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THE ACTIVE TRANSPORT OF FLUORESCEIN BY THE RETINAL VESSELS AND THE RETINA

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SUMMARY

1. The movement of fluorescein across the retinal surface of the rabbit's eye was estimated by measuring the concentration gradient of the dye in the vitreous body. These measurements were made *in vivo* by means of a slit-lamp fluorophotometer, or were taken from frozen sections of enucleated eyes.

2. In the normal eye, fluorescein does not pass from the blood to the vitreous body across any part of the retina. When injected into the vitreous body it passes rapidly out across the entire retinal surface, even against a very large concentration gradient.

3. A variety of metabolic and competitive inhibitors, effective in blocking organic anion transport in the kidney and liver, tend to abolish this unidirectional movement of fluorescein across the retina.

4. The region occupied by the retinal vessels is more sensitive to inhibition than other areas of the retina. Occlusion of the vessels by diathermy prevents the exchange of fluorescein in this region.

5. It appears, then, that there is an active transport of organic anions out of the vitreous body, both by the retinal capillaries and by the retina itself. The latter system is probably located in the pigment epithelium and seems to be carried forward to the rear surface of the iris.

6. Since the walls of the retinal vessels of the rabbit are freely in contact with the vitreous body, the active transport must take place across the capillary endothelial cells themselves. These vessels have structural and permeability characteristics found only in the central nervous system and it is to be presumed that the anion transport system is shared by the capillaries of the brain.

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7. The function of the transport in the retina may be to protect the nervous tissue from toxic materials by preventing their entry from the blood or by removing products of metabolism conjugated as organic anions. Alternatively, the mechanism may be concerned in maintaining the normal adhesion of the retina to the choroid, since retinal detachment was observed to follow its total inhibition.

INTRODUCTION

Studies on the permeability of the retinal capillaries (Ashton, 1965; Cunha-Vaz, Shakib & Ashton, 1966) showed that they offered an extremely high resistance to the passage of certain large molecules, a property which is shared only by the vessels of the brain. It was wished to widen these investigations to more diffusible substances, and fluorescein was chosen because techniques were available for measuring its concentration and concentration gradient in the vitreous body of the living rabbit (Kaiser & Maurice, 1964). The measurement of the gradient in the medium immediately in front of the retina should give a value for the flux of dye across its surface and so lead to a value of the permeability of the surface at the point of observation. The rabbit is particularly suitable for these experiments, since its retinal vessels lie in contact with the vitreous humour virtually free from surrounding glial tissue; furthermore, they are restricted to a limited zone of the retinal surface, and so direct comparisons can be made between vascularized and non-vascularized areas.

It soon became evident that both these areas showed a unidirectional permeability to fluorescein in the direction from the inside of the eye to the blood. The *in vivo* observations on the concentration gradients were then supplemented by measurements taken on frozen sections of the eye, which showed the direction of movement of fluorescein across the boundaries of the entire vitreous body.

The extension of these studies, particularly by the use of competitive inhibitors, showed that a system for transporting organic ions, similar to that in the kidney, moves fluorescein into the blood. It was found that such a mechanism is located both in the retinal capillary endothelium and in some layer of retina proper, probably the pigment epithelium. The existence of an active mechanism which removed iodopyracet from the posterior segment of the eye had been previously shown by Forbes & Becker (1960) but their methods could not determine where it was located.

The function of this active system in the eye remains speculative, but whatever its significance it must almost certainly extend to the brain, because of many similarities between the vessels in the two organs. Indeed,

it has already been suggested by Steinwall (1961) that an organic ion transport system is present at the blood–brain barrier.

The results have been the subject of preliminary communications (Cunha-Vaz & Maurice, 1966*a*, *b*).

METHODS

Adult pigmented rabbits of various breeds were used as the experimental animals.

Under local anaesthesia, fluorescein and other drugs were injected into the vitreous body through a 30-gauge needle inserted into the globe near the equator, and with its point centrally between the retina and lens. No more than 20 μl. of solution were injected at any one time; its composition varied as described in the results section.

Fluorescein was introduced systemically as a 10 g/100 ml. solution in water, in the earlier experiments intravenously and later intraperitoneally. Blood samples were taken from the marginal ear vein into 10 μl. micro-cap pipettes, and were diluted in a known volume of saline before centrifugation.

The proportion of free fluorescein in whole rabbit blood was determined by dialysis. A magnetic stirring rod was inserted in a length of dialysis tubing, and was sealed in with a quantity of saline sufficient to dilate the bag only slightly, thus ensuring a large ratio of surface to volume. The tubing was submerged in heparinized blood stained with fluorescein, and was stirred continuously at 37° C. The fluorescence of the saline was observed in the intact sac from time to time and, after equilibrium was reached, which took about 2 hr, the saline and whole blood were appropriately diluted and their fluorescein levels estimated. In the later experiments the saline was initially stained with fluorescein to the level it was expected ultimately to attain, and was dialysed for an hour only.

In vivo observations. The animals were placed under general anaesthesia with pentobarbitone or urethane and the pupils dilated with atropine and phenylephrine. In order to take measurements across the thickness of the vitreous body it was necessary to eliminate the corneal refraction, and to this end a saline-filled flat contact lens was held on to the cornea with a light suction of 10–20 mm Hg (Text-fig. 1). Greater suction tended to lead to a temporary corneal opacity and to miosis.

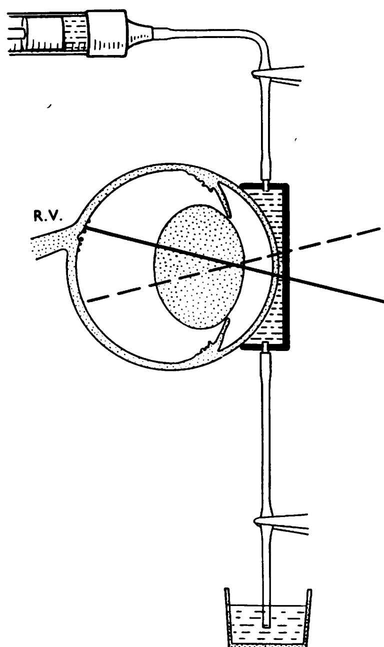
Tracings of the concentration profile across the vitreous body were made with the slit-lamp fluorophotometer of Maurice (1963) by moving its focus from the retina to the lens and recording on an oscilloscope the linear displacement of the instrument and the output from its photocell, as described by Kaiser & Maurice (1964). The position of the retinal surface and, when it could be distinguished, that of the lens was marked on the second channel of the instrument whose trace otherwise served as a base line. By tilting the head of the animal, measurements of the concentration gradient could be made either over the region of the retinal blood vessels or of a corresponding unvascularized area in the lower part of the retina (Text-fig. 1). There was no difficulty in recognizing these areas, and in focusing the microscope and slit-lamp, if the blue filter was removed during the preliminary manoeuvres.

