The Effect of Size and Species on Lens Intracellular Hydrostatic Pressure

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PURPOSE. Previous experiments showed that mouse lenses have an intracellular hydrostatic pressure that varied from 335 mm Hg in central fibers to 0 mm Hg in surface cells. Model calculations predicted that in larger lenses, all else equal, pressure should increase as the lens radius squared. To test this prediction, lenses of different radii from different species were studied.

METHODS. All studies were done in intact lenses. Intracellular hydrostatic pressures were measured with a microelectrode-manometer-based system. Membrane conductances were measured by frequency domain impedance analysis. Intracellular Na^+ concentrations were measured by injecting the Na^+ -sensitive dye sodium-binding benzofuran isophthalate.

RESULTS. Intracellular hydrostatic pressures were measured in lenses from mice, rats, rabbits, and dogs with radii (cm) 0.11, 0.22, 0.49, and 0.57, respectively. In each species, pressure varied from 335 ± 6 mm Hg in central fiber cells to 0 mm Hg in surface cells. Further characterization of transport in lenses from mice and rats showed that the density of fiber cell gap junction channels was approximately the same, intracellular Na^+ concentrations varied from 17 mM in central fiber cells to 7 mM in surface cells, and intracellular voltages varied from -45 mV in central fiber cells to -60 mV in surface cells. Fiber cell membrane conductance was a factor of 2.7 times larger in mouse than in rat lenses.

CONCLUSIONS. Intracellular hydrostatic pressure is an important physiological parameter that is regulated in lenses from these different species. The most likely mechanism of regulation is to reduce the density of open Na^+ -leak channels in fiber cells of larger lenses. (*Invest Ophthalmol Vis Sci.* 2013;54:183–192) DOI:10.1167/iovs.12-10217

Our vasculature convects nutrients to cells of most organs and carries away cellular waste products. Convective flow is required to move solutes when distances are too great for diffusion to be effective. One can estimate diffusion times based on Einstein's famous relationship for one-dimensional diffusion, $x^2 = 2Dt$, where x is the average distance a particle

diffuses in time t , and D is the diffusion coefficient. For biological solutes where $D \approx 10^{-5} \text{ cm}^2/\text{s}$, typical numbers are $t \approx 0.05$ seconds when $x = 10 \mu\text{m}$, whereas $t \approx 2$ months when $x = 10 \text{ cm}$. Thus diffusion is very fast when distance is short, on the order of the size of a cell, but becomes very slow when distance is long, on the order of the size of an organ. As a consequence, most organs are infiltrated by a dense network of capillaries. In the heart, for example, there is approximately one capillary for each myocyte.¹ In contrast, the vertebrate lens lacks a vasculature because the need for transparency precludes the presence of blood vessels, which would scatter light.

To compensate for the lack of a vasculature, the lens appears to generate its own internal circulatory system.² All vertebrate lenses studied have a circulating ionic current that enters at the anterior and posterior poles and exits at the equator.^{3–5} Work reviewed in Mathias et al.⁶ suggests the current is carried by Na^+ . The fiber cell transmembrane electrochemical gradient for Na^+ causes Na^+ to move from the extracellular spaces between fiber cells into the intracellular compartment. This creates an extracellular electrochemical gradient for Na^+ , causing it to move from outside of the lens into extracellular spaces within the lens and flow toward the lens center. This also creates an opposite intracellular electrochemical gradient for Na^+ , causing it to flow back to the lens surface. The intracellular current is directed to the equator by gap junction coupling conductance, which is highest in the peripheral equatorial fiber cells.^{7,8} At the surface, Na^+ is transported out of the lens by the Na/K ATPase, which is concentrated in the equatorial epithelial cells.^{5,9,10}

Model calculations^{6,11} suggest a standing circulating Na^+ flux would be accompanied by a circulation of fluid. Subsequent experiments^{12,13} have been consistent with lens fluid transport; however, solute and fluid fluxes were not measured in lenses from the same species or in the same conditions. The properties of solute transport in the mouse lens have been extensively studied.² Gao et al.¹⁴ therefore used mouse lenses to test the hypotheses: “fluid circulates through the lens; the intracellular leg of fluid circulation is driven by hydrostatic pressure and the flow is through gap junction channels; the fluid flow is generated by membrane transport of sodium.” Intracellular hydrostatic pressure was measured as an indicator of fluid flow in wild type lenses or when gap junction coupling or membrane transport of sodium was altered. As described below, their data provided solid support for these hypotheses.

Based on the above hypotheses, a simplified, but structurally based, model that focuses on the relationship between intracellular water flow and intracellular hydrostatic pressure was developed.¹⁴ Hydrostatic pressure was predicted to scale with the group of parameters $a^2 j_{\text{Na}}/N_j$, where j_{Na} (moles/cm²s) is the average density of fiber cell transmembrane influx of sodium, N_j (cm⁻²) is the number of open gap junction channels per area of fiber cell to cell contact, and a (cm) is

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the lens radius. Using genetically engineered connexin (Cx) knockout/knockin mouse lenses, which were selected because they had normal sodium transport and similar radii, Gao et al.¹⁴ show that the intracellular hydrostatic pressure varies inversely with N_j . In wild type lenses exposed to acute conditions that had been shown to alter j_{Na} , Gao et al.¹⁴ show that the intracellular pressure varies in proportion to j_{Na} .

