The Effect of pH on the Transfer of Fluorescein across the Blood–Retinal Barrier

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The blood–retinal barrier (BRB) might be governed by the same permeability principles as the blood–brain barrier (BBB). For a weak acid like fluorescein, BRB permeability would be proportional to its pH-dependent lipid solubility, according to the pH partition hypothesis. A range of metabolic acidosis was produced in 20 rats by the oral administration of NH₄Cl; six additional rats received normal saline. Four hours later, vitreous fluorophotometry, venous fluorescein values, and arterial pH were measured. Significant linear relationships were found between vitreous fluorophotometry readings and blood hydrogen ion concentrations (p < 0.025) and plasma fluorescein concentrations (p < 0.05). According to the linear relationship, changing the pH from 7.4 to 7.3 or 6.9 would result in an increase in vitreous fluorophotometry reading of 8.5 or 72%, respectively. Since the pH partition hypothesis predicts values of 52 or 640%, our results suggest that the BRB conforms less to the hypothesis than does the BBB. Furthermore, although pH changes of a magnitude able to influence vitreous fluorophotometry readings substantially may occur under experimental conditions in animals, they are unlikely to occur in ambulatory human patients. Invest Ophthalmol Vis Sci 26:1133–1139, 1985

The BRB and BBB have major similarities. The rate of passive inward transport of many molecules into the brain can be interpreted in terms of the pH partition hypothesis. This hypothesis applies to weak acids (such as fluorescein) and bases, that is, molecules that can exist in more than one state of ionization depending on the dissociation of hydrogen ions. According to this hypothesis: (1) the percentage of a weak acid or base in the unionized form depends on the pH; (2) the permeability of the barrier to ions is so small that it can be neglected; and (3) the permeability of the barrier to unionized molecules is proportional to their lipid solubilities.

Grimes et al showed that the octanol/water partition ratio (a convenient estimate of lipid solubility) of fluorescein depended on the pH. Shinowara et al found that acidosis increased penetration of fluorescein across the BRB using fluorescence microscopy of freeze-dried retina. They provided evidence that the BRB was not altered. Rather, the increased penetration was thought to be due to an increase in the percentage of fluorescein in the unionized state in acidosis in accordance with the pH partition hypothesis.

We tested whether penetration of fluorescein from the blood into the vitreous is pH-dependent using vitreous fluorophotometry in rats with metabolic acidosis. Although a relationship was found, the pH partition hypothesis appears to account only partially for the permeability of fluorescein into the vitreous.

Materials and Methods

According to the following protocol, 20 male hooded rats were made acidic. For 24 hr prior to the experiment, the rats were restricted to a fluid diet consisting of a solution of 1.5% NH₄Cl in tap water (pH 5.5) ad lib. On the day of the experiment, the rats were sedated with 0.5 ml/kg of a mixture containing 81 mg/ml ketamine and 1.9 mg/ml acepromazine. Their stomachs were cannulated with a 20-gauge internal diameter tube. From 5 to 10 ml of a solution of 400 millimolar NH₄Cl were administered over a period of 1 hr, which produced a range of blood pH values. The maximum acidosis occurred approximately 2 hr after the administration of the
Fig. 1. Vitreous fluorophotometry scans taken before (dotted line) and 5 min (continuous line), 37 min (dashed line), and 63 min (intermittent line) after injection. The height of the chorioretinal peak (left) decreases with the plasma fluorescein level. The low level to the right is the lens. The vitreous concentration rises with time.

solution. Six rats received 10 ml of normal saline instead of NH₄Cl and had no NH₄Cl added to their drinking water the day before the experiment.

Five hours after initiating the fluid administration (4 hr after initiating the last dose) the rats received (Mydriacyl, Alcon; Fort Worth, TX) and 10% phenylephrine hydrochloride (Neo-Synephrine, Winthrop; New York, NY). 33 mg/kg of 10% sodium fluorescein were given intravenously. One hour later, the animals were anesthetized again with 0.1 ml of ketamine–acepromazine. Vitreous fluorophotometry was then performed. Venous blood was obtained for plasma fluorescein and glucose determinations. Within 30 min after the fluorophotometry, arterial blood was drawn from the carotid artery before fluorescein was injected to determine whether a significant change in pH occurred during the hour that the fluorescein was circulating. Since the change in hydrogen ion concentration averaged less than 2% (even less for pH), the initial arterial blood sample was subsequently omitted. The arterial blood was obtained by exposing the carotid artery on one side, often aided by sectioning the overlying muscle. About 0.5 ml of blood were withdrawn through a 25-gauge needle using a heparinized, glass, 1-ml syringe. Direct pressure was used to attain hemostasis. In the animals from which arterial blood was drawn before finishing the fluorophotometry, the wound edges were reapproximated and gauze moistened with saline solution was applied to the wound.

