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QUANTITATIVE ASSESSMENT OF THE PERMEABILITY OF THE RAT BLOOD–RETINAL BARRIER TO SMALL WATER-SOLUBLE NON-ELECTROLYTES

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SUMMARY

1 The passive permeability of the blood–retinal barrier (b.r.b.) to the water-soluble non-electrolytes, sucrose and mannitol, was determined using a multiple time point–graphical approach as has been used in the assessment of blood-brain barrier (b.b.b.) permeability.

2. The calculated permeability surface area product for the b.r.b. for sucrose was 0·44 ($\pm$0·081 S.E. of mean) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 20$) and for mannitol was 1·25 ($\pm$0·30) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 18$). These values are similar and comparable to those found for the capillaries in the brain ($P > 0·05$) and significantly different from zero ($P < 0·01$).

3. Data on the concentrations of sucrose in different parts of the eye show that the permeability of the blood–retinal barrier, rather than the more permeable blood–aqueous barrier permeability, was being measured by our technique.

INTRODUCTION

The blood–retinal (b.r.b.) closely resembles the blood–brain barrier (b.b.b.) in function (Rapoport, 1976) but is somewhat different in structure. It consists of two barrier sites, the retinal capillary endothelial cells and the retinal pigment epithelial cells whereas the b.b.b. has three sites, the choroid plexus, the cerebral capillary endothelial cells and the arachnoid membrane. Retinal capillary endothelial cells are joined together by tight intercellular junctions (zonulae occludens) (Lasansky, 1967), which are devoid of fenestrae, contain very few pinocytotic vesicles (Shiose, 1970) and are similar ultrastructurally to cerebral capillaries. The retinal pigment epithelial cells have their apical ends connected by rings of tight junctions and separate the retina from the choroid which contains leaky fenestrated vessels. These two cell layers effectively constitute an almost continuous barrier separating the retina from the blood, both in the choroid and in the retinal blood vessels (Cunha-Vaz, 1976).

The b.b.b. retards the entry of most water-soluble molecules from the blood into the brain; the permeation rates of such substances usually depend on their lipid

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solubility (Ohno, Pettigrew & Rapoport, 1978). For some hexoses (Dimattio & Zadunaisky, 1981) and some amino acids (Tornquist & Alm, 1986), facilitated transport has been demonstrated to occur via stereospecific carrier transport systems both in the brain and the retina. It has been suggested that ions and water-soluble non-electrolytes of molecular weight approximately 1000 and larger, cross the cerebrovascular endothelium usually through the intercellular route which includes the tight junctions, but that the transcellular route is followed by water and small non-electrolytes. The basis for each preference is the permeability of the membrane surface for the specific substance as compared to the permeability of the junctional, intercellular region (Wright, Smulders & Tormey, 1972).

Both the b.b.b. and b.r.b. have been shown to be qualitatively similar and to restrict the entry of large protein tracers such as horseradish peroxidase. Quantitative comparison of the two barriers is very limited and although much work has been done on the b.b.b., there is much less on the b.r.b. Most of the studies that have been done suffer from methodological problems which make the results difficult to interpret (Tornquist, 1979; Tornquist, Alm & Bill, 1979; Alm & Tornquist, 1981; Ennis & Betz, 1986).

We used an *in vivo* intravenous technique adapted from a method previously used in the brain (Ohno *et al.* 1978; Gjedde, 1981) to quantitatively assess blood–retinal permeability to sucrose and mannitol (two small uncharged particles). These techniques employ a multiple time point–graphical approach to determine the passive permeability of the blood–retinal barrier (b.r.b.).

METHODS

Surgical procedure

Adult Sprague–Dawley rats, weighing 300–450 g, were anaesthetized with sodium pentobarbitone intraperitoneally (I.P.) (30 mg kg^{-1}), supplemental doses (10 mg kg^{-1} I.P.) being given as needed. Polyethlyene catheters filled with heparinized saline were tied into the right femoral artery and vein. A temperature probe was inserted into the rectum and the animals maintained at 35–37 °C using a heat lamp and temperature feed-back device.

Radiochemicals

[^{14}C]sucrose (specific activity = 4·9 mCi $mmol^{-1}$) and [^{3}H]mannitol (specific activity = 25 mCi $mmol^{-1}$) were obtained from New England Nuclear (Boston MA, U.S.A.). Purity was confirmed by chromatography.

