needle, connected with polythene tubing to a 50 ml. syringe filled with physiological saline, was inserted into the left ventricle. The right auricle was opened by a small cut and the animal was irrigated with saline until all blood was thoroughly washed out. The animal was then perfused for 5–10 minutes with 0.2 per cent. trypan blue in saline; the vascular system was subsequently washed free of dye with saline. This was followed by final irrigation of 10 per cent. formol saline. All the retinæ of these animals were mounted flat and examined under the dissecting microscope. Thick frozen sections were prepared for light microscopy. The other layers of the eyes, brains, hearts, and lungs were also examined.

Colloidal Carbon Injections.—The preparation and the technique used were identical to those described in a previous paper (Ashton and Cunha-Vaz, 1965). The standard dose for intravenous injection was 0.1 ml./100 g. body weight. One hour after injection, when the carbon is expected to have cleared from the circulation, a fatal dose of anaesthetic was administered to the animal and the entire vascular system irrigated *via* the left ventricle with heparinized saline to remove any carbon that might have remained in the lumen of the vessels. Flat preparations of the retinæ were mounted in glycerine jelly, and other retinæ were sectioned in the ordinary way. The iris, choroid, brain, thyroid, and kidneys were also studied by the usual histological methods.

Electron Microscopy.—The material for electron microscopy was obtained from the retinæ of cats and rats, kittens and young rabbits, and from the choroids and irides of rats. The animals were anaesthetized and their eyes were enucleated and rapidly opened by a coronal section through the ora serrata. The vitreous was gently removed; the posterior half of the eye was then cut into four pieces and immersed in chilled 1 per cent. isotonic veronal buffered osmium tetroxide. In order to allow better penetration of the fixative, the retina was separated from the choroid with a thin spatula; fixation was carried out for two hours at 4°C. The retinæ were dehydrated in graded concentrations of alcohol and embedded in Epon or Araldite. Sections, one micron in thickness, were cut from the whole block using a Huxley microtome; these were stained with 1 per cent. alkaline toluidine

blue and studied by light microscopy. Thin sections were cut from selected areas of the block, stained with uranyl acetate in acetic acid, followed by lead citrate, and viewed by AEI EM6 electron microscope.

Findings

Trypan Blue Test.—No trace of the blue dye could be detected in the retinae, either mounted flat and examined by a dissecting microscope, or in thick frozen sections observed by light microscopy. The retinal vessels were also free from any blue colouration. On macroscopical examination, the brains showed a similar total absence of blue staining, except for some regions known to be permeable to trypan blue (area postrema, choroid plexus, pituitary, etc.).

In sharp contrast with the retinae and brains, the other tissues studied (iris, ciliary body, choroid, heart, and lung) were intensely stained with the trypan blue (Fig. 1).

Carbon Injections.—No particles of carbon could be seen in the walls of the retinal vessels, or in the retinal tissue in flat preparations, or in stained histological sections. This was true also of the iris and brain vessels. Carbon particles were seen in the walls of small vessels of the kidney, thyroid, and choriocapillaris.

Electron Microscopy.—The structure of capillaries and small venules of the retinae of adult rats and cats is similar. The diameter of the lumen of the capillaries is less than 8 microns and that of small venules 8 to 20 microns.

The lumina of these vessels are surrounded by endothelial cells, basement membrane, and intramural pericytes. The endothelial cells have villi, membrane-bound vesicles, mitochondria, and occasionally pigment granules. The cell junctions distinctly show

FIG. 1.—Example of an experiment with trypan blue showing the deeply-stained heart and lungs (2), and choroid (3) in contrast with the brain (1) and the retina (4) which remain colourless. The specimens are from a 9-day-old kitten.

dense junctional complexes, with narrowing of the intercellular space and marked condensation of the subjacent cytoplasmic matrix. The basement membrane external to the intramural pericytes is complete, thick, and appears electron dense. The intramural pericytes are surrounded by basement membrane and contain organelles similar to those found in the endothelial cells (Fig. 2). There is no true

FIG. 2.—Retinal capillary of a rat, showing thick and continuous basement membrane (BM) filling the spaces between the capillary cells (En—endothelial cells; IP—intramural pericyte) and the neighbouring glial cells (G). Well-formed junctional complexes (JC) can be seen between the non-fenestrated endothelial cells. Uranyl acetate followed by lead citrate. $\times 20,000$.

perivascular space and the plasma membranes of the neighbouring glial cells (predominantly Müller cells) are in close contact with the basement membrane; the distance between them measures only 150 to 200Å. This lack of perivascular space is characteristic also of the brain. The structure of the capillaries and venules in immature retinæ of kittens (Fig. 3) and young rabbits (Fig. 4, overleaf) differs from that of the adult animal.

