

Human Retinal Molecular Weight Exclusion Limit and Estimate of Species Variation

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PURPOSE. To determine the maximum size of molecule capable of freely diffusing across human retina, referred to as the retinal exclusion limit (REL), and the location of any sites of high resistance to diffusion. To assess the degree of interspecies variation in the REL of three animals commonly used to model human disease.

METHODS. Trephines of human neuroretina were mounted in a modified Ussing chamber. FITC-dextran of various molecular weights (MWT) were dissolved in phosphate-buffered saline, and the rate of transretinal diffusion was determined over 24 hours with a spectrophotometer. The theoretical REL was calculated by extrapolating the linear relationship between the rate of diffusion and $\log(\text{MWT})$. In separate experiments to determine the sites of barrier to diffusion, FITC-dextran with a MWT greater than the calculated REL were applied to either the inner or outer retinal surface, processed as frozen sections, and viewed with a fluorescence microscope. Experiments to determine the REL were repeated in bovine, porcine, and rabbit retina.

RESULTS. The REL in human tissue was 76.5 ± 1.5 kDa (6.11 ± 0.04 nm). The inner and outer plexiform layers formed the sites of highest resistance to diffusion. The REL in pigs, cattle, and rabbits were 60 ± 11.5 , 78.5 ± 20.5 , and 86 ± 30 kDa, respectively (5.68 ± 0.45 , 6.18 ± 0.61 , and 6.38 ± 0.88 nm).

CONCLUSIONS. In humans, the inner and outer plexiform layers are sites of high resistance to the diffusion of large molecules, resulting in an REL of 76.5 kDa. There was only moderate interspecies variation in the REL of the animals studied, suggesting that they provide adequate models for the study of human transretinal macromolecular diffusion. (*Invest Ophthalmol Vis Sci.* 2003;44:2141–2146) DOI:10.1167/iovs.02-1027

The diffusion of molecules across the neuroretina may influence the clinical appearance and course of several diseases of the retina. Many conditions, such as diabetic and hypertensive retinopathy are characterized by abnormal intraretinal collections of protein, blood, and lipid. Any sites of high resistance to diffusion will influence the distribution of these molecules within the layers of the retina, and may alter the time course of their removal. Similarly, retention of high-molecular-weight (MWT) proteins within the retina, either from leaky retinal capillaries or damage to the outer blood-retinal barrier, may exert an osmotic force that could cause or aggravate retinal edema.

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Supported by the Special Trustees of Guys and St. Thomas' Hospital and the Anne Allerton Trust.

Submitted for publication October 6, 2002; revised November 22, 2002; accepted November 27, 2002.

Disclosure: **T.L. Jackson**, None; **R.J. Antcliff**, None; **J. Hillenkamp**, None; **J. Marshall**, None

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Intraretinal barriers to diffusion will also influence the success of intravitreal drug delivery to the outer retinal layers and subretinal space,^{1–4} and the pharmacokinetics of intravitreal drug removal.^{4,5} Attempts to introduce agents into the vitreous cavity from outside the eye using iontophoresis⁶ or osmotic pumps⁷ must also overcome any intraretinal barriers to diffusion. Despite the potential importance of transretinal macromolecular diffusion in both experimental and clinical science, the principal intraretinal barriers to diffusion have not been fully characterized in humans or in the animals that are routinely used to model human disease.

The purpose of the present study was to determine the sites of resistance to transretinal macromolecular diffusion in human retina and the resultant retinal exclusion limit (REL)—that is, the maximum size of molecule capable of freely diffusing across the retina. In addition, we sought to determine the interspecies variation in REL in three animals commonly used in eye research.