The arms of the fluorophotometer were locked at an angle of 25° to each other, but even with a fully dilated pupil it was not possible to observe more than a limited area of vitreous around the posterior pole of the eye without the iris obstructing either the light pathway from the slit-lamp or to the microscope. To achieve the necessary working distance the normal photometric microscope objective was replaced by a lower power, × 1·5. A very small sensitive window in the mirror of the instrument was used such that the volume of fluid in which measurements were taken, corresponding to the intersection of the optical paths of the slit-lamp and microscope, was a parallelopiped approximately 1·5 mm high, 0·04 mm wide, and 1·7 mm deep.

The absorption of blue light by the fluorescein, an extinction coefficient of 0·01/mm for

10^{-6} g/ml. solution with the slit-lamp filter employed (Ilford 622), could cause a drop in the reading from the lens to the retina. The experiments were planned to reduce this absorption to less than 30 %, equivalent to a uniform concentration of 3×10^{-6} g/ml. throughout the vitreous body.

In vitro observations. The animal was killed, a suture passed through the limbus at the 12 o'clock position, and the eyeball enucleated. The aqueous humour was allowed to run out through a needle hole and the eye was immediately frozen in solid CO_2. Using the suture as a guide, it was then divided by a saw-cut in a vertical antero posterior plane about 4 mm to one side of the medial section. On the stage of a freezing microtome a brass ring, 24 mm in diameter, was frozen. The larger segment of the frozen eye was placed in this ring, cut surface downward, and some 10 % gelatine solution at 37° C was poured into the space



Text-fig. 1. Diagrammatic vertical cross-section of eye and contact lens, showing arrangement of syringe and saline reservoir used in applying lens with controlled suction. The continuous and interrupted lines indicate how the head is rotated from the horizontal to move the focus of the fluorometer through the vitreous humour in front of the retinal vessels (R.V.) or retina proper.

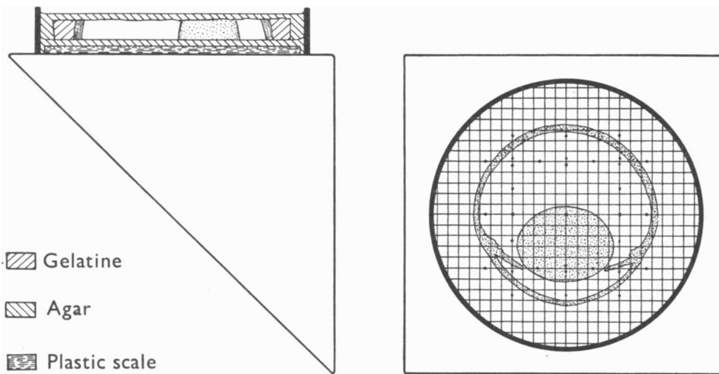
between them where it quickly gelled and froze. The eye was then sectioned with the microtome until the median plane was exposed. The brass ring was then warmed with the fingers and removed, and excess gelatine trimmed with a knife on the microtome stage.

Meanwhile, a flat sheet of agar gel, about 1 mm thick, had been prepared to receive the section. This was prepared by pouring some 2 % agar solution into a brass ring cemented on to the upper surface of a 45° glass prism with $1\frac{1}{2}$ in. (3·8 cm) square faces. After the agar had set, the prism was cooled in a refrigerator. Some 2 % agar solution at 56° C was poured into the brass ring, and immediately the frozen section of the eye was pushed down through it

and held with its flat surface in contact with the layer of gel below until the liquid had set; this took less than a minute and was accelerated by placing some small pieces of solid CO_2 on the upper surface.

The section of the eye was now completely surrounded with gel (Text-fig. 2*a*), and when it melted there was little tendency for the vitreous body to become stirred. The fluorescein dissolved in the humour quickly diffused into the thin sheet of gel lying next to it, and readings could be taken in this layer by reflexion from the hypotenuse of the prism. This gave a more uniform background than the frozen and remelted vitreous body.

To enable measurements to be taken systematically over the entire surface of the section, a thin plastic sheet engraved with a grid of mm squares lay on the surface of the prism under the gel. The corners of 5 and 10 mm squares were marked so that they could be rapidly identified as the rows of squares were scanned (Text-fig. 2*b*). A sheet of paper was marked



Text-fig. 2 (*a*) Illustration of how the frozen section of the eye is mounted in gelatine and agar gel for measurement of the concentration contours. (*b*) Appearance of the section, square grid and reference marks in the front surface of the prism.

out with this system of squares before the experiment, and the outline of the vitreous chamber and the position of the retinal vessels could be drawn upon it during the 20 min allowed for the section to melt and the fluorescein to equilibrate between it and the agar gel.

Occlusion of retinal vessels. The retinal vessels were closed at the optic nerve head by diathermy. The animal was placed under general anaesthesia, and the eye was kept proptosed by means of a clamp on the lids. A conjunctival flap was lifted up and a small circle of diathermy burns made on the exposed sclera in the region of the pars plana. A hole was cut in the sclera with a needle point in the centre of this circle. The plane contact lens was attached to the cornea, and a fine diathermy needle was introduced through the hole and guided on to the disk under direct observation with a Zeiss operating microscope.

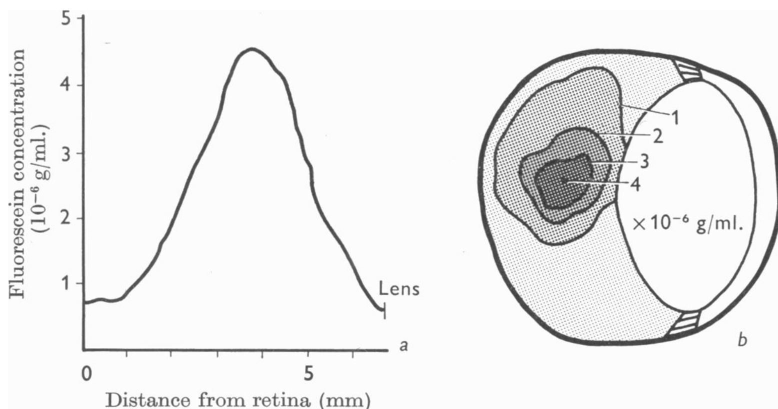
With the least necessary diathermy current, all arterial and venous trunks were occluded at their point of entry on both sides of the disk, care being taken to avoid damage to the underlying nerves as far as possible. The needle was withdrawn when the circulation appeared to be at a standstill in all branches of the retinal vessels. The experiments were started shortly after the operation. The hole was left unclosed and some vitreous leakage inevitably occurred.

The establishment of a chronic occlusion was not practicable; either the circulation partially re-established itself, or vitreous haemorrhages developed at a later stage.

RESULTS

In vivo observations were carried out on more than 120 eyes, and measurements were taken from forty frozen sections. All the major conclusions set out below were supported by at least four *in vivo* observations and two frozen sections.