Procedure

[^{14}C]sucrose and/or [^{3}H]mannitol, 200–250 μCi of each, was injected into the femoral venous catheter at time zero, followed by a 300 μl saline flush. Sequential arterial samples (50 μl) were taken at regular intervals via the arterial catheter, their exact timing depending on the final time point chosen for each experiment. The blood samples were put into heparinized tubes, spun in a microcentrifuge (Beckman Instruments, Oxnard, CA, U.S.A.), and 20 μl aliquots from each sample placed in a scintillation vial. Rats were killed between 1 and 45 min after the start of each experiment. The brain and eyes were rapidly removed. An incision was made into the eye with a microscalpel through the sclera behind the ciliary body. The incision was extended 360 deg using Van Ness scissors, and the cornea, lens and vitreous were removed *en bloc*. The retina was dissected free from the choroid and sclera and the optic nerve was cut, the entire process taking less than 1 min. A sample of occipital cortex was taken from the brain.

Retina and brain samples were put into pre-weighed scintillation vials and digested overnight at 50 °C in 1 ml Protosol (New England Nuclear). All samples including plasma samples were prepared

for scintillation counting by adding 10 ml of Ready Solv–MP scintillation cocktail (Beckman Instruments, Fullerton, CA, U.S.A.). ^{3}H and ^{14}C counting were performed with a Beckman LS 9000 liquid scintillation counter.

Calculations

For the situation when a test substance moves unidirectionally from plasma into brain tissue and the experimental time is long enough to allow adequate mixing in the intravascular compartment, Patlak, Blasberg & Fenstermacher (1983) modified the analysis of the bolus method used by Ohno *et al.* (1978) and showed that the following relation holds:

$$A_m/C_p(T) = (PA)\left[\int_0^T C_p\,dt/C_p(T)\right] + V,$$

where A_m = concentration in tissue at time T (final time point) $C_p(T)$ = concentration in plasma at time T, V = functional volume of test solute that rapidly and reversibly exchanges with the plasma per unit mass of tissue and is calculated graphically, and PA = capillary permeability–surface area product.

Capillary permeability–surface area product can be determined from experimental data under conditions of changing plasma concentrations, using the above equation and graphical analysis (Patlak *et al.* 1983). Each rat is used as a single point on the graph. For each rat, the integrated plasma concentration is obtained by plotting the sequential arterial plasma counts against time (in seconds) and the area under the curve is calculated. This value,

$$\int_0^T C_p\,dt,$$

is then divided by the final plasma concentration obtained in the same rat and plotted on the x-axis against the ratio of the tissue concentration to the final plasma concentration on the y-axis. Patlak *et al.* (1983) showed that these points fall on a curve but there is a linear portion and the best-fit line for these points can be drawn by least-squares analysis of the points. From the above equation, it can be seen that the capillary permeability–surface area product value is the slope of the linear regression line and V is the intercept of the line with the y-axis, as it is the amount in the tissue before diffusion has occurred out of the intravascular compartment. This relation was developed by Bradbury & Kleeman (1967) and has been used by several other investigators (Baños, Daniel, Moorhouse & Pratt, 1973; Sarna, Bradbury & Cavanagh, 1977; Gjedde, 1981), to determine capillary permeability–surface area product values for different substances in the brain.

Dissection of eye for measurement of gradient within the eye

We chose two time points on the linear part of the curve at which unidirectional flux was occurring and backflux was calculated to be absent. Sucrose was labelled with two different isotopes (^{3}H and ^{14}C) and time points of 5 and 20 min chosen. The same procedure of arterial sampling was followed and the results analysed by the reiterative method (Ohno *et al.* 1978). Both eyes were enucleated at 20 min after injection, one being rapidly frozen in dry ice and the other dissected fresh as above. The frozen eye was dissected into layers as depicted in Fig. 1. When the eye was frozen solid, the cornea was cut through 360 deg, removed and the aqueous humour removed as a solid ice ball. The lens and vitreous were removed *en bloc*, again as an ice ball from the remaining tissue. The vitreous behind the lens was cut free from the rest of the sample and labelled posterior vitreous. The rest of the vitreous and lens was thawed and the lens was removed. This constituted the anterior vitreous sample. The retina was thawed and dissected from the choroid and sclera: it was more difficult to separate the retina from the choroid after freezing and subsequent thawing than when they were dissected fresh. Each sample was weighed, digested overnight in Protosol and counted as above.