METHODS

Human Studies

Estimation of Molecular Weight Retinal Exclusion Limit. The REL was estimated by determining the rate of transretinal diffusion of fluorophore labeled dextrans. Fifteen pairs of eyes were obtained from the UK Transplant Support Service (Bristol Eye Bank, Bristol). The median age of the donors was 68 years and the median postmortem time, 45 hours. After removal of the cornea, iris, and vitreous, the eyecups were examined under a dissecting microscope, and eyes with visible retinal disease were excluded. A total of 32 8-mm trephines of neuroretina were taken from the area centered around the macula. Pilot experiments were conducted using unfixed human retina, but tissue could not be reliably mounted in the Ussing chamber without perforating the retina. Tissue was therefore fixed in 0.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium phosphate buffer (final pH 7.2) for 1 hour before being mounted in a modified Ussing chamber⁸ with a 6-mm interchamber aperture. The internal limiting membrane (ILM) of the retina was positioned facing a half-chamber containing 0.412 mM fluorescein isothiocyanate (FITC)-labeled dextran. Separate experiments were conducted using dextrans of 4.4, 9.5, 19.5, 42, 71, 77, and 167 kDa (Sigma, Poole, UK), dissolved in phosphate-buffered saline (PBS; sodium chloride 120 mmol/L, potassium chloride 2.7 mmol/L, phosphate buffer 10 mmol/L, pH 7.4 at 25°C; Sigma), with penicillin 100,000 U/L, streptomycin 100 mg/L, and amphotericin B 250 $\mu\text{g/L}$ (Sigma). The second half-chamber contained PBS, antibiotic, and antimycotic alone. Care was taken to ensure that small air bubbles did not form in any part of the assembly. The fluid volume in each half-chamber was kept identical throughout the mounting procedure and during subsequent fluid removal, to prevent the risk of a harmful hydrostatic pressure gradient developing across the sample. If retinal holes were accidentally created during the mounting procedure, then FITC could be seen immediately streaming through the hole into the second half-chamber, and the experiment was discarded. The locking screws that held the assembly together were tightened using a torque-range screwdriver (RS Components, Corby, UK). The minimum torque required to prevent leakage around the interchamber aperture was applied (30 cN/m). The tops of the chambers were sealed with insulation tape to prevent evaporation and a small glass-encased magnetic stirrer was inserted into the bottom of

each chamber. Experiments were conducted at 25°C, protected from ambient illumination.

After 24 hours, the fluid was simultaneously removed from both chambers, frozen at -40°C, and then batch processed to determine the rate of transretinal diffusion of FITC-dextran. The absorbance at 490 nm was determined for each sample with a spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). The concentration of FITC-dextran was then calculated from predetermined standard curves for each dextran. Separate experiments were conducted for each MWT dextran, and the mean rate of diffusion was plotted against the natural log of MWT (*x*-axis). A log scale was chosen, because pilot studies indicated that the rate of diffusion showed an exponential decline with increasing MWT. Hence, by plotting the rate of diffusion against the log of MWT, a straight-line graph was created. This straight line was extrapolated and the antilog of the point where it crossed the *x*-axis was taken as the theoretical REL. The natural log was selected as the most appropriate log scale.⁹ RELs were also calculated using the Stokes-Einstein radius of each dextran. The rate of diffusion displayed an inverse linear relationship with the molecular radius, and an REL could be calculated without log transformation.

Assessment of Intraretinal Barriers to Diffusion. The site of resistance to transretinal diffusion was assessed by examining retinal tissue that had been exposed on one surface to an FITC-dextran of a mass above the REL calculated as just shown. Retinal trephines (8 mm) were obtained from the same eyes by using the same tissue preparation, mounting techniques, and experimental conditions. Experiments differed, in that an Ussing chamber with a 3-mm interchamber aperture was used. Retinal tissue was oriented so that the ILM faced a half-chamber containing an FITC-dextran at least one SD above the calculated REL. Separate experiments were conducted using an FITC-dextran that was nearest to, but below, the REL. Both these experiments were then repeated with the photoreceptor layer facing the FITC-dextran. Tissue was removed after 24 hours' exposure, placed in embedding compound (BDH Laboratory Supplies, Poole, UK), and frozen in a eutectic solution of isopentane, cooled in a liquid nitrogen bath. Semithin (7 μ m) sections were cut on a cryotome (Anglia Scientific Instruments, Cambridge, UK), placed on gelatin-coated slides, coverslipped, viewed with a fluorescence microscope (Orthoplan; Leica, Milton Keynes, UK), and photographed (Ektachrome 320T; Kodak, Rochester, NY).

Animal Studies

The degree of interspecies variation in REL was determined using porcine, bovine, and rabbit retinas. Hybrid pigs aged 8 to 9 months, Friesian cows aged 18 to 24 months, and New Zealand White rabbits aged 6 to 9 months were selected. Porcine and bovine eyes were obtained from a local slaughterhouse and transported on ice. Transit time ranged from approximately 60 to 90 minutes. Rabbit eyes were harvested from research animals killed by other investigators in the same research facility. Experiments were started within 16 hours of enucleation. The provisions of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were adhered to.