Background. The threshold at which fluorescein can no longer be measured in the various media of the eye is generally not limited by the sensitivity of the fluorophotometer, but by the diffusion of light by the tissues themselves or by their auto-fluorescence. The vitreous body is itself sufficiently clear to allow fluorescein to be determined in it at concentrations well below 10^{-8} g/ml. However, interference from the lens, the retina



Text-fig. 3. Intravitreal injection of 10 μl. of 0·01 % fluorescein. (*a*) *In vivo* recording taken 15 min after the injection. (*b*) Concentration contours from same eye frozen 30 min after the injection.

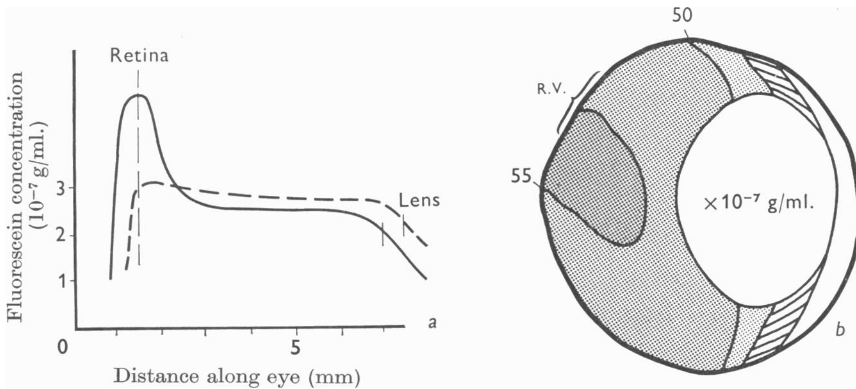
and, above all, the myelinated nerve fibres of the retina raised this threshold at the boundaries of the vitreous body in the *in vivo* measurements. When fluorescein was present, further errors resulted from the excitation of additional fluorescence locally in the dye by blue light reflected from the nerve fibres. Because the angle between the arms of the fluorophotometer had to be made small for the retinal surface to be seen, the interference caused by the surface raised the threshold for about 1 mm into the vitreous body.

The background scatter from the agar and neighbouring interfaces limited the sensitivity of the frozen section technique and raised the threshold to about 1×10^{-7} g/ml.

Validity of methods. Two tests were devised to check the accuracy of the

methods in representing known distributions of fluorescein within the eye. In the first a comparison of the two methods was made on a small volume of fluorescein solution very shortly after it had been injected into the centre of the vitreous body; if the original depot of dye was small enough and it had not had sufficient time to diffuse to the lens or retina, it would be expected to have assumed a Gaussian distribution. Both methods agreed in giving approximately the right form (Text-fig. 3), and it is clear that no gross distortion was introduced by the process of freezing and melting.

In the second test an attempt was made to establish a uniform concentration throughout the vitreous. To this end the dye was allowed to diffuse for a few hours in the living eye and then for a further period after



Text-fig. 4. (*a*) *In vivo* recordings taken from eye 24 hr after injection of 20 μl. of 0·015 % fluorescein together with inhibitor, and 7 hr after death of animal. Recordings taken over retinal vessels (R.V.) and retina proper indicated by continuous and interrupted lines respectively (see Text-fig. 1). This convention is followed in all following diagrams which are redrawn from the original records. (*b*) Contours from eye frozen 6 hr after intravitreal injection of 20 μl. of 0·25 % fluorescein with inhibitor and 3 hr after death of the animal.

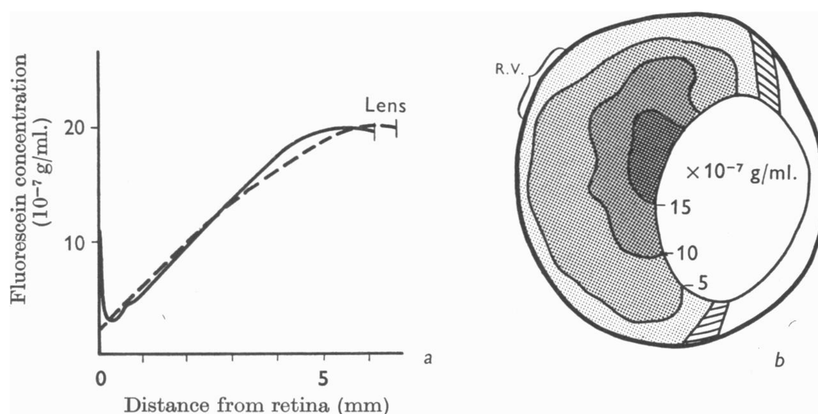
the animal was killed. The closure of the pupil that, in the rabbit, normally follows death could be prevented by a previous retrobulbar injection of anaesthetic.

The concentration contours (Text-fig. 4*b*) showed no evidence of disturbances of the uniform distribution of fluorescein that could be attributed to freezing. There was a slight drop in concentration in the anterior portion of the vitreous body but this could well have been a result of an incomplete assumption of the diffusion equilibrium. The *in vivo* recordings gave a flat response through most of the thickness of the vitreous body, indicating that no change in level was introduced by the optical power of the crystalline lens of the eye (Text-fig. 4*a*). The photographic trace does not cut off square at the retinal and lens surfaces as it should with an ideal recording

system. This is largely, again, because of the small angle between the arms of the fluorophotometer which results in a sliding average of the concentration across a depth of 1–2 mm of the vitreous being recorded by the instrument.

Loss of fluorescein from the eye

Normal eye. Immediately after the fluorescein is injected it may be seen in the vitreous body as a discrete mass, but it spreads rapidly and its outlines become diffuse. After 1–2 hr a rising concentration may be detected in the anterior chamber, and after 4–5 hr the fluorescence of the aqueous takes up a constant ratio to that of the vitreous humour, though this is



Text-fig. 5. Intravitreal injection of 15 μl. of a solution of 0·06 % fluorescein in saline. (*a*) *In vivo* recordings taken 6 hr after the injection. (*b*) Contours from eye frozen 7 hr after the injection.

well below 1 %. It may be assumed that the fluorescein has assumed a fixed pattern of distribution within the vitreous body by this time.

If the quantity injected was not too large, the distribution found in frozen sections was always as shown in Text-fig. 5*b*. This picture was confirmed by the *in vivo* observations (Text-fig. 5*a*). The concentration was greatest immediately behind the lens and it dropped to a low value around the entire retinal surface. This value seemed close to zero; whether this was actually so could not be confidently established owing to the uncertainties which attend measurements near the surfaces. In four out of five frozen sections, however, the concentration immediately in front of the retinal vessels appeared slightly lower than elsewhere on the surface. Prolongation of the *in vivo* curves suggested that they would in any case reach zero concentration less than 1 mm behind the retinal surface. This

indicates that the minimum value of the outward permeability of this surface is equivalent to that of a layer of vitreous humour 1 mm thick. There is evidence that molecules even as large as serum albumin move relatively unhindered within the vitreous body of the rabbit (Maurice, 1959) and so the diffusion rate of fluorescein in this cavity may be taken as its value in free solution, 6×10^{-6} cm^2/sec at 37° C (Kaiser & Maurice, 1964). The outward permeability of the retinal surface to the dye must, then, be greater than 0·2 cm/hr.