The theoretical dependence on a^2 arose because of the force/flow relationship in spherical geometry. The purpose of the present study was to experimentally determine the relationship between lens radius and intracellular hydrostatic pressure. To obtain a significant variation in lens size, we used lenses from different species. However, the theoretical dependence on a^2 assumes that all lenses have the same density of gap junction channels and the same average sodium influx per unit area of fiber cell membrane, properties which are not guaranteed to be constant among different species. Thus, in the smaller lenses from mice and rats, where electrophysiological studies are possible, we have tried to integrate our hydrostatic pressure data with data on other transport properties.

Glossary

a	(cm)	Lens radius.
C_s	(F/cm ²)	Surface cell membrane capacitance.
c_m	(F/cm ²)	Fiber cell membrane capacitance.
D_{Na}	(cm ² /s)	Effective intracellular diffusion coefficient for Na ⁺ .
G_{DF}	(S/cm ²)	Coupling conductance per area of cell-to-cell contact for differentiating fibers.
G_{MF}	(S/cm ²)	Coupling conductance per area of cell-to-cell contact for mature fibers.
g_m	(S/cm ²)	Fiber cell membrane conductance.
G_s	(S/cm ²)	Surface cell membrane conductance.
J_{Na}	(moles/cm ² s)	Radial intracellular flux of Na ⁺ .
$\bar{j}_{Na}$	(moles/cm ² s)	Average fiber cell transmembrane influx of Na ⁺ .
K	(mm Hg s/cm ² mole)	A group of parameters that are thought to not vary with lens species or size.
N_j	(cm ⁻²)	Density of open fiber cell gap junction channels.
p_i	(mm Hg)	Intracellular hydrostatic pressure.
r	(cm)	Radial distance from the lens center.
R_{DF}	(Ω cm)	Effective intracellular resistivity of differentiating fibers.
R_{MF}	(Ω cm)	Effective intracellular resistivity of mature fibers.
R_e	(Ω cm)	Effective extracellular resistivity.
S_m/V_T	(cm ⁻¹)	Surface area of membrane per unit volume of tissue.
u_i	(cm/s)	Radial intracellular water flow velocity.
$\Delta[Na^+]_i$	(M)	The change in intracellular Na concentration between the lens center and surface.
$\Delta\psi_i$	(Volts)	The change in intracellular voltage between the lens center and surface.
A_i	(cm ² /s mm Hg)	The effective intracellular hydraulic conductivity.

MATERIALS AND METHODS

All animals were euthanized in accordance with the ARVO statement for The Use of Animals in Ophthalmic and Vision Research, then the eyes were removed and placed in a Sylgard-lined Petri dish filled with solution containing (in mM) NaCl 137.7, NaOH 2.3, KCl 5.4, CaCl₂ 2,

MgCl₂ 1, HEPES 5, glucose 10, pH 7.4 (normal Tyrode). The lens, suspended by its zonules to a uveal sclera ring of tissue, was dissected from the eye.¹⁴ Each lens was transferred and pinned by its sclera to the bottom of a chamber with a Sylgard base. The chamber was mounted on the stage of a microscope and perfused with normal Tyrode solution.

Measurement of Intracellular Pressure in the Intact Lens

Intracellular hydrostatic pressures were measured as described in Gao et al.,¹⁴ so only a brief description is given here. The microelectrode resistance (1.5–2.0 MΩ when filled with 3 M KCl) was recorded in solution outside of the lens by injecting serial current pulses and recording the amplitudes of the responding voltage steps, which indicated the resistance of the tip of the electrode. When the electrode was inserted into the lens, positive intracellular pressure pushed cytoplasm into the tip, causing the resistance to increase. A side port on the electrode holder was connected by plastic tubing to a mercury manometer, which allowed adjustment of the pressure within the electrode. The pressure within the electrode was increased to push cytoplasm back into the fiber cell. When pressure within the electrode was equal to intracellular pressure, cytoplasm was just pushed out of the electrode, and the amplitude of the electrode resistance returned to its original value measured in the bathing solution. This was the recorded value of intracellular pressure.

Within each lens, the intracellular pressures were recorded at four to five different depths along a track that started at the surface, 45° between the posterior pole and equator, and led to the lens center. The position of the tip of the microelectrode was recorded at each location where the pressure was measured. The data from 6 to 10 lenses were pooled.

After pressure measurements were completed, the electrode was removed from the lens and its resistance was remeasured in the bathing solution to check for clogging or breakage of the electrode tip. Data were accepted only if the electrode resistance had not changed significantly. Generally, the final resistance was within 0.3 MΩ of the initial resistance, unless breakage or clogging caused more than an order of magnitude change.