Although it was not possible to reliably mount intact unfixed human tissue in the Ussing chamber, fresh animal tissue supported on filter paper could be mounted without manifest damage in approximately 90% of cases. Unfixed tissue was therefore used in all experiments, except those used to determine the effect of fixation itself. Because of the large interspecies variation in posterior pole retinal anatomy, midperipheral, inferior retinal trephines were used. Dextran of 9.5, 19.5, 42, and 77 kDa were selected. For each MWT dextran, separate experiments were conducted on tissue from at least three animals per species. Dextran diffusion studies were similar to those in human tissue, except that a Ussing chamber with a 3-mm interchamber aperture was used, tissue was supported on hardened, ashless filter paper with a 20- to 25- μ m pore size (no. 541; Whatman, Maidstone, UK) and 5 mmol/L glucose and 0.01% bovine serum albumin (Sigma) were added to both half-chambers to support the metabolism of unfixed tissue. Unlike human studies, the rate of transretinal dextran diffusion was determined using three samples over the 24-hour period,

rather than a single sample at the end of the experiment. Experiments were repeated in triplicate for each animal species using 0.412 mM fluorescein (Chauvin Pharmaceuticals, Ltd., Essex, UK) instead of FITC-dextran.

Control Experiments for Human and Animal Studies

To determine the effect of fixation on the rate of dextran diffusion, the experiments on bovine retina were repeated, using fixed tissue. Bovine retina was fixed the same as human tissue, but experimental conditions were otherwise unchanged. Dextran of 9.5, 19.5, 42, and 77 kDa were used to calculate the REL.

The FITC-dextran was analyzed using gel filtration chromatography to detect the presence of any free (unconjugated) fluorescein that might result in a falsely high estimate of REL. Samples were run through a separation column (Sephadex G-100; Sigma), and serial specimens were processed on a spectrophotometer to determine whether the elution pattern matched that of the dextran or free fluorescein.

The stability of the FITC-dextran bond was also assessed in conditions matching those of the human and animal experiments. Three dextran were chosen (9.5, 19.5, and 77 kDa). These were placed in one half-chamber of the Ussing chamber with a 6-mm interchamber aperture, separated from PBS in the second half-chamber by a regenerated cellulose dialysis membrane with an 8-kDa exclusion limit (BioDesign Inc., Carmel, NY). The samples were covered and stirred overnight and then analyzed for the presence of free fluorescein, as just described.

The effect of experimental conditions and sample processing on the absorbance of the FITC-label was also assessed. Two batches (lots 86H5034 and 98H5086) of 42-kDa FITC-dextran were compared. Solutions were protected from ambient illumination as per experimental conditions and then stored at 25°C, 4°C, or -40°C with five freeze-thaw cycles (-40°C/+25°C). Experiments were repeated in triplicate. The degree of fading of the FITC label was assessed using the spectrophotometer, with five readings taken over a 2-week period.

The filter paper used to support the unfixed animal retina was assessed to determine its effect on the movement of dextran between the two half-chambers. Experiments were conducted using the same conditions and measurement techniques as were used in the animal experiments, except that filter paper alone was interposed between the half-chambers. Five different MWT dextran were used. These experiments were also used to determine whether dextran diffusion was impeded by an unstirred layer on the surface of the interchamber membrane. The integrity of tissue clamped in an Ussing tissue has been determined previously.⁸

RESULTS

The theoretical RELs extrapolated from the straight-line portion of the graph plotting rate of diffusion against the natural log (Ln) of MWT were as follows: humans 76.5 ± 1.5 kDa (Figs. 1, 2), pigs 60 ± 11.5 kDa, cattle 78.5 ± 20.5 kDa, and rabbits 86 ± 30 kDa (Figs. 3, 4). The RELs calculated using Stokes-Einstein radius instead of ln(MWT) were: humans 6.11 ± 0.04 nm ($R^2 = 0.896$), pigs 5.68 ± 0.45 nm ($R^2 = 0.6974$), cattle 6.18 ± 0.61 nm ($R^2 = 0.863$), and rabbits 6.38 ± 0.88 nm ($R^2 = 0.914$). The transretinal diffusion of free fluorescein (MWT 376 kDa) was rapid in all animal species (pigs 6.30 ± 1.73 μ mol $\cdot$ h⁻¹ $\cdot$ mm⁻² $\times 10^{-2}$, cattle 3.31 ± 2.89 μ mol $\cdot$ h⁻¹ $\cdot$ mm⁻² $\times 10^{-2}$, and rabbits 11.07 ± 1.10 μ mol $\cdot$ h⁻¹ $\cdot$ mm⁻² $\times 10^{-2}$). When the data were added to the graphs used to extrapolate REL, the linear relationship between rate of diffusion and ln(MWT) was still evident ($R^2 = 0.815$ - 0.981).