RESULTS

Each animal was used as a single time point for calculating the results. The integration factor with time for the concentration in the plasma was calculated by

computer analysis from the plasma curves using the counts from the individual arterial samples taken during each experiment where t is the time in seconds after the radioactive tracer was injected that the experiment was terminated. A_m was calculated by dividing the counts in the tissue by the wet weight. C_p was calculated from the counts in the final plasma sample divided by 0·02 since the sample size was 20 μl.

Plots of A_m/C_p (t) against $\int_0^T C_p\,dt/C_p(t)$ for each animal, for the occipital cortex and the retina are shown in Fig. 2 for sucrose and in Fig. 3 for mannitol. The data

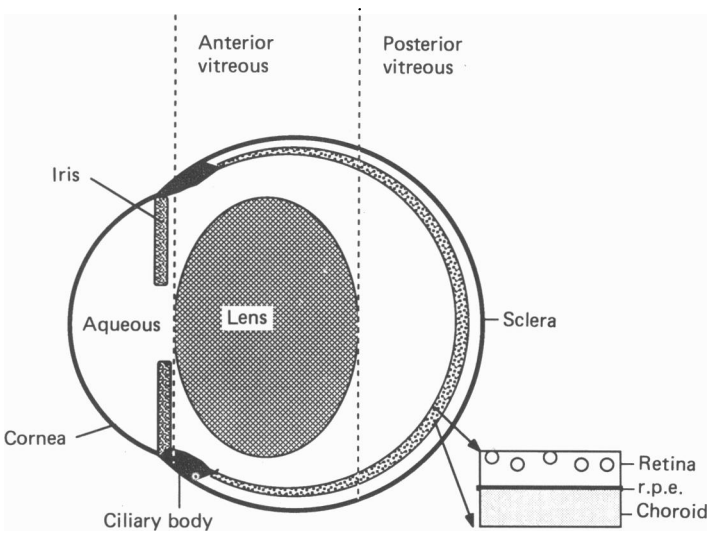

Fig. 1. Schematic drawing of whole rat eye to show anatomy and to define layers used for analysis of gradient within eye. Retinal pigment epithelial cells, r.p.e.

were fitted by least-squares analysis to provide the linear regression lines in the Figures. Capillary permeability–surface area product and V were calculated from the slope and the intercept with the y-axis, respectively, of the linear regression line. Slopes used to calculate capillary permeability–surface area products were significantly greater than zero ($P < = 0{\cdot}01$).

Results are expressed as the means ±standard errors. Capillary permeability–surface area products for the retinal capillaries were 0·44 ($\pm 0{\cdot}081$) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 20$, $r = 0{\cdot}76$) for sucrose and 1·25 ($\pm 0{\cdot}30$) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 18$, $r = 0{\cdot}75$) for mannitol. For the vascular volume, $V = 0{\cdot}02$ ($\pm 0{\cdot}024$) ml g^{-1} for sucrose and 0·0356 ($\pm 0{\cdot}0071$) ml g^{-1} for mannitol. Capillary permeability–surface area products for the capillaries of the occipital cortex were 0·476 ($\pm 0{\cdot}038$) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 25$, $r = 0{\cdot}93$) for sucrose and 0·98 ($\pm 0{\cdot}29$) $\times 10^{-5}$ ml g^{-1} s^{-1} ($n = 21$, $r = 0{\cdot}61$) for mannitol, both of which are similar to previously published results (Ohno *et al.* 1978). For the vascular volume of the occipital lobe, $V = 0{\cdot}008$ ($\pm 0{\cdot}001$) ml g^{-1} for sucrose and 0·20 ($\pm 0{\cdot}007$) ml g^{-1} for mannitol, again agreeing with other investigators. There was no significant difference between the capillary permeability–surface area products obtained for the retinal and cerebral capillaries ($P > 0{\cdot}05$).