It is evident from the concentration contours that the principal movement of fluorescein is outward across the entire retinal surface. A lesser gradient anteriorly towards the vitreous-aqueous humour boundary is also seen. A rough forward prolongation of this gradient suggests the concentration would still be substantially above zero at the border of the ciliary processes.

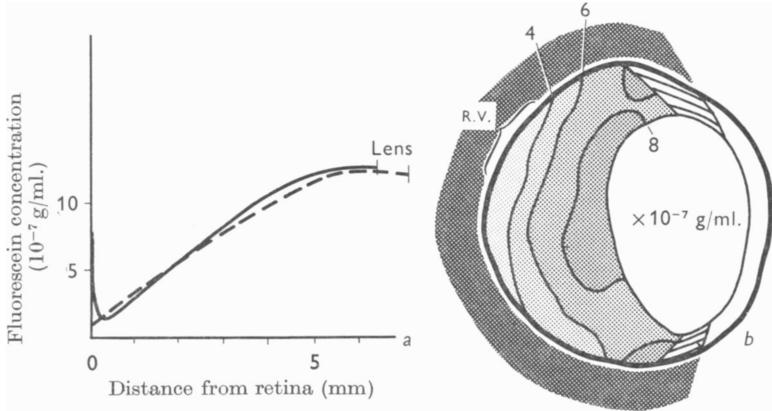
Uphill gradient. The pattern of fluorescein distribution within the frozen vitreous body remained essentially unchanged when very high concentrations of the dye were maintained in the plasma as a result of intravenous or intraperitoneal injection. Technically, it was difficult to determine the value of the greatest possible blood-vitreous concentration step, for low levels of dye in the vitreous body were required for this purpose, thus aggravating the problem, referred to previously, of determining the concentration at the retinal surface. Again, although the amount of fluorescein that could be permitted in the blood was limited by the danger of saturation entry, the concentrations that were obtained stained the aqueous humour so heavily that *in vivo* observations were extremely difficult. A significant entry of the dye into the vitreous body from the ciliary region also occurred.

In several frozen sections, the gradient in the vitreous body showed an outwards movement across the retinal surface, including the vascularized region, when the concentration of fluorescein at the surface was apparently 100 times less than that of the unbound dye in the plasma (Text-fig. 6).

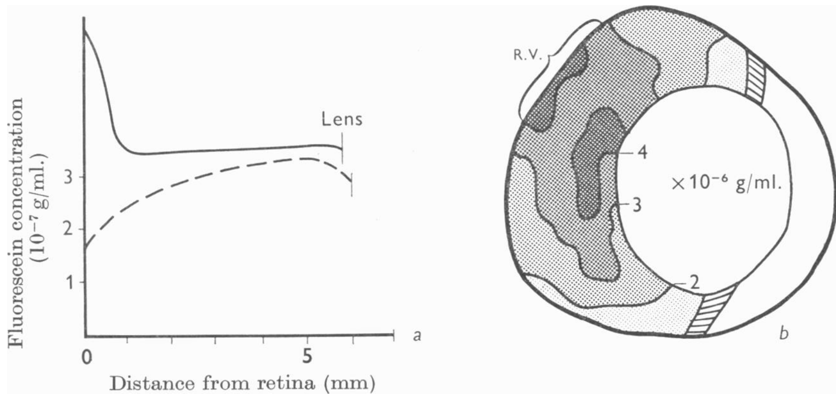
Inhibited movement. The pattern of fluorescein distribution within the vitreous body was modified in a typical manner under conditions designed to inhibit the active transport of anions, and no meaningful differences could be distinguished with our techniques between any of a variety of inhibitors employed. These were: the intravitreal injection of enzymatic inhibitors (dinitrophenol and fluoride) and of competitive inhibitors (iodopyracet, benzyl-penicillin and probenecid), the systemic administration of Benemid (probenecid), and the intravitreal injection of very large doses of fluorescein.

Within a wide range of concentration of inhibitor, the fluorescein distribution across the frozen section was as shown in Text-fig. 7. It is evident

that the movement of fluorescein out of the eye in the region of the retinal vessels has been blocked. In other areas of the retina a substantial flux out of the vitreous appears to continue. The gradient seemed generally to be less than in the normal eye, and in many frozen sections and *in vivo*



Text-fig. 6. Intravitreal injection of 20 μl. of 0·025 % fluorescein. Intraperitoneal injection of 5 ml. 10 % fluorescein 3 hr later (concentration of free fluorescein in the blood 2×10^{-5} g/ml.). (*a*) *In vivo* recordings taken 4 hr after the intraperitoneal injection. (*b*) Contours from eye frozen 5 hr after the intraperitoneal injection.



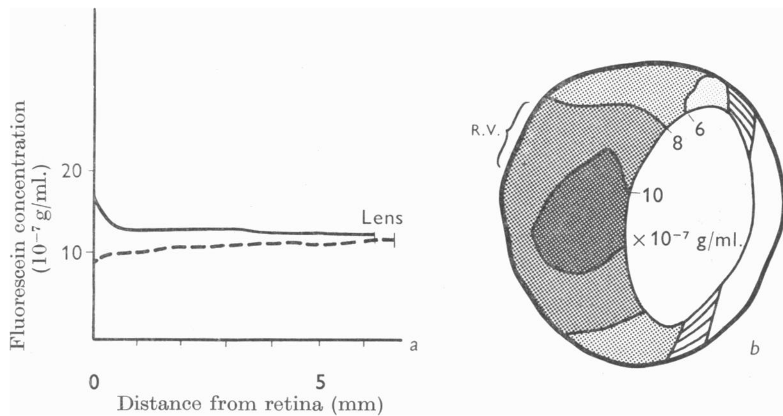
Text-fig. 7. (*a*) *In vivo* recordings taken 17 hr after the injection of 20 μl. 0·7 M iodopyracet in a solution of 0·015 % fluorescein. (*b*) Contours from eye frozen 8 hr after the injection of 20 μl. of $0{\cdot}75 \times 10^{-2}$ Benemid in a solution of 0·06 % fluorescein.

observations the concentration at the retinal surface had dropped to about one half that immediately behind the lens. Very roughly, the resistance of the retina appeared equivalent to 4 or 5 mm of vitreous humour so that its permeability was of the order of 0·05 cm/hr.

Under these conditions the forward gradient of the dye appeared

relatively more important. Nevertheless, the concentration of dye at the vitreous-aqueous interface, estimated by extrapolation, had not fallen as far as in the normal eye; corresponding to this, the concentration measured in the aqueous humour was very much raised above its normal value.

With very high concentrations of inhibitor, the distribution pattern could be made to change further, and the passage of fluorescein could be blocked over the entire retinal surface (Text-fig. 8). Sufficiently large inhibiting concentrations, within the limitations imposed on the volumes of fluid that could be injected intraocularly or systemically, could only be



Text-fig. 8. Intravitreal injection of 15 μl. of 1 M benzylpenicillin in a solution of 0·03 % fluorescein. (*a*) *In vivo* recordings taken 24 hr after the injection. (*b*) Contours from eye frozen 25 hr after the injection.