Vitreous fluorophotometry was performed with a slit-lamp based fluorophotometer, as previously described. To illustrate the progression of fluorophotometry profiles in the hour after injection, scans of a normal rat eye from another experiment are shown (Fig. 1). The fluorophotometry scans had a high chorioretinal peak value and a low value for the lens. The chorioretinal peak declined along with the plasma fluorescence with time. The vitreous fluorescence sloped down from the chorioretinal peak, became relatively flat in the middle portion of the vitreous by 1 hr, and then sloped sharply downward at the lens. The midvitreous measurement was taken at the midpoint of the rather straight segment between the chorioretinal peak and the posterior aspect of the lens. The person interpreting the scan did not know the pH state of the animal.

In other studies from our laboratory, we found that the native fluorescence in the midvitreous before injection was 3 ± 1 ng eq/ml. This was considered small enough to be neglected in the present study. The spread function of the chorioretinal peak was evaluated 2–8 min after injection when very little fluorescein was in the midvitreous. The spread function in the midvitreous was 5 ± 3% of the chorioretinal peak. Therefore, we subtracted 5% of the chorioretinal peak value from the midvitreous value in scans taken 1 hr after injection. This amounted to 15.3 ± 8.3% of the midvitreous value. The mean of the two eyes of each animal was used for data analysis.

Heparinized venous blood was centrifuged and the total plasma fluorescein, after diluting it 100 times with phosphate buffer (pH = 7.4), was determined using the fluorophotometer. The plasma glucose level was measured using a Beckman II glucose analyzer (Beckman; Fullerton, CA), and the arterial pH was determined using a Corning 168 pH/blood gas analyzer (Corning; Medfield, MA).
The investigations conformed to the ARVO Resolution on the Use of Animals in Research.

**Results**

Data obtained from 20 animals receiving NH₄Cl and six animals receiving saline are shown in Table 1. The arterial pH ranged between 7.283 and 6.886 in the animals receiving NH₄Cl and between 7.562 and 7.366 in the animals administered saline.

The relation between the midvitreous fluorophotometry value and blood hydrogen ion concentration is shown in Figure 2. A linear equation was fit to the data by the method of least squares. The equation was midvitreous fluorophotometry value = 99 + 1.2 × 10⁻³ × blood hydrogen ion concentration. The coefficient of determination (R²) was used to calculate a t statistic. This permitted us to test the hypothesis that a correlation was present between the two factors. A p value of less than 0.025 was obtained. Therefore, we rejected the null hypothesis and concluded the linear regression was statistically significant at the p < 0.05 level.

No significant linear regression was found between the plasma fluorescein concentration and the blood hydrogen ion concentration.

The mean plasma glucose values were 185 ± 60 and 169 ± 17 mg/dl in the rats administered NH₄Cl and saline, respectively. These were not different statistically.

**Discussion**

This study identified two factors that correlated with penetration of fluorescein from the blood into the vitreous: (1) the concentration of fluorescein in the blood; and (2) the pH of the blood. The importance of the first factor is intuitively obvious. The second factor could produce an effect by altering the barrier and/or changing fluorescein molecules. The morphologic integrity of the barrier cannot be evaluated in the present study. However, in a recent report, Shinowara et al found the barrier to be intact in metabolic and respiratory acidosis using morphologic methods. Regardless of the morphologic or functional state of the barrier, an effect of pH on fluorescein molecules leading to altered penetration across the barrier should occur on the basis of the pH partition hypothesis.

The magnitude of these pH-dependent changes can be of consequence. Fluorescein exists in aqueous solutions as either a monocation, an uncharged molecule, a monoanion, or a dianion. A change in pH from 7.4 to 7.3 produces a 52% increase in the lipid soluble, unionized form. A change in pH from 7.4 to 6.9 produces a 640% increase in the unionized fluorescein. The exact percentage of fluorescein in the unionized form at any pH is given in Appendix I.