FIG. 3.—Retinal capillary of a 1-day-old kitten, showing only scattered areas of a minute amount of basement membrane material between the capillary cells (En) and the adjacent glial fibres (G). A junctional complex can be seen between the thick and continuous endothelial cells. Uranyl acetate followed by lead citrate. $\times 14,500$.

In the kitten, the basement membrane is either incomplete or totally absent, and also at this stage intramural pericytes are poorly differentiated from the endothelial cells. In short, the newly formed capillaries consist of one or more layers of endothelial cells with patchy or no basement membrane. These cells have more vesicles and mitochondria than are seen in endothelial cells of mature cats; they are especially rich in rough surface endoplasmic reticulum and RNA particles. The junctional complexes between the endothelial cells of kittens and young rabbits (Fig. 5, overleaf) and the arrangement of the perivascular glial cells is similar to that of adult animals.

The growing capillaries in immature rabbits lie free in the vitreous near the surface of the retina and only endothelial cells can be recognized surrounding the lumen (Fig. 4). The basement membrane is absent and there are no neighbouring glial cells.

Iris and Choriocapillaris.—The small vessels of the iris have a continuous endothelial lining which is attenuated in places. The intercellular junctions show increased density of the apposing membranes of the endothelial cells, but there is variable

FIG. 4.—Retinal capillary of a 9-day-old rabbit. The vessel is free in the vitreous and shows little or no basement membrane material. Vitreous filaments (V.F.) can be seen in contact with the capillary cells (En). Between the thick continuous endothelial cells there are well-defined junctional complexes (JC). ILM—internal limiting membrane of the retina.

Uranyl acetate followed by lead citrate. $\times 15,000$.

FIG. 5.—Higher magnification of the junctional complex seen in Fig. 4, showing increased cytoplasmic density of the endothelial cells (En) in the area of the junction with narrowing of the intercellular space. Compare with intercellular junction in Fig. 6.

Uranyl acetate followed by lead citrate. $\times 60,000$.

narrowing of the intercellular space and no condensations in the subjacent cytoplasm (Fig. 6). A continuous basement membrane surrounds the vessels and involves the intramural pericytes.

FIG. 6.—Part of an iris capillary, showing continuous endothelium (En), the basement membrane (BM), and an intercellular junction (J). A relatively wide intercellular space ($\sim 150 \text{ \AA}$) is maintained throughout the intercellular junction, and the endothelial cells show an increased density of the apposing membranes in the junctional area without involvement of their subjacent cytoplasm.

Uranyl acetate followed by lead citrate. $\times 60,000$.

The choriocapillaris has extremely thin endothelial cells with numerous fenestrations. The intercellular junctions are similar to those of the iris vessels, but the basement membrane, which completely surrounds the endothelial cells and the occasional intramural pericyte, is thinner and has low electron density (Fig. 7).

FIG. 7.—Vessel of the choriocapillaris in a rat, showing thin fenestrated (F) endothelial cells (En). The basement membrane material (BM) is continuous but scarce. $\times 16,000$.

Discussion

Our experiments in adult rabbits, cats, and rats confirm the findings of others (Schnaudigel, 1913; Palm, 1947; Ashton, 1965) that trypan blue, when injected into the circulation of the adult rabbit, leaves the retinal tissue and its vessels unstained in a similar way to the central nervous systems in contrast with other tissues and vessels of the body which stain intensely blue.

Similarly, the vessels of the adult retina were found to be equally impermeable to carbon particles, and this applied also to the vessels of the brain and iris, whereas the other vessels studied—choriocapillaris, and vessels of the kidney and thyroid—were permeable to carbon. Both these barriers to trypan blue and carbon particles were found to be present at a very early stage in retinal vessels of embryonic type (kitten, ratling, and young rabbit) when the vessels are still invading the retina, and it will be recalled that Grazer and Clemente (1957) demonstrated a barrier to trypan blue in developing cerebral vessels. Provided that the vessels of both these tissues are structurally comparable, some light might be shed on the barrier question as a whole by considering in what way they may differ from vessels elsewhere, while the less complex structure of the developing retinal vessels may further narrow the problem.

Our electron microscopical observations of the small retinal vessels of the adult animal showed that these vessels are formed by a continuous layer of thick endothelial cells with abundant organelles in their cytoplasm. These cells are strongly held together by *constant and conspicuously dense junctional complexes*, which do not appear to have been previously recognized in studies on retinal vessels. The endothelial lining rests upon a thick basement membrane which engulfs the intramural pericytes and is in direct contact with the glial tissue. This structure of the retinal capillaries is, therefore, very similar to that of the brain capillaries (Farquhar and Hartmann, 1956; Maynard, Schultz, and Pease, 1957; Pease, 1958).