The effect of fixation on the rate of dextran diffusion across bovine retina is shown in Figure 5. The rate of diffusion across fixed tissue was less than that in unfixed tissue, and the REL was increased from 78.5 to 100.5 ± 8.1 kDa (6.65 ± 0.14 nm).

Regeneron Ex. 2014

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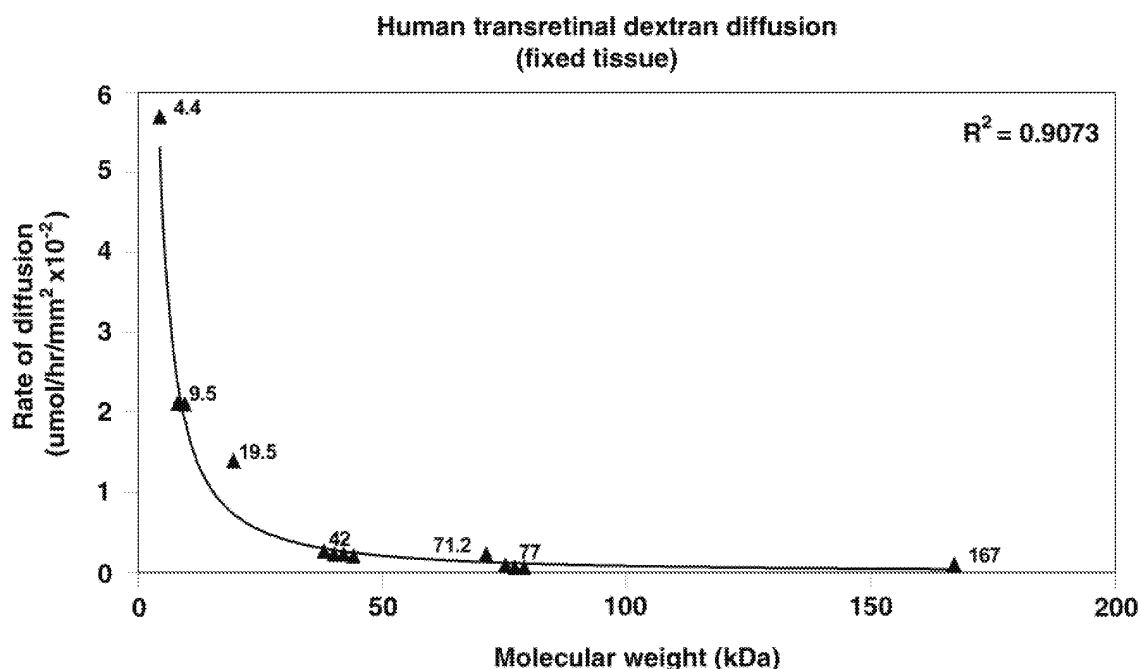


FIGURE 1. Graph shows the rate of transretinal dextran diffusion versus the molecular weight of each dextran. Tissue was fixed in 0.5% glutaraldehyde/3% paraformaldehyde.

When human retina was mounted in the Ussing chamber with the photoreceptor layer facing a half-chamber containing high-MWT (167 kDa) FITC-dextran, frozen sections (Fig. 6) indicated that the transretinal diffusion of the FITC label was largely arrested at the outer plexiform layer (OPL). When tissue was mounted with the ILM facing the FITC-dextran, diffusion was arrested at the level of the inner plexiform layer (IPL). Retinal tissue was uniformly stained when an FITC-dextran below the REL was used.

No free fluorescein was detected in the FITC-dextran stock solutions or after exposure to experimental condi-

tions. There was no measurable fading of the FITC label in any of the control experiments. Dextran movement through the filter paper was rapid and did not relate to MWT. The mean transretinal movement of the 9.5 kDa FITC-dextran was $26.5 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mm}^{-2} \times 10^{-2}$; 19.5 kDa, $35.5 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mm}^{-2} \times 10^{-2}$; 42 kDa, $38.8 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mm}^{-2} \times 10^{-2}$; 77 kDa, $35.2 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mm}^{-2} \times 10^{-2}$; and 148 kDa, $34.5 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mm}^{-2} \times 10^{-2}$. These rates were, on average, 61 times more rapid than in the retina of the rabbit, the species with the fastest transretinal diffusion.