The Distribution of $[Na^+]_i$ in the Intact Lens

Intracellular sodium concentration was measured using a dual wavelength spectrometer system as described in Wang et al.¹⁵ SBFI (sodium-binding benzofuran isophthalate, 0.2 mM) was dissolved in the pipette solution. This solution was injected into fiber cells at different depths in the lens by advancing the microelectrode along its track towards the lens center. The ratios of emission at 360/380 nm excitation were compared to Na calibration curves that were determined at seven depths into the lens, as was described for intracellular calcium.¹⁶ The ratios versus intracellular sodium concentration, $[Na^+]_i$, were determined with Equation 1.

$$R = \frac{K_d R_{\min} + [Na^+]_i R_{\max}}{K_d + [Na^+]_i}, \quad (1)$$

where R is the experimentally measured ratio at a given depth in the lens, whereas K_d , $R_{\min}$, and $R_{\max}$ were determined by the calibration curves at the same depth. The value of K_d was essentially independent of depth, but the maximum and minimum ratios, $R_{\max}$ and $R_{\min}$, respectively, varied significantly because the lens absorbs light in a wavelength- and depth-dependent manner.

Intracellular Impedance of Intact Lenses

Impedance was measured as described in Mathias et al.¹¹ A centrally placed intracellular microelectrode was used to inject a stochastic current composed of sinusoidal frequencies between 0 Hz and 5 KHz.

A peripherally placed intracellular microelectrode was used to record the induced voltage. Both signals were sent to a fast Fourier analyzer (Hewlett Packard, Palo Alto, CA) where the intracellular impedance was calculated in real time. Figure 1 is a structurally based equivalent circuit model of the lens.^{17,18} This model was used to curve fit the data using the Levenberg-Marquardt nonlinear curve-fitting algorithm.^{19,20} The overall impedance of the lens (see Fig. 1) is

$$Z(j\omega) = R_s(r) + Z_L(j\omega) \quad (2)$$

The overall resistance at any frequency is given by the magnitude of the complex impedance $|Z(j\omega)|$, and the phase is given by $\theta(\omega) = \tan^{-1}(\text{Im}(Z(j\omega))/\text{Re}(Z(j\omega)))$.

The impedance is frequency dependent, but at frequencies above a few hundred Hz, the magnitude of the impedance asymptotes to a frequency-independent “series resistance” (R_s in Fig. 1 or Equation 2). The “series resistance” represents the intracellular resistance due to gap junctions between the point of recording the voltage and the surface of the lens.²¹ By advancing the microelectrode along its track toward the lens center, the series resistance at several depths in each lens can be recorded, and similar data would then be pooled from several lenses to determine gap junction coupling conductance. Thus the high frequency impedance gives a fairly direct measure of gap junction coupling conductance without resorting to nonlinear curve fitting. The relationship between resistance ($R_s(r)$ Ω) and underlying effective intracellular resistivities (R_{DF} and R_{MF} $\Omega\text{-cm}$) has two forms, depending on whether one is recording in the outer differentiating fibers (DF) or inner mature fibers (MF):

$$R_s(r) = \begin{cases} \frac{R_{DF}}{4\pi} \left(\frac{1}{r} - \frac{1}{a} \right) & b \leq r \leq a \\ \frac{R_{DF}}{4\pi} \left(\frac{1}{b} - \frac{1}{a} \right) + \frac{R_{MF}}{4\pi} \left(\frac{1}{r} - \frac{1}{b} \right) & 0 \leq r \leq b \end{cases} \quad (3)$$

Equation 3 was derived for current flow in a conductive sphere with a point source of current at its center; hence the $1/r$ dependence, where r is the distance from the lens center and a is the lens radius. In mouse and rat lenses, the location $r = b$ ($b \approx 0.85 a$) is the transition between differentiating fibers (DF; $r \geq b$), which have organelles, and mature fibers (MF; $r \leq b$), which lack organelles.²¹ It is also the site of extensive posttranslational modification of fiber cell membrane proteins, including the Cxs.²¹ As a consequence, R_{DF} is less than R_{MF} and there is a change in the slope of the resistance versus location curve at $r = b$.

The frequency-dependent component, $Z_L(j\omega)$, depends on the two parallel pathways for current to leave the lens: (a) by crossing surface cell membranes $Y_s(j\omega)$ S/cm² and (b) by crossing fiber cell membranes to enter the extracellular spaces and exit the lens $Y_e(j\omega)$ S/cm². Viz:

$$Z_L(j\omega) = \frac{1}{4\pi a^2 (Y_s(j\omega) + Y_e(j\omega))} \quad (4)$$

$$Y_s(j\omega) = G_s + j\omega C_s \quad (5)$$

$$Y_e(j\omega) = \frac{\gamma}{R_e} \left(\coth \gamma a - \frac{1}{\gamma a} \right) \quad (6)$$

$$\gamma = \sqrt{R_e \frac{S_m}{V_T} (g_m + j\omega c_m)} \quad (7)$$

G_s and g_m (S/cm²) are, respectively, the surface and fiber cell membrane conductances; C_s and c_m (F/cm²) are, respectively, the surface and fiber cell membrane capacitances; R_e (Ω cm) is the effective resistivity of the extracellular spaces within the lens; and S_m/V_T (cm⁻¹) is the average surface area of fiber cell membrane per volume of tissue. The parameter $j = \sqrt{-1}$, and ω represents an arbitrary

FIGURE 1. An equivalent circuit representation of the lens.¹⁸ The resistance $R_s(r)$ is due to a point source of current in a central fiber cell. The frequency dependent impedance $Z_L(j\omega)$ is due to the structure of the lens syncytium and electrical properties of the constituent cell membranes. These components are defined in the text.

sinusoidal frequency. The input resistance of the lens, $R_{in} = Z_L(0)$, is useful because it represents the effects of membrane conductances and lens size in a model-independent manner. In the Results section, R_{in} is indicated on the impedance data in Figure 6 and is listed in Table 2.