Some instructive conclusions can be drawn from an examination of the ultra-structure of the vessels which behaved differently from the retinal vessels towards the tracer substances used. In the case of the carbon particles, it can be seen that all the vessels studied, which do not present a barrier to their passage have definite structural differences from the retinal vessels. Thus, the choriocapillaris and small vessels of the thyroid (Monroe, 1953; Ekholm, 1957) and kidney (Farquhar and Palade, 1959; Rhodin, 1960; Farquhar, Wissig, and Palade, 1961), have very thin endothelial cells with numerous cytoplasmic discontinuities; the basement membrane is also very thin and irregular, and surrounding the vessel wall there is loose connective tissue.

The vessels of the iris, which like the retinal and cerebral vessels are impermeable to carbon particles in visible amounts, have a structure similar to the small vessels of the muscles in general. They have endothelial cells of medium thickness, and the intercellular junctions do not present condensations in the subjacent cytoplasm.

Regarding trypan blue, our experiments showed that all vessels studied, even the iris vessels, allowed its passage and the only exceptions were the retinal and brain vessels.

Before attempting to interpret the various factors involved, our electron microscopical observations of the developing retinal vessels of kittens and young rabbits will first be considered. In both these immature animals the permeability of the retinal vessels is similar to that of the adult vessels, but the vascular structure is simpler. In the kitten, the retinal vessels lie in a compact glial tissue with thick endothelium and invariably present well-defined attachment structures, but the basement membrane is tenuous and incomplete and may, therefore, be eliminated as a possible site for the blood-retinal barrier to carbon particles and trypan blue. In the young rabbit also, the retinal vessels consist solely of simple tubes of endothelial cells with dense and constant junctional complexes but are entirely devoid of basement membrane or perivascular glia. Thus only two possible anatomical sites remain for the blood-retinal barrier to carbon particles and to trypan blue, namely, the endothelial cells and the attachments between them (Fig. 8).

FIG. 8.—That the blood-retinal barrier to trypan blue and carbon is probably located within the endothelium and its junctional complexes is shown by three anatomical situations (a, b, c) in which the barrier was shown to be present.

The role played by these structures in the blood-retinal barrier to the two different tracers studied can be better interpreted in the light of the electron microscopical findings in the vessels which were permeable to the tracers examined. For instance, carbon particles normally pass through the vessel walls of the choriocapillaris, kidney, and thyroid, where the endothelial cells present numerous fenestrations (Fig. 9, opposite).

It is logical to assume that these endothelial pores permit a relatively unrestricted passage of carbon particles, whereas a barrier is presented by the thick and continuous endothelial cells of the small vessels of the iris, retina, and brain (Moore and Ruska, 1957).

There is also other evidence which can shed some light on this subject. Majno and Palade (1961) showed that histamine applied locally after an intravenous injection of carbon particles caused the endothelial cells of most venules of the body to separate from each other, providing intercellular gaps through which the particles passed. Histamine applied in the eye produces this effect in the iris vessels, making

Normal visceral capillary
(kidney, thyroid, choriocapillaris)
No barrier to carbon particles
Fenestrated endothelium
Wide intercellular junctions

FIG. 9.—Electron microscopy shows that capillaries which normally allow the free passage of carbon particles possess a fenestrated endothelium.

them permeable to carbon particles, but does not increase the permeability of the retinal or cerebral vessels in this way (Ashton and Cunha-Vaz, 1965) (Fig. 10). Here, also, the endothelial cells and their attachments would appear to be involved and, although it is probably unjustifiable to treat them as separate entities, it is feasible that the reactivity to histamine of the endothelial cells of the retina (and brain) differs in some fundamental way from that of endothelium elsewhere in the body, or that the intercellular junctions of the retinal endothelium might be too firmly adherent to permit openings.

FIG. 10.—Electron microscopy shows that the *free* passage of carbon through vessels with nonfenestrated endothelium, as occurs in some vessels after histamine injection, depends upon opening of the intercellular junctions (*a, b*). These do not open in the brain and retina (*c, d*), indicating peculiar intercellular junctions or a different reactivity of the endothelium of these vessels.

Regarding trypan blue the same two possibilities remain (Fig. 11, overleaf). Either the special structure of the attachments between the endothelial cells prevents the diffusion of the dye through the intercellular junctions, or the endothelial cells of the retinal and cerebral vessels behave differently from other endothelial cells of the

FIG. 11.—That the blood-retinal barrier to trypan blue may be related to tighter intercellular junctions of the endothelium is suggested by the structure and behaviour of three types of capillaries (a, b, c). Alternatively or additionally, the endothelium of the retina may have functional peculiarities not revealed by electron microscopy.

body and exclude the trypan blue. Preliminary studies suggest that an active mechanism may also be involved (Cunha-Vaz and Maurice, 1965).