Human retinal exclusion limit (fixed tissue)

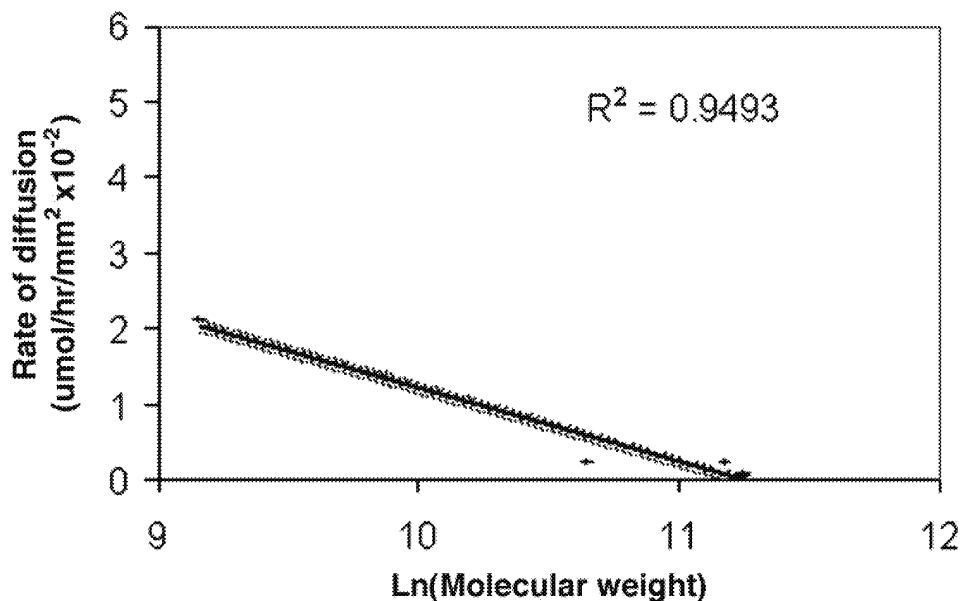


FIGURE 2. Data represent only the straight-line portion of the graph plotting the rate of diffusion versus the natural log (Ln) of molecular weight of each dextran. Data points are therefore those from the experiments using the 9.5, 19.5, 42, 71.2 and 77 kDa dextrans. Data from the 4.4 and 167 kDa-dextran experiments shown in Figure 1 were excluded. *Solid line:* linear trend; *dotted line:* ± 1 mean SD. Error bars, ± 1 SD.

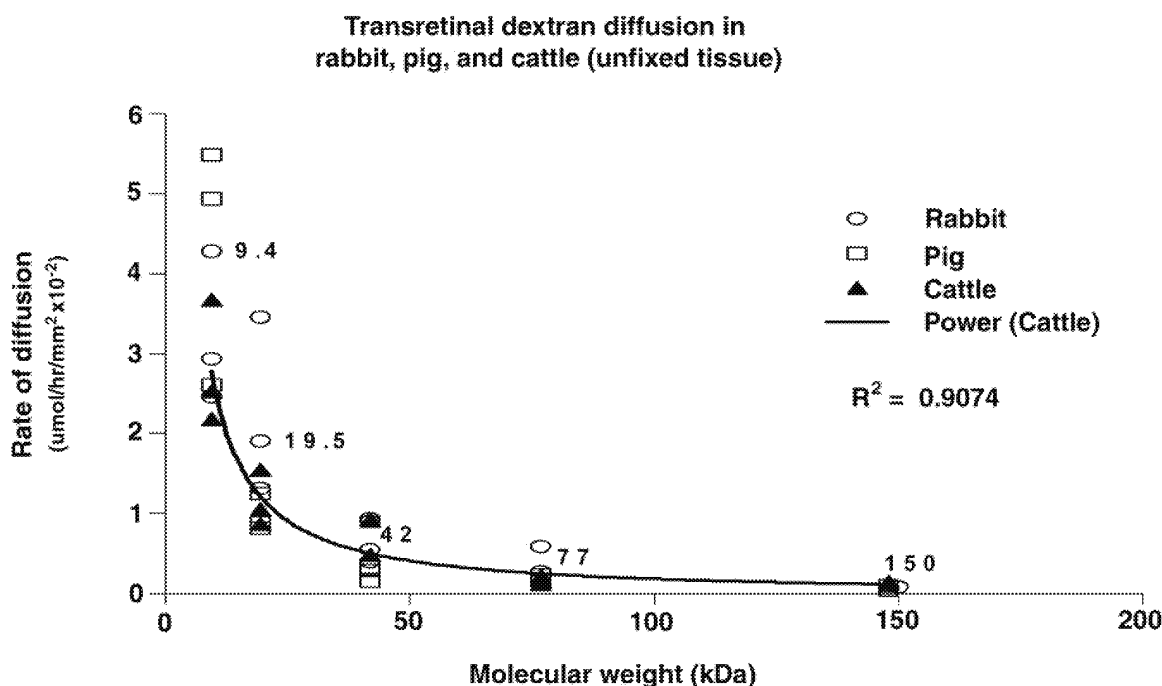


FIGURE 3. Graph shows the rate of transretinal dextran diffusion versus the molecular weight of each dextran. Unlike human experiments, rabbit, pig, and cattle tissue were unfixed.