Data Analysis and Statistics

Results are presented as mean $\pm$ standard deviation. Differences between two groups were assessed with the Student's *t*-test.

MODEL CALCULATIONS

When embarking on these studies, we anticipated that larger lenses would have higher intracellular hydrostatic pressures; however, the data in Figure 2 show that this is not the case. The uniform pressures were surprising, and their implications for lens transport were not obvious. The model calculations presented here focus on the relationship between lens radius and intracellular fluxes. These calculations are not intended to be exact; rather, they are intended to be useful in understanding the implications of our data. The models include a number of approximations, but the models are not arbitrary as they are based on lens structure and require mass balance; that is, the total flux of Na⁺ or water into fiber cells filling the volume of the lens equals the total efflux leaving surface cells.

Approximations

The lens circulation described in the Introduction involves radial fluxes between the center and surface of the lens (r dimension) and also angular fluxes around the lens (θ dimension). The resulting equations can be simplified by averaging over the θ dimension and focusing on the radial dependence. The data presented in this paper describe radial gradients that were recorded halfway between the posterior pole and equator, essentially at the location of the angular average of the gradients, so comparison of this “angular average” model with data should be appropriate.

The Methods section describes the change in the number of open gap junction channels that occurs at the DF to MF transition, but this change has only a small effect on model calculations of radial gradients, and no effect on our conclusions, so it is neglected for simplicity.

The circulation appears to be driven primarily by Na transport²; hence, the model calculations here focused on Na⁺ and neglected the roles of other ions. Na⁺ moves into fiber cells and down its transmembrane electrochemical gradient, which depends on transmembrane voltage and the Nernst potential for Na⁺. Thus Na influx is logarithmically related to Na concentration. The equations are, therefore, nonlinear, and their general solutions would require extensive calculations that are beyond the scope of this paper. One particular special case, however, can be easily solved. Assume Na influx (j_{Na}) is

uniform at all radial locations with a value that is the radial average of the actual influx. The resulting equations can then be simply integrated to obtain a quadratic dependence on r for the radial gradients in intracellular hydrostatic pressure, voltage, and Na concentration. These quadratic models fit the data surprisingly well.

Water Flow

Our data suggest that the intracellular hydrostatic pressure at the lens center ($p_i(0)$ mm Hg) is the same in lenses of different sizes from several different species, so it is useful to derive the radial variation in pressure in terms of $p_i(0)$. Following the analysis presented in Gao et al.,¹⁴ the intracellular hydrostatic pressure is approximately:

$$p_i(r) = p_i(0)(1 - r^2/a^2) \quad (8)$$

where

$$p_i(0) = K \frac{a^2 j_{\text{Na}}}{N_j} \quad (9)$$

K is a constant that depends on lens fiber cell structure and single gap junction channel hydraulic conductivity, factors that are not expected to vary with size or species. The experimental observation is that $p_i(0)$ is the same in lenses with very different radii; hence the prediction is that $N_j/j_{\text{Na}} \propto a^2$. However, in the Results, we show that N_j is the same in mouse and rat lenses, so it is probably the same in the even larger lenses from rabbit and dog. The implication is that $j_{\text{Na}} \propto 1/a^2$. What is the implication for the effect of size on lens water flow?

Water flow velocity ($u_i(r)$ cm/s) depends on the pressure gradient (i.e., the derivative of p_i with respect to r). Viz:

$$u_i(r) = -A_i \frac{dp_i(r)}{dr} \quad (10)$$

The experimental finding that N_j is independent of size or species implies that A_i is also independent of size or species, so water flow will depend on the size dependence of the gradient in intracellular pressure. Differentiating Equation 8 and evaluating the gradient at the lens surface where water flow velocity is maximal gives:

$$-\frac{dp_i(a)}{dr} = \frac{p_i(0)2}{a} \quad (11)$$

Thus, when $p_i(0)$ is independent of lens radius, the water flow velocity at the lens surface is decreased in the larger lenses in proportion to $1/a$.

Total water flow leaving the lens is given by $4\pi a^2 u_i(a)$, which increases in the larger lenses in proportion to a . Thus, as size increases, the volume of water flow increases but the velocity of flow decreases.