Whatever the mechanism involved, our experimental evidence points clearly to the endothelial membrane of the retinal vessels as the site of the blood-retinal barrier to carbon particles and to trypan blue, whether it be structural or functional, or both. It does not, of course, exclude the role of other factors in the blood-retinal barrier to different substances.

Summary

The permeability of the retinal vessels of rabbits, cats, and rats to trypan blue and colloidal carbon was studied and compared with that of other vessels of the body.

It was found that the retinal vessels, in common with those of the brain, showed a characteristic impermeability to these substances, which points to the existence in the retina of a Blood-Retinal Barrier, comparable to the well-known Blood-Brain Barrier.

This barrier to trypan blue and colloidal carbon was also found to be present at a very early stage in the immature retinal vessels of the kitten, ratling, and young rabbit.

In order to elucidate this problem, an attempt was made to relate the electron microscopical appearance of the vessels studied to their permeability to carbon and trypan blue. It was found that only two possible anatomical sites could be involved, namely the endothelial cells and the attachments between them, and it was shown that a particular type of tight junctional complex exists between the retinal endothelial cells.

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REFERENCES

- ADLER, F. H. (1959). "Physiology of the Eye", 3rd. ed., p. 338. Mosby, St. Louis.
- ASHTON, N. (1965). *Trans. ophthal. Soc. U.K.*, **85**, 199.
- and CUNHA-VAZ, J. G. (1965). *Arch. Ophthal. (Chicago)*, **73**, 211.
- BAKAY, L., and HAQUE, I. U. (1964). *J. Neuropath. exp. Neurol.*, **23**, 393.
- BROMAN, T. (1949). "The Permeability of the Cerebrospinal Vessels in Normal and Pathological Conditions". Munksgaard, Copenhagen.
- CRONE, C. (1960). *Acta physiol. scand.*, **50**, Suppl. 175, p. 33.
- CUNHA-VAZ, J. G., and MAURICE, D. (1965). *J. Physiol. (Lond.)*, **183**, 42P.
- DE ROBERTIS, E., and GERSCHENFELD, H. M. (1961). *Int. Rev. Neurobiol.*, **3**, 7.
- EHRlich, P. (1885). "Das Sauerstoff-Bedürfniss des Organismus: eine Farben-analytische Studie". Hirschwald, Berlin.
- EKHOLM, R. (1957). *Z. Zellforsch. mikr. Anat.*, **46**, 139.
- FARQUHAR, M. G., and HARTMANN, J. F. (1956). *Anat. Rec.*, **124**, 288.
- and PALADE, G. E. (1959). *Ibid.*, **133**, 378.
- , WISSIG, S. L., and PALADE, G. E. (1961). *J. exp. Med.*, **113**, 47.
- GÄRTNER, W. (1927). *Z. Biol.*, **86**, 115.
- GOLDMANN, E. E. (1913). "Vitalfärbung am Zentralnervensystem". Reimer, Berlin.
- GRAZER, F. M., and CLEMENTE, C. D. (1957). *Proc. Soc. exp. Biol. (N.Y.)*, **94**, 758.
- GRÖNTOFT, O. (1954). *Acta path. microbiol. scand.*, Suppl. 100.
- JEPPSSON, P. G. (1962). *Acta neurol. scand.*, **38**, Suppl. 160.
- KROGH, A. (1946). *Proc. roy. Soc. B.*, **133**, 140.
- MAJNO, G., and PALADE, G. E. (1961). *J. biochem. biophys. Cytol.*, **11**, 571.
- MAYNARD, E. A., SCHULTZ, R. L., and PEASE, D. C. (1957). *Amer. J. Anat.*, **100**, 409.
- MONROE, B. G. (1953). *Anat. Rec.*, **116**, 345.
- MOORE, D. H., and RUSKA, H. (1957). *J. biophys. biochem. Cytol.*, **3**, 457.
- PALM, E. (1947). *Acta ophthal. (Kbh.)*, **25**, 29.
- PEASE, D. C. (1958). Quoted by R. D. Tschirgi—"The blood-brain barrier", in "Biology of Neuroglia", ed. W. F. Windle, p. 130. Thomas, Springfield, Ill.
- RHODIN, J. A. G. (1960). In "Edema, Mechanisms and Management", ed. J. H. Moyer and M. Fuchs. Saunders, Philadelphia.
- RODRIGUEZ, L. A. (1955). *J. comp. Neurol.*, **102**, 27.
- RODRIGUEZ-PERALTA, L. (1962). *Anat. Rec.*, **142**, 273.
- SCHNAUDIGEL, O. (1913). *V. Graefes Arch. Ophthal.*, **86**, 93.
- SPATZ, H. (1933). *Arch. Psychiat. Nervenkr.*, **101**, 267.
- WALTER, F. K. (1933). *Ibid.*, **101**, 195.