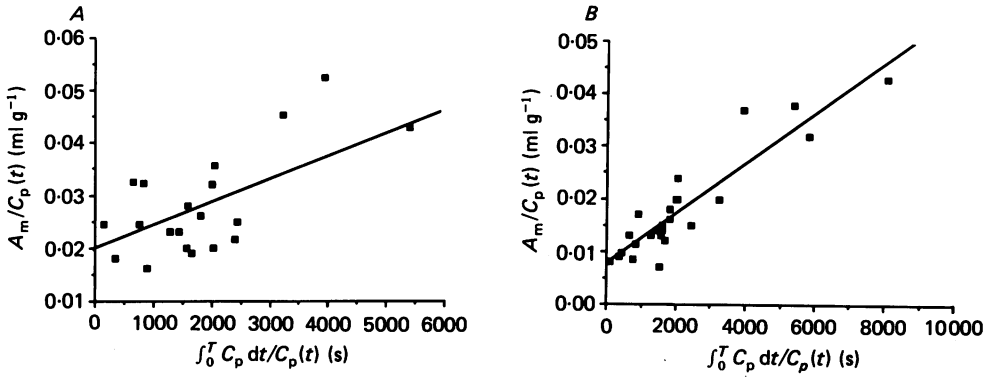

Fig. 2. Graphs show tissue–plasma concentrations of sucrose in retina (*A*) and occipital cortex (*B*) plotted against plasma concentration of sucrose integrated with time, for each rat and the best-fit line drawn by linear-regression analysis of the points. The slope of the line is the capillary permeability–surface area product, and *V*, the intercept of the line with the *y*-axis.

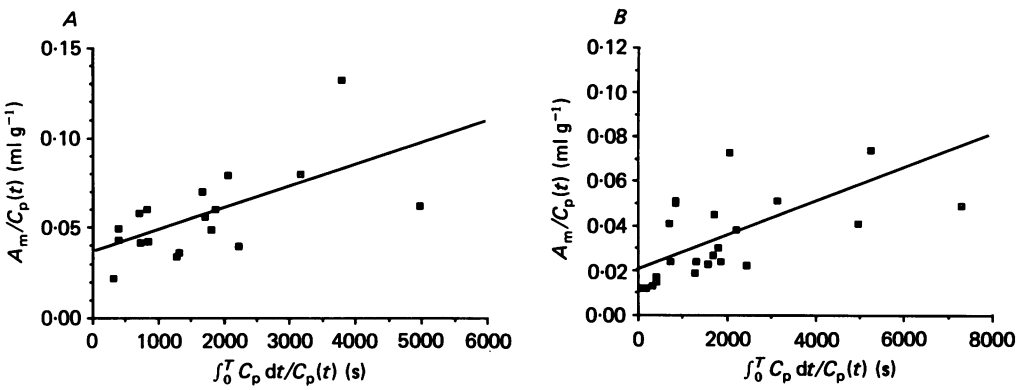

Fig. 3. Graphs show tissue–plasma concentrations of mannitol in retina (*A*) and occipital cortex (*B*) plotted against plasma concentration of mannitol integrated with time, for each rat and the best-fit line drawn by linear-regression analysis of the points. The slope of the line is the capillary permeability–surface area product, and *V*, the intercept of the line with the *y*-axis.

Gradient of radioactivity within the eye

To ensure that radioactivity within the retina was not coming via the aqueous humour from the ciliary body which has a high permeability to small non-electrolytes such as sucrose (Pederson & Green, 1973), the eye was dissected in layers from front to back (see Methods) and the concentration of radioactivity in each layer determined. The extracellular space of the retina was taken as 31% of the wet weight (Ames & Nessbett, 1966), and the concentration of tracer found in the retina minus that remaining in the vascular space of the retina was assumed to be extracellular. The concentration in the extracellular space of the fresh retina was compared to that in the frozen retina, the aqueous, anterior vitreous and posterior vitreous. Ratios

comparing the concentrations of each of these layers to the concentration in the fresh retina were determined.

Frozen retinae contained a much higher concentration of radioactivity than did freshly dissected retinae. When the vascular volume was calculated (using the reiterative approach), it was also found to be extremely high, suggesting that during freezing and thawing the retina had become contaminated from the choroid. Sucrose concentrations of the other layers of the frozen eye, which were essentially fluids rather than tissues, were compared to the concentration found in the fresh retina and the results shown in Table 1. Although a higher concentration of tracer was found in the aqueous than the retina (retina: aqueous concentration = $1:1{\cdot}9\pm0{\cdot}9$), concentrations in the more posterior layers, in particular the posterior vitreous which adjoins the retina, were found to be less (retina: anterior vitreous = $1:0{\cdot}5\pm0{\cdot}2$ and retina: posterior vitreous = $1:0{\cdot}28\pm0{\cdot}18$).