Na Flux

Define $\Delta[\text{Na}^+]_i = [\text{Na}^+]_i(0) - [\text{Na}^+]_i(a)$ and $\Delta\psi_i = \psi_i(0) - \psi_i(a)$. Following the analysis in Wang et al. (2009):

$$[\text{Na}^+]_i(r) = (1 - r^2/a^2)\Delta[\text{Na}^+]_i + [\text{Na}^+]_i(a) \quad (12)$$

$$\psi_i(r) = (2 - r^2/a^2)\Delta\psi_i + \psi_i(a) \quad (13)$$

Data presented in Results suggest that $\Delta[\text{Na}^+]_i$, $[\text{Na}^+]_i(a)$, $\Delta\psi_i$ and $\psi_i(a)$ are each independent of lens size or species. What is the implication for the effect of size on lens Na flux?

The radial flux of Na^+ ($J_{\text{Na}}(r)$ moles/(cm² s)) is driven by a linear combination of the forces of diffusion, conduction, and advection. Viz:

$$J_{\text{Na}}(r) = -D_{\text{Na}} \left(\frac{d[\text{Na}^+]_i}{dr} + \frac{F}{RT} [\text{Na}^+]_i \frac{d\psi_i}{dr} \right) - A_i [\text{Na}^+]_i \frac{dp_i}{dr} \quad (14)$$

Both D_{Na} and A_i depend on N_j and other parameters that are expected to be independent of lens radius or species. Since our experimental data suggest that N_j is independent of lens size or species, the size dependence of J_{Na} arises through the size dependence of the gradients. Differentiating Equations 12 and 13 and evaluating the gradients at the lens surface where Na flux velocity is maximal gives:

$$J_{\text{Na}}(a) \propto \frac{1}{a} \quad (15)$$

Thus, when $[\text{Na}^+]_i(a)$, $\Delta[\text{Na}^+]_i$, $\Delta\psi_i$, and $p_i(0)$ are each independent of lens radius, the Na flux velocity at the lens surface ($J_{\text{Na}}(a)$) is predicted to decrease in the larger lenses in proportion to $1/a$.

Total Na^+ leaving the lens is given by $4\pi a^2 J_{\text{Na}}(a)$, which increases in the larger lenses in proportion to a . Thus, just as for water flow, as size increases, the amount of Na^+ leaving the lens increases but the velocity of flow decreases.

Mass balance dictates that the total Na^+ leaving the surface cells of the lens equals the total Na^+ entering fiber cells throughout the lens volume. Viz:

$$4\pi a^2 J_{\text{Na}} = \frac{4}{3} \pi a^3 \frac{S_m}{V_T} j_{\text{Na}} \quad (16)$$

Since the surface-to-volume ratio of a fiber cell is not expected to depend on size or species of lens, combining Equations 15 and 16 leads, once again, to the conclusion

$$j_{\text{Na}} \propto \frac{1}{a^2}. \quad (17)$$

In the case of water flow, this conclusion was based on the assumption that transmembrane water flow is proportional to j_{Na} . Here, we arrive at the same conclusion based entirely on experimental data and the requirement for mass balance.

RESULTS

As discussed in the Introduction, intracellular hydrostatic pressure in the lens is expected to vary in proportion to the group of parameters $a^2 j_{\text{Na}}/N_j$.¹⁴ Other parameters, such as cell size and gap junction single channel hydraulic conductivity, are predicted to affect intracellular pressure, but we think it is unlikely these factors vary between lenses from different species. Gao et al.¹⁴ demonstrated intracellular hydrostatic pressure varied with j_{Na}/N_j in mouse lenses of similar radii. Here, we are measuring hydrostatic pressure in lenses of very different radii, but from different species. Nevertheless, hydrostatic pressure should still depend on j_{Na}/N_j , even if either j_{Na} or N_j is not the same in lenses from different species. Our initial expectation was that they would be similar in lenses from different species, hence the pressures would be significantly higher in lenses with larger radii. Figure 2 illustrates the remarkable observation that hydrostatic pressure measured in the center of lenses of different sizes from different species was always the same.

FIGURE 2. Intracellular hydrostatic pressures in lenses of different sizes from different species. The radii of the different lenses were (cm): mouse (0.11 ± 0.002 , $n=6$); rat (0.22 ± 0.002 , $n=10$); rabbit (0.49 ± 0.018 , $n=8$); dog (0.57 ± 0.019 , $n=6$). The approximate ages of the animals were (months): mouse 2 to 3; rats 22; rabbits 22; dogs 22. (A) When radial distance from the lens center is normalized to the lens radius, the intracellular hydrostatic pressure gradients are indistinguishable. (B) When the intracellular hydrostatic pressure gradients are graphed as a function of actual distance from the center of the different lenses, it can be seen that the gradient is steepest in the smallest lens (mouse) and decreases monotonically with increasing lens size.

The pressure in surface cells of all lenses was essentially zero, so the intracellular pressure gradient is determined by the pressure at the center ($p_i(0)$), which did not vary appreciably between these lenses of very different sizes. On average, $p_i(0)$ was 335 ± 6 mm Hg. Indeed, when pressure was graphed as a function of normalized distance from the lens center (r/a), the combined data from 36 lenses from four species all fell on the same curve (Fig. 2A).