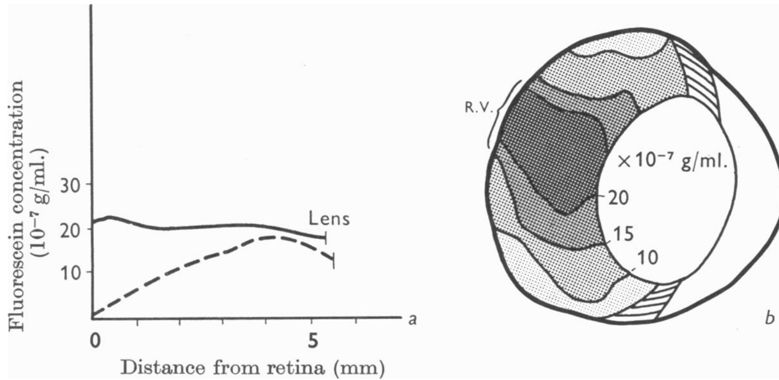
obtained with a near-saturated penicillin solution, or with a combination of systemic Benemid and topical inhibitor. It is difficult to estimate the degree of flatness of these profiles with accuracy, but assuming that a drop in concentration of 5 % per millimetre towards the retina could have been detected with certainty, a maximum value for the retinal permeability of 1×10^{-2} cm/hr may be calculated.

Ophthalmoscopic examination revealed no abnormalities, either short or long term, when moderate doses of inhibitor were used (with the exception of cataracts caused by dinitrophenol). A rapid detachment of the retina was provoked, however, by the large doses of penicillin required to cause total inhibition of the fluorescein transfer.

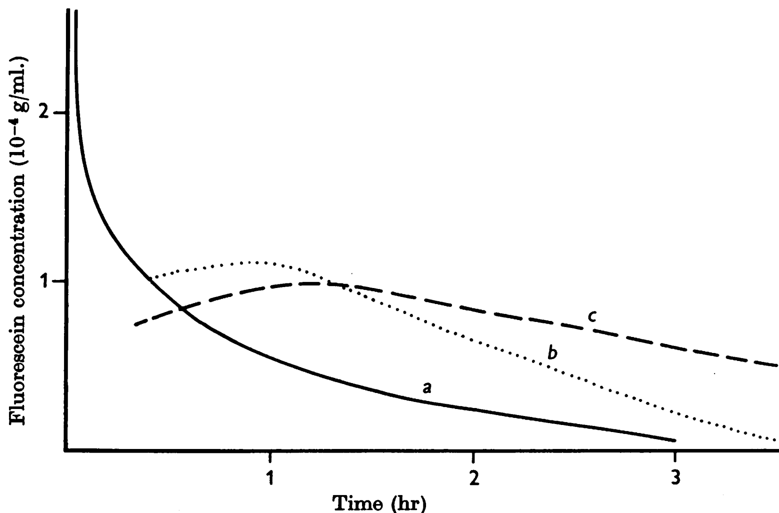
Occluded retinal vessels. When fluorescein was injected into the vitreous body of an eye in which the retinal circulation had been occluded, it was found that it assumed a distribution around these vessels similar to that in the inhibited eye (Text-fig. 9). In other regions the gradient was similar to that in the uninhibited eye.

Fluorescein penetration

Normal eye. After single intravenous or intraperitoneal injections of fluorescein solution, the blood concentration follows patterns exemplified in Text-fig. 10. The more uniform blood levels obtained from intraperitoneal injections made the results easier to interpret quantitatively,



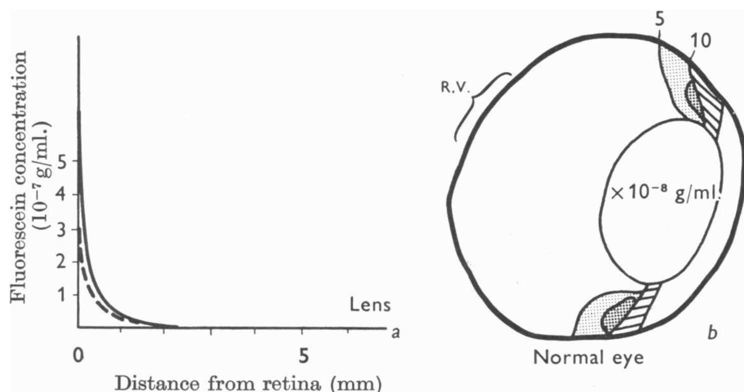
Text-fig. 9. Occlusion of the retinal vessels. Intravitreal injection of 20 μl. of 0·06 % fluorescein. (*a*) *In vivo* recordings taken 5 hr after the injection. (*b*) Contours from eye frozen 5 hr and 30 min after the injection.



Text-fig. 10. Variation of fluorescein concentration in the blood with different modes of administration. (*a*) Intravenous injection of 1 ml. 10 % solution. (*b*) Intraperitoneal injection of 1 ml. 10 % solution. (*c*) Intraperitoneal injection of 1 ml. 10 % solution after systemic administration of 400 mg Benemid. Ordinate: total fluorescein concentration in whole blood. Abscissa: time after injection.

and only the experiments in this group will be described. The proportion of free fluorescein in the whole blood, as determined by dialysis, fell in the range 20–25 % in experiments covering a range of blood concentrations of 10^{-6}–10^{-4} g/ml. These figures correspond to those obtained by Goldmann (1950) by the rapid ultrafiltration of rabbits' blood before it clotted.

In the untouched eye, or one in which an inadequate concentration of inhibitor was injected, the penetration of fluorescein across the retinal surface was not detectable either by observation *in vivo* or in frozen sections (Text-fig. 11). This confirms the observations of Bleeker (1963). Our *in vivo*



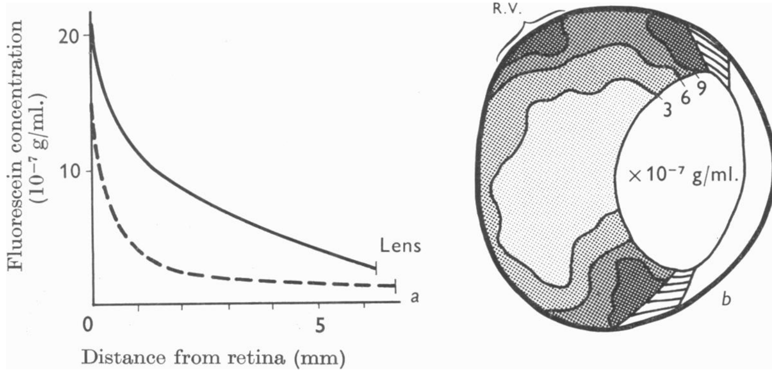
Text-fig. 11. Intraperitoneal injection of 3 ml. 10 % fluorescein. (*a*) *In vivo* recordings taken 1 hr and 45 min after the injection. (*b*) Contours from eye frozen 2 hr after the injection.

measurements showed that the level in the vitreous body remained below 10^{-8} g/ml. when the free fluorescein in the blood was in the region of 10^{-4} g/ml. for many hours. These observations suggest an inwards retinal permeability no greater than about 10^{-5} cm/hr, if the theoretical treatment described in the next section is applied. A small entry into the most anterior portion of the vitreous body, originating from the region of the ciliary body, could be seen in the frozen sections.