TABLE 1. Concentration gradient of sucrose across the eye

Retina	Posterior vitreous	Anterior vitreous	Aqueous
1	$0{\cdot}28\pm0{\cdot}18$	$0{\cdot}5\pm0{\cdot}2$	$1{\cdot}9\pm0{\cdot}9$

Concentrations of sucrose in each layer were compared to those in the freshly dissected retina. The ratios obtained for each eye ($n = 6$) were then averaged and the means $\pm$ standard errors shown.

The weights of the fresh retinae with attached posterior vitreous, following dissection were compared to weights of the retina and vitreous when dissected separately to see how much contamination of the retina there was by vitreous in the fresh samples. The fresh retinal specimens weighed 0·021 g ($\pm0{\cdot}004$, $n = 25$), and when dissected separately from the vitreous weighed 0·011 g ($\pm0{\cdot}001$, $n = 5$). The total weight of the vitreous was 0·05 g ($\pm0{\cdot}005$, $n = 5$).

DISCUSSION

In this study we measure the permeability of the b.r.b. to the water-soluble non-electrolytes sucrose and mannitol, and demonstrate that the permeability–surface area product is very low and similar to that of the b.r.b. with mannitol being more permeant than sucrose. Cerebral capillaries have been shown to have very low capillary permeability–surface area products by many other investigators and the method used in this study for the occipital cortex gives similar values to those achieved by others (Ohno *et al.* 1978), demonstrating that the technique we used was sound. The retinal capillary endothelial cells are joined together by tight junctions that are as tight as those found in the cerebral capillaries. Because the b.r.b. consists of retinal capillaries and retinal pigment epithelium, it is likely that the intercellular junctions of the retinal pigment epithelial cells are as tight as the capillary endothelial cells. In this study, we cannot separate the relative contributions of the endothelial cells and the retinal pigment epithelial cells.

The non-pigmented epithelium of the ciliary body is leaky to small non-electrolytes such as sucrose. Multicompartmental analysis as well as *in vitro* measurements show

that the ciliary epithelium of the rabbit eye is a leaky layer with leaky tight junctions (Cole, 1966; Pederson & Green, 1973), whereas the retina has very tight junctions as does the brain. Hence it was important to show that tracer accumulating in the retina did not diffuse from the ciliary body through the vitreous but entered through the b.r.b. By showing that the concentration gradient of the tracers within the eye was from the retinal extracellular space to the posterior vitreous, we demonstrated that tracer within the retina probably crossed the b.r.b. and did not come through the vitreous. It is also likely that some tracer in the posterior vitreous, some of which remains attached to the dissected retina, entered from the retina and therefore lowered retinal radioactivity. Hence the capillary permeability–surface area product values we obtained for the retina, are underestimated.

Ennis & Betz (1986) recently found that the capillary permeability–surface area product of the b.r.b was four times that of the b.b.b. whereas our results demonstrate approximate equality. There are several possible reasons for the difference. We removed the retina under direct vision after the anterior segment of the eye, including the ciliary body, had already been removed, and thus minimized contamination of the retina by the ciliary body. Intraocular fluids drain from the eye when it is opened in this way including a large percentage of vitreous. We estimated how much vitreous contaminated the fresh retina by weighing each of the entire vitreous and retina from frozen eyes after dissecting them separately and comparing these weights to values of the freshly dissected retina. We calculated that most of the vitreous was removed during our dissection of the retina. Furthermore, because of the concentration gradient, it effectively lowers the permeability–surface area product by increasing the weight of the retinal sample. However, this would seem to be at maximum a factor of 2 and we feel that this is acceptable as attempts to remove this adherent posterior vitreous would be likely to damage the retina and produce much larger errors.

Now that the method to isolate the retina and the b.r.b. from the rest of the eye has been worked out and the base-line values for retinal permeability have been determined, it should be possible to look at the effect of metabolic perturbations and inflammatory mediators on the blood–retinal barrier. This is of clinical importance, as break-down of the b.r.b., at the level of the retinal blood vessels or the r.p.e., occurs in many pathological conditions (Cunha-Vaz, 1979), including diseases such as diabetes mellitus and intraocular inflammation.

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