In Figure 2B, the pressures are graphed as a function of actual distance from the lens center, and one can see that the intracellular pressure gradient decreases as size increases. As derived in the previous section, when $p_i(0)$ is the same in lenses of differing radii, water flow velocity, which depends on the gradient in intracellular hydrostatic pressure, decreases in proportion to $1/a$, consistent with the data in Figure 2B. Moreover, total water flow leaving the lens surface depends on the surface area times the flow velocity. Total water flow therefore increases in the larger lenses in proportion to a . Thus as size increases, the volume of water flow increases but the velocity of flow decreases.

As stated at the outset, we previously showed that in mouse lenses of similar sizes, pressure varied reciprocally with N_j and directly with j_{Na} .¹⁴ Thus the implications of the data shown in Figure 2 are that, in larger lenses from different species, either N_j increases as a^2 , or j_{Na} decreases as a^2 , or some combination of both such that j_{Na}/N_j decreases as a^2 . Impedance studies are limited to smaller lenses, so we could not test these possibilities in lenses from all the species represented in Figure 2; however, the impedance of rat and mouse lenses can be measured and compared. The value of a^2 for a rat lens is approximately four times greater than that for a mouse lens, so there should be a significant difference in some measurable parameters.

A Comparison of N_j in Mouse and Rat Lenses

As described in Methods, the high frequency series resistance is a fairly direct measure of the intracellular resistance between the point of recording and the surface of the lens.¹⁸ This resistance depends on the effective intracellular resistivity, whose value is dominated by gap junctions, which limit cell to cell ion flow. There is a change in the effective intracellular resistivity at the differentiating fiber (DF) to mature fiber (MF) transition ($r = b$) due to loss of open Cx50 channels in the MF.^{21,22}

The results of measuring R_s in mouse and rat lenses and the curve fits of Equation 3 to each data set are shown in Figure 3. In each species, we assume that $b = 0.85a$ as this has been

shown to be a good approximation for the location at which gap junction coupling conductance changes in mouse and rat lenses.²¹ As can be seen in Figure 3, the resistance curves, when graphed as a function of normalized radial location, are not very different, though some difference is predicted by Equation 3 based on the different values of radius (a cm) in lenses from these two species.

The values of effective intracellular resistivity (R_{DF} and R_{MF}) depend inversely on the density of open gap junction channels, but are not directly affected by lens size, though there could be differences in lens fiber cell gap junction expression between the mouse and rat. Table 1 summarizes the best fit values of effective intracellular resistivity, and the coupling conductances per area of cell-to-cell contact (G_{DF} and G_{MF} S/cm²). $G = 1/wR$, where $w = 3 \mu\text{m}$ is the approximate value of fiber cell width, which is the approximate radial distance between gap junction plaques. The value of coupling conductance should be directly proportional to N_j . In order to account for the pressure data, the coupling conductance in rat would have to be 4-fold greater than in mouse, and that is obviously not the case. Assuming the density of fiber cell gap junction channels is independent of lens size or species, then the pressure data imply that the average fiber cell membrane influx of Na^+ should vary as $j_{Na} \propto 1/a^2$.

A Comparison of Na^+ Transport in Mouse and Rat Lenses

If the uniform intracellular hydrostatic pressure at the lens center of approximately 335 mm Hg is due to regulation of Na^+

FIGURE 3. The intracellular series resistance (R_s) between the normalized radial location (r/a) and the lens surface $r/a = 1$. The R_s data are from 12 mouse and 10 rat lenses. For the rat lenses, $a = 0.22 \pm 0.003$ cm ($n=10$), whereas for mouse lenses $a = 0.11 \pm 0.007$ cm ($n=12$); hence the ratio $(a(\text{rat})/a(\text{mouse}))^2 = 4$. The smooth curves are the best fit of Equation 3 to the data.

TABLE 1. A Comparison of Gap Junction Coupling in Rat and Mouse Lenses

	a (cm)	R_{DF} (K Ω -cm)	R_{MF} (K Ω -cm)	G_{DF} (S/cm ²)	G_{MF} (S/cm ²)
Rat	0.22	4.0	6.4	0.83	0.52
Mouse	0.11	3.6	4.1	0.93	0.81

transport across species, then the radial intracellular Na^+ flux should decrease as $1/a$ (see Equation 17) as did the radial intracellular water flow. Three factors drive J_{Na} : the intracellular Na^+ -concentration gradient drives diffusion; the intracellular voltage gradient drives conduction; and the intracellular pressure gradient drives advection (see Equation 16). We have already determined that the center-to-surface change in hydrostatic pressure is the same in mouse and rat lenses, but the rat lens radius is twice that of the mouse lens; hence the pressure gradient and water flow velocity in rat lenses is less than that in mouse lenses by a factor of 2 ($a(\text{rat})/a(\text{mouse}) = 2$). Figure 4 shows that the center-to-surface change in intracellular Na^+ concentration is the same in mouse and rat lenses, but the average radius is 2.2 times larger in rat (for $[\text{Na}^+]_i$ measurements $a(\text{rat})/a(\text{mouse}) = 2.2$); hence radial diffusion of Na^+ is slower in rat by a factor of 2.2.