Interpolating according to the linear regression, which was fit to the data in our studies, a reduction in pH from 7.4 to 7.3 would be associated with an increase in the vitreous fluorophotometry reading of 8.5%. A reduction from pH 7.4 to 6.9 would result in an increase of 72%. While these changes are substantial, they are less than those that would have been predicted by the pH partition hypothesis. A number of factors could be responsible for this.

First, relatively small molecules such as fluorescein may be able to pass between cells and across the BRB

<table>
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<tr>
<th>Rat*</th>
<th>Midvitreous fluorophotometry value (ng eq/ml)</th>
<th>Plasma fluorescein concentration (µg/ml)</th>
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* Rats 1–20 received NH₄Cl, while rats 21–26 did not.
† Fluorophotometry values are expressed in fluorescence units equivalent to the fluorescence of fluorescein concentrations at pH = 7.4.
Fig. 2. The relations between the midvitreous fluorophotometry value and blood hydrogen ion concentration and pH are presented.

to some extent even if large molecules such as horseradish peroxidase cannot. This transport would not depend on the pH. Processes such as pinocytosis, transcellular channels, or specialized transport systems also could allow penetration of molecules independent of lipid solubility and pH.

Second, biologic membranes actually have finite permeabilities for ionized species. These are ignored by the pH partition hypothesis. The percentages of fluorescein in the unionized and monoanionic forms at pH 7.4 are $1.7 \times 10^{-2}$ and 17, respectively. Thus, if the permeability of the barrier to the monoanion were one thousandth of the unionized form, the contribution of the monoanion to the total permeation of fluorescein would be the same as that of the unionized form. In fact, the octanol/water partition coefficients of organic unionized species are usually between 1,000 and 10,000 times those of their corresponding ionized species.

Contribution to permeation by the monoanionic and dianionic forms of fluorescein would reduce the pH dependence of transport. This is because a change in pH near the physiologic pH causes changes in their percentages of the total that are smaller than that of the unionized species. For example, the monoanion constitutes 17% of the total fluorescein at pH 7.4 and 39% at pH 6.9, a change of 130%. This compares to a change of 640% for the unionized species for this change in pH. Appendix II shows that, based on data in the literature, the amounts of the ionized species of fluorescein that enter the octanol phase may be substantial, suggesting that some fluorescein ions may cross the barrier in vivo as well.

Third, the transport of fluorescein during the process of aqueous secretion probably is less dependent on pH than transport by lipid solubility across the BRB. While some diffusion of fluorescein must occur from the posterior chamber into the vitreous, vitreous fluorophotometry readings, as performed in our studies, have generally been considered to reflect the function of the BRB. This is supported by the contours of the fluorophotometry scans at 1 hr after injection, which are flat or show a declining concentration gradient from the retina to the lens. In contrast, fluorescein injected into the rat posterior chamber demonstrates a declining gradient from the lens to the retina (unpublished data).

Fourth, as the pH falls below 7.4, the fluorescence
of a given concentration of fluorescein decreases. For example, there is an 11% reduction in fluorescence of a constant concentration of fluorescein as the pH falls from 7.4 to 7.0.18

The fraction of fluorescein bound to plasma proteins is pH-dependent in the pH range we studied. According to Rockey and Li,19 a change in pH from 7.3 to 6.9 decreases the binding of fluorescein about 5% and increases the amount of fluorescein available to cross the barrier. Thus, this factor would tend to enhance a correlation between pH and vitreous fluorophotometry levels.

The rather weak relation of pH to vitreous fluorophotometry readings is consistent with two previous studies from our laboratory on rats and monkeys.8,20 These showed lipid solubility to be much less of a determinant of transport into the vitreous than would have been predicted by the pH-partition hypothesis.

Our results have implications for the interpretation of previous work concerning the permeability of the BRB. Rapoport and co-workers presented data that suggested that hypercapnia caused a breakdown of the rat BRB.21 In their report, the pH was reduced to values as low as 6.9. These investigators have recently concluded from further studies that the increased passage of fluorescein across the BRB in hypercapnia is due to pH-dependent changes in the dissociation state of fluorescein rather than to changes in the barrier itself.4

Several groups have shown an increase in vitreous fluorophotometry values in diabetic rats of 74% or greater as compared to normal rats.13-17 In our preliminary studies, the arterial pH in streptozotocin diabetic rats was decreased to approximately 7.2, whereas it was approximately 7.4 in normal rats. Based on the present study, this difference in pH would account for an increase in vitreous fluorophotometry readings of only about 20%. Furthermore, Vine et al found no difference in arterial pH between normal and streptozotocin diabetic rats.22 Thus, pH-dependent changes may contribute to, but do not provide the major explanation for, increased vitreous fluorophotometry values in experimental diabetes.