DISCUSSION

This study suggests that molecules with a MWT greater than 77 kDa do not freely diffuse across fixed human retina. The REL of 77 kDa was obtained by extrapolation of the straight-line portion of a graph plotting the rate of dextran diffusion against the natural log of MWT. It therefore represents a theoretical rather than absolute limit. As can be seen in Figure 1, dextrans larger than 77 kDa passed across the retina, but the rate of diffusion was much reduced relative to lower-MWT dextrans.

There is limited and sometimes contradictory literature available on the relationship between MWT and transretinal diffusion. Experiments in squirrel monkey¹⁰ found that horseradish peroxidase (44 kDa) diffused rapidly across the retina, but experiments in rhesus monkey found that it did not.¹¹ Studies of antibodies delivered into the vitreous cavity of rhesus monkey⁴ found that Fab antibodies (48 kDa) diffused across the retina, but full-length antibodies (148 kDa) did not. This suggests that the REL in rhesus monkey is between 48 and 148 kDa.

Experiments in rabbit found that horseradish peroxidase diffused across the retina¹² but albumin¹² (67 kDa) and tissue plasminogen activator (69 kDa)¹³ did not. This suggests that the REL in rabbit is between 44 and 67 kDa, but other researchers¹⁴ have found that albumin passes across rabbit retina, suggesting an REL greater than 67 kDa.

The present findings are consistent with dextran diffusion studies undertaken by Marmor et al.,¹⁵ who used a micropipette to inject carboxyfluorescein and FITC-dextrans (10, 70, and 150 kDa) separately into the subretinal space of rabbits through a transretinal route. They then observed the leakage of the fluorescent labels into the vitreous cavity and out of the eye. As they noted, the experimental design prevented accurate assessment of the surface area of retinal diffusion, the concentration of agent in the subretinal bleb, and the stability of the agents in vivo. In addition, they could not exclude removal of the agent by phagocytosis from RPE or Müller cells. Nonetheless, they demonstrated that the 10-kDa dextran passed relatively rapidly through the retina, whereas the 70-

kDa dextran moved more slowly. After an initial decrease in concentration, possibly from back-flow through the injection site, the concentration of subretinal 150 kDa showed a negligible decline over 3 days.

One strength of the present study is that the concentration of FITC-dextran was known, as was the surface area of diffusion. This facilitated a quantitative measurement of diffusion per unit concentration and surface area. In addition, isolated intact retina was used, preventing dextran loss from either puncture sites, or phagocytosis. Control experiments were also conducted to establish the stability of the agents in experimental conditions.

One weakness is that the effect of fixation on the REL of human retina could not be determined. Studies using unfixed human retina were unsuccessful because of problems mounting intact tissue in the Ussing chamber. Experiments using bovine retina with relatively short postmortem times suggested that fixation results in a 28% increase in REL. It is possible that this simply reflects experimental error; however, the rate of diffusion of each of the four dextrans was reduced, suggesting that this may represent a real phenomenon.

Fixation may alter the REL by a number of mechanisms. Fixation involves cross-linkage of amino groups and results in tissue shrinkage that may alter the channels through fixed tissue. Further, tissue integrity is better preserved, cellular swelling reduced, and active transport mechanisms are not present. If fixation of human tissue had a similar effect to that in cattle tissue, the REL of unfixed human retina would be 60 kDa. To accurately quantify the effect of fixation on human retina would require further experiments using unfixed tissue with short postmortem times. Such experiments may be possible, but harvesting adequate numbers of freshly enucleated human donor eyes is notably more difficult than in animals.