Figure 5 illustrates the intracellular voltage recorded from mouse and rat lenses. Though the data have significant variability, when plotted as a function of normalized radial location (r/a), they all fall around the same smooth curve. Thus the center-to-surface change in intracellular voltage is also the same in mouse and rat lenses, and hence the voltage gradient and conduction of Na^+ are also less in rat by a factor of 2.

Since our data suggest each component that drives J_{Na} is reduced by approximately a factor of 2 in rat, it follows that J_{Na} is slower in rat than mouse lenses by a factor of 2. For mass balance, the total Na flux leaving surface cells of the lens must equal the total Na flux entering fiber cells throughout the volume of the lens (see Equation 16). Invoking mass balance, our data on Na^+ transport, like the pressure data, imply that $j_{\text{Na}} \propto 1/a^2$, or the average influx of Na^+ for fiber cells must be approximately a factor of 4 smaller in the rat than the mouse.

Transmembrane influx of Na^+ is driven by the transmembrane electrochemical gradient for Na^+ and scales with the membrane Na^+ conductance.

$$j_{\text{Na}} = -\frac{g_{\text{Na}}}{F}(\psi_i - \psi_e - E_{\text{Na}}) \quad (18)$$

The radial dependence of $[\text{Na}^+]_i$ and ψ_i have been measured (Figs. 4 and 5), and their average values are the same in lenses

from mice and rats. The transmembrane electrochemical gradient depends also on the average extracellular voltage and Na concentration. Though we have no way to measure extracellular voltages and Na^+ concentrations, model calculations¹¹ in connection with the intracellular values presented here suggest that the average transmembrane electrochemical gradient for Na^+ should be approximately the same in rat and mouse lenses. A 4-fold reduction in j_{Na} in rat relative to mouse would therefore most likely be due to a significant reduction in g_{Na} .

The fiber cell membrane protein responsible for g_{Na} is not known, so it is not possible to directly test this idea by Western blotting or immunostaining. However, whole lens impedance studies provide a comparison of fiber cell membrane conductance g_m (S/cm²) in rat and mouse lenses. When epithelial cells differentiate into fiber cells, they rapidly lose all of their K^+ channels, so fiber cell membrane conductance is selective for Na^+ and Cl^- .²³

$$g_m = g_{\text{Na}} + g_{\text{Cl}} \quad (19)$$

If there is a 4-fold difference in g_{Na} between rat and mouse lenses, there should be a measurable difference in g_m .

A Comparison of Impedance Data from Mouse and Rat Lenses

When a sinusoidal current of angular frequency ω (radians/s) is applied to a linear circuit, the responding voltage is a sinusoid of the same frequency. Assuming that the applied current is given by $i(t) = I \sin \omega t$, then the responding voltage is described by $v(t) = \sin(\omega t + \theta)$. The magnitude of the frequency-dependent resistance of the circuit is $R(\omega) = V(\omega)/I$. There is also a phase shift ($\theta(\omega)$ radians) between the applied current sinusoid and the responding voltage sinusoid. The Methods section describes how the complex frequency-dependent impedance of the lens is measured using Fourier theory. The resulting data presented in Figure 6, however, are exactly the same as intuitively described above, if one replaces the magnitude of the complex impedance with $R(\omega)$. These data were curve fit with a structurally based equivalent circuit

FIGURE 4. A comparison of intracellular sodium concentration gradients in lenses from rats and mice. The radii of the different lenses are (a cm): mouse (0.10 ± 0.003 cm, $n = 10$); rat (0.22 ± 0.003 cm, $n = 8$). (A) When radial distance from the lens center is normalized to the lens radius, the intracellular sodium concentration gradients are indistinguishable. (B) When the intracellular sodium concentration gradients are graphed as a function of actual distance from the center of the mouse and rat lenses, it can be seen that the gradient is steepest in the mouse lens and decreases by a factor of 2.2 in the rat lenses, since the rat lenses were 2.2 times larger than the mouse lenses.

FIGURE 5. A comparison of the intracellular voltage (ψ_i volts) in lenses from mice and rats. The voltage is graphed as a function of normalized distance from the lens center. The average radius of the mouse lenses was 0.11 ± 0.007 cm ($n = 12$), whereas that of the rat lenses was 0.22 ± 0.003 cm ($n = 10$). Since the average rat lens radius was a factor of 2 larger than that of the mouse lens, whereas the voltage change between the center and surface was approximately the same, the voltage gradient in the rat lenses was approximately half that in the mouse lenses.

representation of the lens to obtain the parameter values presented in Table 2.

The physical interpretations of the parameters listed in Table 2 are as follows. R_{in} is the experimentally measured input resistance (see Fig. 6). In terms of our equivalent circuit model of the lens given in Equation 4, $R_{in} = Z_L(0)$. G_s is the surface cell membrane conductance, which represents the total conductance of the surface layer of cells normalized to the surface area of the lens. g_m is the fiber cell membrane conductance per unit area of membrane. R_e is the effective extracellular resistivity, which depends on the volume fraction and tortuosity of the extracellular spaces.² The parameters in Table 2 are determined by nonlinear curve fitting of impedance data, which are similar to those shown in Figure 6. Of particular interest is the value of g_m , which is a factor of 2.7 times smaller in rat lenses than mouse lenses. As described in the Discussion section, this difference could be due to a 4-fold smaller value of g_{Na} in fiber cells from rat lenses.