Abnormally increased vitreous fluorophotometry values occur in human diabetic patients.23-28 Since the pH can fall to 7.0 or lower in diabetic ketoacidosis, one might speculate that increased fluorophotometry values might be due to subclinical pH alterations in
human diabetics. However, metabolically controlled patients with diabetes do not have significant deviations in the pH of their blood.29 This type of patient has been studied in most previous fluorophotometry investigations on diabetes.

In conclusion, the present experiments demonstrate that pH alterations can influence the penetration of fluorescein into the vitreous. We have attempted to discuss quantitatively how one factor contributing to this is a change in the ionic composition of fluorescein in accordance with the pH partition hypothesis, even if the barrier itself is unaltered. However, our results suggest that factors other than the pH partition hypothesis contribute to penetration of fluorescein from the blood into the vitreous. Our data also point out that pH alterations are unlikely to account fully for the changes observed by vitreous fluorophotometry in diabetic patients or animals.

Key words: pH, fluorescein, blood–retinal barrier, vitreous fluorophotometry, lipid solubility

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Appendix I

Four species of fluorescein have been shown to exist in aqueous solutions. The respective pKa (the negative logarithm of the dissociation constant) values are 2.2, 4.4, and 6.7.10 The fraction of the total fluorescein in the unionized form as a function of the hydrogen ion concentration is shown as30:

\[ U = \frac{[H^+] K_u}{[H^+]^2 + [H^+] K_c + K_c K_u} \]  

(1)

where \( U = \frac{\text{fraction of unionized fluorescein}}{\text{total fluorescein}} \), \([H^+]\) is the hydrogen ion concentration, \(K_c = 10^{-2.2}\), \(K_u = 10^{-4.4}\), and \(K_u = 10^{-6.7}\). The fraction of the entire fluorescein existing as the cation, monoanion, or dianion, respectively, equals the first, third, or fourth term in the denominator on the right side of the equation over the whole denominator. The fraction times 100% gives the percentage in each form.

Appendix II

It is possible to test the assumption of the pH partition hypothesis that the ionic forms of fluorescein contribute negligibly to the partition ratio of the total fluorescein. If the lipid solubility is negligible for all but the unionized form, we can write:

\[ R = U P_u \]  

(2)

where \( R = \frac{\text{partition ratio for total fluorescein}}{\text{fraction of unionized fluorescein}} \), strictly speaking, one cannot apply the term partition coefficient to a mixture of species). \( U = \frac{\text{fraction of fluorescein in the unionized form}}{\text{total fluorescein}}\), and \( P_u = \frac{\text{partition coefficient for the unionized species}}{\text{fraction of unionized species}} \). At \( pH = 8.03 \), \( U = 1.0 \times 10^{-5} \), \( R \) at this \( pH \) was given by Grimes et al as 0.03. Therefore, \( P_u = 3000 \). At \( pH = 6.42 \), \( U = 6.2 \times 10^{-3} \), which when multiplied by \( P_u \) gives 19. However, according to Grimes et al, \( R \) at this \( pH \) was 38. Thus, their data imply: (1) that the unionized fluorescein does not fully account for the amount of fluorescein entering the octanol phase; and (2) that in the physiologic \( pH \) range, the ionic forms (probably mainly the monoanion) contribute considerably to the total partition ratio and, presumably, to permeation through biologic membranes. For such circumstances, an extended pH partition hypothesis has been proposed.2 The total permeability of a continuous lipid membrane to a weak acid is proportional to a partition parameter equal to the sum of the products of the fraction of each permeating species and its corresponding partition coefficient. For fluorescein, which has four species, the equation is:

\[ R = C P_c + U P_u + M P_m + D P_d \]  

(3)

where \( R \) is the partition parameter for total fluorescein; \( C \), \( U \), \( M \), and \( D \) are the fractions of total fluorescein in the cationic, unionized, monoanionic, and dianionic forms, respectively; and \( P_c \), \( P_u \), \( P_m \), and \( P_d \) are the partition coefficients of the cationic, unionized, monoanionic, and dianionic forms of fluorescein, respectively.

References

11. Peyman GA and Bok D: Peroxidase diffusion in the normal