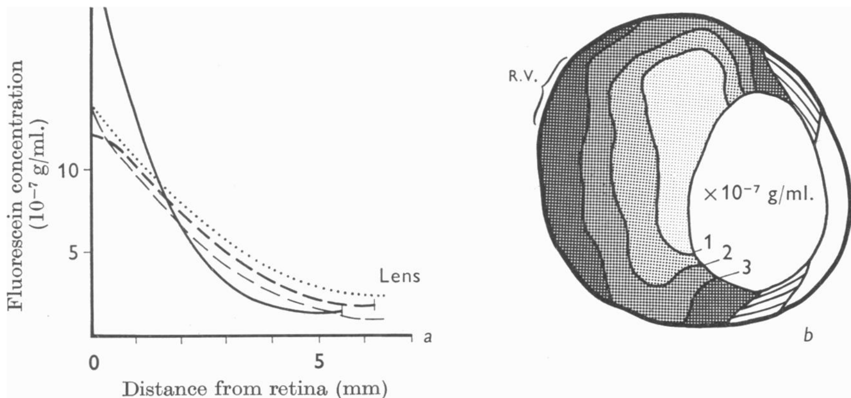
Inhibited eye. When the eye was pre-treated with an adequate dose of inhibitor, fluorescein was found to enter over all the retinal surface after an intraperitoneal injection of the dye. The entry pattern was similar whether metabolic or competitive inhibitors were injected into the eye or whether Benemid was administered systemically (Text-fig. 12). Very high concentrations of fluorescein in the blood forced an entry by some mechanism of saturation, which gave a similar distribution.

With intravitreal inhibition, in general, the rate of entry appeared greater over the region of the retinal vessels than over the rest of the surface. By

contrast, the entry was more uniform over the whole retinal surface when systemic Benemid was given or the large intravitreal concentration of penicillin was produced that was found sufficient (p. 477) to block completely the movement of fluorescein out of the retina (Text-fig. 13). The



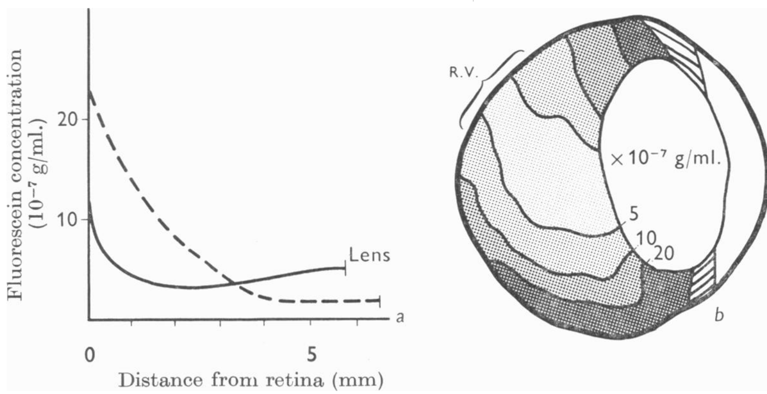
Text-fig. 12. Intravitreal injection of 20 μl. of $0{\cdot}75 \times 10^{-2}$ M benemid. Intraperitoneal injection of 5 ml. 10 % fluorescein 2 hr later. (*a*) *In vivo* recordings taken 2 hr and 30 min after the fluorescein injection. (*b*) Contours from eye frozen 3 hr after the fluorescein injection.



Text-fig. 13. Intravitreal injection of 15 μl. of 1 M benzylpenicillin. Intraperitoneal injection of 3 ml. 10 % fluorescein 5 hr later. (*a*) *In vivo* recordings taken 2 hr and 30 min after the fluorescein injection. Fine dotted and dashed lines show curves calculated for spherical and plane models as described in text, with arbitrarily chosen vertical scale. (*b*) Contours from eye frozen 3 hr after the fluorescein injection.

profile of the concentration within the vitreous body conformed approximately to that which would be expected if the dye were liberated at the retinal surface at a constant rate, diffused through the vitreous humour

at the rate of movement found in free solution, 6×10^{-6} cm²/sec, and met a barrier to further progress at the lens surface. The geometry of the rabbit's eye, a curved lens surface placed 6–7 mm from the spherical retina of 8 mm radius, leads to a very difficult computation. For the present purpose two simple approximations were used between which the true condition might be expected to fall. In one, the retina and lens were taken as large plane sheets $6\frac{1}{2}$ mm apart and, in the other, the retina was assumed to be a complete sphere $6\frac{1}{2}$ mm in radius. The distributions at any time, corresponding to these approximations, can be interpolated between the examples plotted by Crank (1956). The experimentally determined distribution of the fluorescein concentration was generally similar, sometimes closely so, to one or other of these curves (Fig. 13).



Text-fig. 14. Occlusion of the retinal vessels. Intraperitoneal injection of 400 mg of Benemid. Intraperitoneal injection of 5 ml. of 10% fluorescein at the same time. (*a*) *In vivo* recordings taken 1 hr after the injection. (*b*) Contours from eye frozen 1 hr and 30 min after the injection.

From seven experiments the retinal permeability in the fully inhibited eye lay in the range $3{\cdot}5$–18×10^{-3} cm/hr (median value $5\frac{1}{2} \times 10^{-3}$ cm/hr). It is interesting that the average permeability of the retinal surface to the sodium ion, which does not seem to be actively transported, has been calculated to be 1×10^{-2} cm/hr (Maurice, 1957).

Occluded retinal vessels. When fluorescein was administered systemically to an eye in which the retinal vessels had been occluded and that had also been treated with competitive inhibitor, the entry over the region of the vessels was found to be less than elsewhere (Text-fig. 14). In the inhibited untouched eye, on the other hand, entry is greater in this region (Text-fig. 12). It could not be determined how much the entry from the area of the vessels was reduced by occlusion because the fluorescein penetrating the vitreous body rapidly encroached on the narrow region they occupy.

DISCUSSION

General. In the normal eye, the rapid outflux of fluorescein across the boundary of the vitreous, even against a high concentration gradient, compared with the great resistance to its penetration from the blood points to an active transport system operating over the entire surface of the vitreous chamber. This is confirmed by the action of inhibitors, both metabolic and competitive, which tend to make the passage of fluorescein across the barrier more equal in the two directions. The rough calculations, given earlier, suggest that the barrier may lose all its directional properties under inhibition.