By acquiring freshly enucleated eyes, it was possible to estimate the REL in unfixed porcine, bovine, and rabbit retinas. The REL in these species cannot be directly compared to the results from human tissue, due to differences in methodology, but they suggest that there is only moderate interspecies vari-

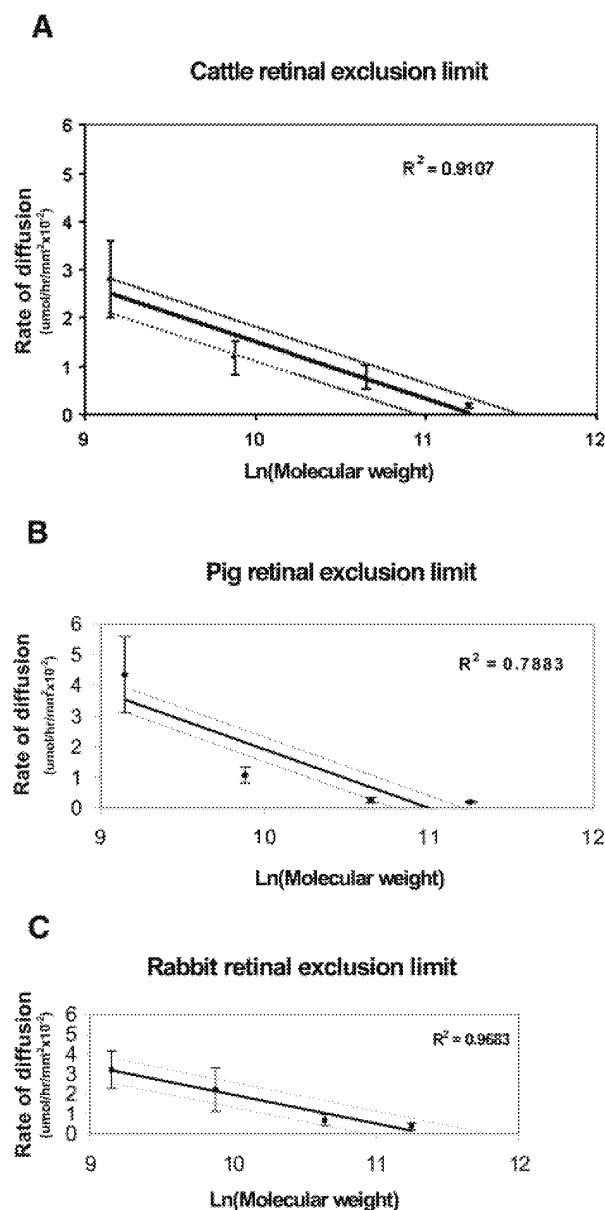


FIGURE 4. Data in the three graphs represent only the straight-line portion of the graph plotting the rate of diffusion versus the natural log (Ln) of molecular weight (9.5, 19.5, 42 and 77 kDa dextrans) for unfixed retina in (A) cattle, (B) pigs, and (C) rabbits. *Solid line*: linear trend line; *dotted line*: ± 1 mean SD. Error bars, ± 1 SD.

ation, particularly when the REL is calculated by using the Stokes-Einstein radii.

The rate of diffusion could be related to both the MWT and Stokes-Einstein radius of each dextran. There was a slightly higher correlation with the former, but, as expected, the results were generally similar. Molecular size may be more important than MWT *in vivo*, but the Stokes-Einstein radius provides only a theoretical estimate of size based on MWT. For experimental studies such as this, MWT has the advantage of being an absolute rather than a theoretical parameter.

The IPL and OPL were both found to be sites of high resistance to the diffusion of macromolecules across the retina (Fig. 6). This finding differs from a study in New Zealand albino rabbits¹² that found the external limiting membrane is a barrier to the transretinal diffusion of albumin, although studies of Dutch belted rabbits¹⁴ suggested that albumin can cross the

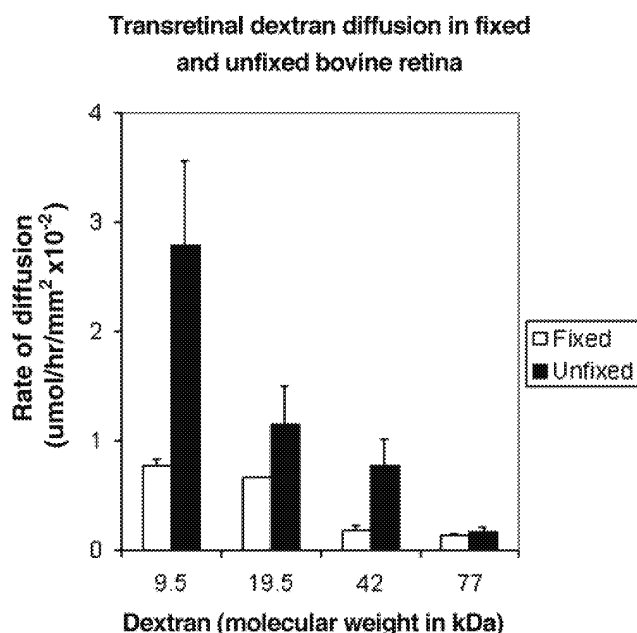


FIGURE 5. Comparison of rate of transretinal dextran diffusion in fixed and unfixed bovine retinal tissue, according to molecular weight.