The nature of the competitive inhibitors which have been found effective: iodopyracet, benzyl-penicillin and Benemid, suggests an analogy with the organic anion transport systems in the kidney and liver, and it may be assumed that all such ions will be actively transported outwards across the retinal surface.

TABLE 1. Concentration of inhibitor in the vitreous body required to inactivate the transport mechanism for fluorescein. These were calculated on the basis that the injected material was distributed uniformly throughout a vitreous body of average dimensions 1·7 ml. Generally, each concentration step of an inhibitor ($\sqrt{10}\times$) was tested on one eye only, and thus the table indicates the levels of inhibition only roughly.

The inhibitory concentrations appeared to be the same whether movement in or out of the eye was being considered. In the case where the dye penetrated the barrier by saturation of the mechanism, the necessary concentration of dye either in the vitreous barrier or free in the blood seemed to be of the same order of magnitude

Inhibitor	Inhibitory concentrations (M)
Fluorescein (saturation)	3×10^{-4}
Iodopyracet	1×10^{-3}
Benemid	3×10^{-5}
Dinitrophenol	1×10^{-5}
NaF	3×10^{-5}

No attempt was made to determine accurately the threshold concentrations for the inhibitors, but their order of magnitude, shown in Table 1, corresponds to that found in the kidney and liver.

Evidence for the action of such a system in the posterior segment of the eye was previously offered by Forbes & Becker (1960), who showed that the rate at which iodopyracet left the vitreous body was very much reduced by saturation or competitive inhibition. They made no attempt to determine in which region of the vitreous surface this active movement was located.

In the present investigation it was possible to identify the zones where the transport was located in the normal eye and to observe regional dif-

ferences of behaviour under inhibition. Interest was mainly centred on the retinal vessels, and these will form the principal topic of discussion; the remainder of the retina, and the region of the iris and ciliary body will then be treated in separate sections.

Retinal vessels. The retinal vessels of the rabbit fan out in a horizontal band lying on top of a strip of myelinated nerve fibres. Only a few short capillary loops dip down between the nerve fibres. Underneath the fibres the retina has the same structure as in other regions. The results of this paper show that the fluorescein transport system in the vascularized zone is different from that elsewhere in its sensitivity to inhibition. The marked reduction of fluorescein transport and exchange when the retinal vessels are occluded makes it clear, furthermore, that they are responsible for the greater part of the anion transport in this region. It seems that the layer of nerve fibres prevents any significant exchange of fluorescein between the vitreous body and the underlying retina. That the fibres would present a strong barrier to diffusion is suggested by the close packing of their myelin sheaths, but a number of glial cells that penetrate across the layer prevent this being anticipated with certainty. The conclusion that the vessels are engaged in active transport gains support from the results of the intravitreal injection of another organic anion, trypan blue, which may be seen to stain the retinal vessels in the normal eye, but not in an eye treated with a competitive inhibitor.

In the rabbit, the walls of most retinal vessels are seen with the electron microscope (Pl. 1) to be completely free from surrounding glia and in direct contact with the vitreous humour (Ashton, 1965; Cunha-Vaz *et al.* 1966). If the vessels are responsible for the active transport of fluorescein, then the endothelial cells must be the site of this activity because they form the only complete cellular layer interposed between the blood and vitreous body. The only other structure which could form a barrier to the passage of the dye is the basement membrane.

This concept is supported by the same electron microscopical studies, which show that the blood–retinal barrier to large molecules is located in the endothelial layer of the retinal vessels (Ashton, 1965; Cunha-Vaz *et al.* 1966). The barrier is formed by a tightly packed layer of endothelial cells joined together by junctional complexes which completely seal the intercellular spaces. This arrangement was not observed in other vessels of the body, with the sole exception of the brain vessels (Shakib & Cunha-Vaz, 1966).

Relevant to this point is a study (Rodriguez-Peralta, 1962) of the retinal barrier to fluorescent cationic dyes, the diaminoacridines. In the rat, histological observations showed a one-way permeability from the vitreous humour to the blood, the barrier to the movement in the opposite direction being the inner cell membrane of the retinal vascular endothelium.

The impermeability of the retinal vessels to fluorescein in the blood, as well as their distinctive structure, is shared only by the capillaries of the brain. Indeed, previous studies have shown many similarities between the blood–brain barrier and the blood–retinal barrier (Palm, 1947; Ashton & Cunha-Vaz, 1965; Ashton, 1965). It has been shown, furthermore, that large doses of iodopyracet will bring about a break-down of the barrier offered by the brain capillaries (Broman & Olsson, 1948).

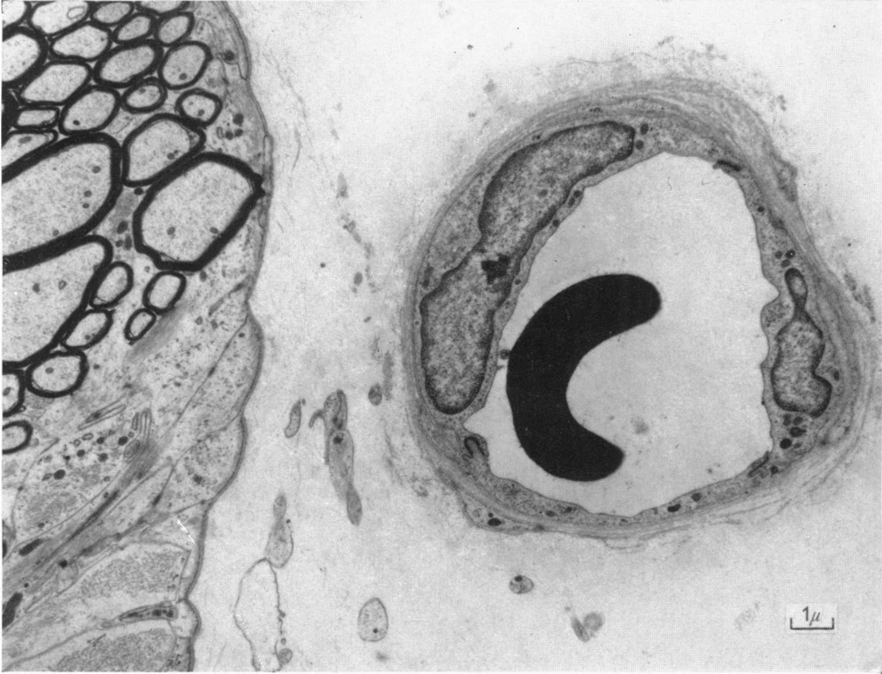
The possibility of an active process being involved in the blood–brain barrier has already been proposed by Steinwall (1961). This author was impressed by the finding that most of the substances which have been used as indicators of the blood–brain barrier, e.g. organic anions like fluorescein, are secreted by the liver and kidney from the blood into the urine and bile by a mechanism of unidirectional transport. He proposed that the impermeability of the blood–brain barrier to these substances could depend, at least in part, upon a similar mechanism of unidirectional transport functioning in the opposite direction, that is, from brain to blood. The experimental studies of Steinwall were, however, seriously complicated by the fact that he could interfere with the transport mechanism only 'from behind', that is, by applying inhibitors to the lumen of the cerebral blood vessels. This procedure would correspond in the kidney to forcing the inhibitors backward into the tubular lumen—a method that is very seldom practised by renal physiologists.

The similarity between the structure and the permeabilities of the capillaries of the brain and the retina supports the view that an active transport mechanism for organic anions is present in the former, and suggest, further, that it is located in the endothelial cells of the vessel walls.

Retina proper. It is evident that an active transport system for organic anions operates also over the surface of the rabbit retina in those regions which are free from retinal vessels. The results suggest that the active process may operate at two levels, one of which is blocked only by very high concentrations of inhibitor.

Which layers of the retina are the seat of this process cannot be decided from the present evidence. However, studies of the penetration of trypan blue from the blood into the retina showed it to be stopped at the pigment epithelium (Cunha-Vaz *et al.* 1966), which suggests that this layer is concerned in the organic anion transport. It is interesting to note, again, that a one-way permeability to diaminoacridines, from the vitreous into the blood, has been demonstrated in the pigment epithelium cell membrane facing the retina by Rodriguez-Peralta (1962).

Ciliary region. The virtual absence of fluorescein from the aqueous humour after its intravitreal injection in the normal eye, contrary to what



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would be expected from the concentration gradient in the anterior vitreous body, suggests that active transport out of the eye continues in the anterior uvea. On the other hand, the normal entry of fluorescein from the blood in this region indicates another process in action distinct from that found over the rest of the retinal surface.

There is an anatomical basis for this double mechanism in the presence in this region of both iris and ciliary body. The passage of the dye through the ciliary processes, from the blood to the posterior chamber, has been observed directly, in the cat, by van Alphen & Macri (1966). At the same time, the iris is better situated to absorb the last traces of fluorescein diffusing into the anterior chamber. This suggests the hypothesis that under normal conditions the entry from the blood occurs through the ciliary body only, and that the posterior surface of the iris, probably the pigment epithelium, behaves similarly to regions of the retina embryologically related to it and actively transports fluorescein out of the eye.

Function of the transport mechanism. The transport mechanism in the blood–retinal barrier, both at the level of the retinal vessel walls and of the pigment epithelium, is situated so as to prevent the penetration of organic anions from the blood into the retinal tissue and to remove any already present. Organic anions are known to be toxic to the brain, for convulsions or paralysis have been seen to develop when they enter the central nervous system (Broman & Olsson, 1948; Bassett, Rogers, Cherry & Gruzhit, 1953). A transport mechanism similar to that described here has been proposed by Steinwall (1961) to protect the central nervous system tissue against the penetration of these substances, and it is probable that this protection would be extended to the retina. The removal of toxic products of retinal metabolism by means of conjugation with glucuronic acid or sulphate is one way in which this mechanism could operate. These products are known to be actively transported by the kidney as are other organic anions.

The formation of retinal detachments under the action of very high concentrations of competitive inhibitor suggests that the anion transport mechanism has a part to play in maintaining normal retinal attachment to the pigment epithelium. This possibility must also remain speculative pending further experimental investigations.

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REFERENCES

ASHTON, N. (1965). The blood-retinal barrier and vaso-glial relationships in retinal disease. *Trans. ophthal. Soc. U.K.* **85**, 199–229.

ASHTON, N. & CUNHA-VAZ, J. G. (1965). Effect of histamine on the permeability of the ocular vessels. *Archs Ophthal. N.Y.* **73**, 211–223.

BASSETT, R. C., ROGERS, J. S., CHERRY, G. R. & GRUZHIT, C. (1953). The effect of contrast media on the blood-brain barrier. *J. Neurosurg.* **10**, 38–47.

BLEEKER, G. M. (1963). *Variation in Depth in the Anterior Chamber of the Eye.* Amsterdam: Van Campen.

BROMAN, T. & OLSSON, O. (1948). The tolerance of cerebral blood vessels to a contrast medium of the Diodrast group. *Acta radiol.* **30**, 326–342.

CRANK, J. (1956). *The Mathematics of Diffusion*, 1st edn. pp. 59 and 93. Oxford: Clarendon Press.

CUNHA-VAZ, J. G. & MAURICE, D. M. (1966*a*). Fluorescein transport by retinal vessels. *J. Physiol.* **183**, 42*P*.

CUNHA-VAZ, J. G. & MAURICE, D. M. (1966*b*). Fluorescein transport by retinal vessels. *Proc. XX int. Cong. Ophthal., Munich.* (In the Press.)

CUNHA-VAZ, J. G., SHAKIB, M. & ASHTON, N. (1966). Studies on the permeability of the blood-retinal barrier. I. On the existence, development and site of a blood-retinal barrier. *Br. J. Ophthal.* **50**, 441–453.

FORBES, M. & BECKER, B. (1960). The transport of organic ions by the rabbit eye. II. *In vivo* transport of iodopyracet (Diodrast). *Am. J. Ophthal.* **50**, 867–873.

GOLDMANN, H. (1950). Über Fluorescein in der menschlichen Vorderkammer. *Ophthalmologica, Basel* **119**, 65–95.

KAISER, R. J. & MAURICE, D. M. (1964). The diffusion of fluorescein in the lens. *Expl Eye Res.* **3**, 156–165.

MAURICE, D. M. (1957). The exchange of sodium between the vitreous body and the aqueous humour. *J. Physiol.* **137**, 110–125.

MAURICE, D. M. (1959). Protein dynamics in the eye studied with labelled proteins. *Am. J. Ophthal.* **47**, 361–367.

MAURICE, D. M. (1963). A new objective fluorophotometer. *Expl Eye Res.* **2**, 33–38.

PALM, E. (1947). On the occurrence in the retina of conditions corresponding to the 'blood–brain barrier.' *Acta ophthal.* **25**, 29–35.

RODRIGUEZ-PERALTA, L. A. (1962). Experiments on the site of the blood-ocular barrier. *Anat. Rec.* **142**, 273.

SHAKIB, M. & CUNHA-VAZ, J. G. (1966). Studies on the permeability of the blood-retinal barrier. IV. Junctional complexes of the retinal vessels and their role on the permeability of the blood-retinal barrier. *Expl Eye Res.* **5**, 229–234.

STEINWALL, O. (1961). Transport mechanisms in certain blood–brain barrier phenomena: a hypothesis. *Acta psychiat. neurol. scand.* suppl. **150**, 314–318.

VAN ALPHEN, G. W. H. M. & MACRI, F. J. (1966). Entrance of fluorescein into aqueous humor of cat eye. *Archs Ophthal. N.Y.* **75**, 247–253.

EXPLANATION OF PLATE

PLATE 1

Section of a retinal vessel seen in the electron microscope. The direct contact of the vitreous humour with the endothelial cells, and the tight cellular junctions should be noted. Osmium tetroxide fixed tissue embedded in araldite. Sections stained with uranyl acetate and lead citrate.