external limiting membrane. The finding also differs from those in a study in rhesus monkey⁴ that suggested that the ILM acts as a barrier to the diffusion of full-length antibodies (148 kDa). Although antibodies have a different molecular configuration to dextrans, the fact that they are more globular would be expected to enhance retinal penetration. Variation in net surface charge is also unlikely to explain this difference.¹²

The present results are consistent with a study¹¹ in rhesus monkey that demonstrated that the OPL acts as a barrier to the diffusion of peroxidase, and studies of human retina that found that the IPL and OPL represent a barrier to the movement of

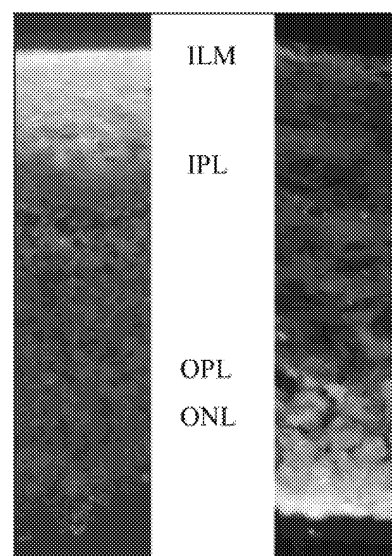


FIGURE 6. Fluorescence micrograph (gray-scale image) of frozen sections of fixed human neuroretina. *Left*: tissue exposed to a 167-kDa FITC-dextran on the inner retinal surface for 24 hours. *Right*: tissue exposed to FITC-dextran on the outer retinal surface. Although faint fluorescence was visible throughout the retinal substrate, fluorescence was predominantly arrested at the IPL or OPL. Retinal thickness measured approximately 150 μm .

water across the retina.¹⁶ As noted by the authors, such a barrier may be expected, given the anatomic structure of the IPL and OPL. Whatever the principal barriers to diffusion are, they should be regarded as relative rather than absolute, because in the present study large-MWT dextrans diffused across the retina, albeit at a much reduced rate.

An REL of 77 kDa in humans correlates with several clinical observations. The hard exudates observed in hypertensive and diabetic retinopathy are characterized by intraretinal deposits of serum and glial breakdown products.¹⁷ Given the large size of these macromolecules, it is unsurprising that they may persist for many months. Their intraretinal distribution at the level of the OPL¹⁷ is also consistent with the results of this study. Only the inner two thirds of the retina is vascularized,¹⁸ and breakdown of the inner blood-retinal barrier leads to exudation within this area. Retinal bulk fluid flow is predominantly in the inner to outer direction.¹⁸ If the OPL acts as a barrier to diffusion in vivo, then it is unsurprising that these deposits collect in the OPL, which may act as a form of molecular sieve.

The presence of intraretinal barriers to diffusion may also influence the pattern of intraretinal edema. It is known that arterial hypertension,¹⁹ diabetes,²⁰ and intraocular surgery¹¹ can damage the inner blood-retinal barrier and allow high-MWT proteins such as albumin to leak into the interstitial space. The present findings suggest that these proteins would not easily pass through the IPL and OPL. They would therefore exert an osmotic force that tends to retain water. Retention of water may be further aggravated by the low hydraulic conductivity of these structures.¹⁶ Pathologic studies of cystoid macular edema^{21,22} are consistent with this hypothesis. These indicate that retinal vascular disease leads preferentially to fluid accumulation in the inner nuclear layer, whereas breakdown of the outer blood-retinal barrier leads to fluid collection in the outer retina (Henle's layer).

This study indicates that molecules larger than 77 kDa do not freely diffuse across fixed human retina. The IPL and OPL represent the principal barriers to diffusion, although the resultant REL is relative, rather than absolute. Studies in unfixed porcine, bovine, and rabbit retinas showed relatively similar RELs. This interspecies consistency suggests that they may serve as appropriate models for the study of human disease. These results may assist in the design of novel strategies^{6,7} to deliver macromolecular therapeutic agents^{1-4,13,23,24} to ocular tissue and may help in the understanding of some common clinical observations.

Acknowledgments

The authors thank Ali Hussain, PhD, and Andrea Hodgetts, PhD, for providing invaluable advice on experimental design and conduct.

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