DISCUSSION

The central hydrostatic pressure in lenses of significantly different sizes from different species was shown to be 335 ± 6 mm Hg. This was a surprising result, as the central hydrostatic pressure was expected to increase with lens size. We have investigated the question: How do lenses of differing sizes maintain the central hydrostatic pressure at a uniform value? Our hypothesis on this question is discussed below. We have also considered the implications for the circulation of fluid in these lenses of different sizes. The conclusion is that fluid flow velocity is proportional to $1/a$, so the larger the lens, the slower the flow. This conclusion was also surprising, as one would expect that the greater the distance for advection of nutrients, the greater the need for velocity. This brings to mind the question: Why is the central hydrostatic pressure independent of size when this causes reduced advection of nutrients in larger lenses? Some speculations on this question are also discussed below.

FIGURE 6. Impedance data (circles) and best fit (lines) using the model in Figure 5. **A.** Data and best fit for a typical rat lens. The radius of the lens was 0.22 cm and the recording was made at 0.14 cm from the lens center. **B.** Data and best fit for a typical mouse lens. The radius of the lens was 0.12 cm and the recording was made at 0.07 cm from the lens center. Note the difference in y-axis scaling between mouse and rat lens data.

How Is the Central Hydrostatic Pressure Regulated?

The most widely accepted hypothesis on fluid transport by biological tissues is that transmembrane fluid flow, through osmosis, passively follows transmembrane transport of salt. Results in Gao et al.¹⁴ show that hydrostatic pressure in the lens varies in proportion to transmembrane Na^+ transport, supporting this hypothesis for fluid flow in the lens. It is therefore not surprising that the results presented here suggest regulation of Na^+ influx (j_{Na}) in the lens is ultimately responsible for maintaining a uniform central hydrostatic pressure of 335 mm Hg in lenses of different sizes from different species.

The factors responsible for transmembrane Na^+ influx are presented in Equation 18. Our data suggest that the average transmembrane electrochemical gradient for Na^+ is approximately the same in rat and mouse lenses, suggesting that the membrane Na^+ conductance (g_{Na} S/cm²) may vary as $1/a^2$. Since the rat lens radius is approximately 2 times larger than that of the mouse lens, g_{Na} in mouse lenses should be approximately 4 times larger than in rat lenses. Though we cannot directly measure g_{Na} , nonlinear curve fitting of impedance data provided an estimate of fiber cell membrane conductance $g_m = g_{Na} + g_{Cl}$.²³ There is a significant difference in the best fit values of g_m from rat and mouse lenses, with the average conductance in the mouse lenses being a factor of 2.7 times larger than that in the rat.

Table 3 shows that the 2.7-fold difference in g_m between rat and mouse could indeed account for a 4-fold higher g_{Na} in mouse than rat. The parameters with an asterisk in Table 3 have values based on experimental measurements, whereas the values of g_{Na} , g_{Cl} , and j_{Na} are estimates. With regard to g_{Cl} and g_{Na} , the values were based on the assumption that Cl-channel expression is the same in fiber cell membranes of the mouse and rat lens, but Na-channel expression is 4-fold greater in the mouse than in the rat, with the constraint that the experimental value of $g_m = g_{Na} + g_{Cl}$. There are no data to

TABLE 2. A Comparison of Impedance-Derived Parameters in Rat and Mouse Lenses

	a (cm)	R_{in} (KΩ)	G_s (mS/cm ²)	g_m (μS/cm ²)	R_e (KΩ-cm)
Rat ($n = 10$)	0.22 ± 0.002	0.9 ± 0.34	1.3 ± 0.78	2.2 ± 1.23	28.1 ± 10.87
Mouse ($n = 30$)	0.11 ± 0.003	3.9 ± 0.63	0.3 ± 0.25	6.0 ± 2.48	21.7 ± 8.18

TABLE 3. Hypothetical Values of g_{Na} and g_{Cl} That Are Consistent with the Experimental Values of g_m and Produce a 4-Fold Greater j_{Na} in Mouse Than Rat

	a^* (cm)	g_m^* ($\mu S/cm^2$)	g_{Na} ($\mu S/cm^2$)	g_{Cl} ($\mu S/cm^2$)	j_{Na} (pmole/(cm ² s))
Rat	0.22	2.2	1.26	0.96	0.76
Mouse	0.11	6.0	5.04	0.96	3.04

support our assumption on g_{Cl} ; it is just as likely to differ in rat and mouse lenses, but Table 3 shows one way in which all experimental numbers could add up. If we make an educated guess on average values for the components of the Na^+ transmembrane electrochemical gradient ($\psi_i = -54$ mV, $\psi_e = -42$ mV, and $E_{Na} = +60$ mV), then the values of g_{Na} in Table 3 give the values shown for j_{Na} . Our working hypothesis is: fiber cell g_{Na} is regulated across species to maintain a species-independent central hydrostatic pressure of 335 mm Hg.

The central pressure is probably not regulated through feedback, since in the Cx knockout/knockin mouse lenses, the pressure varied inversely with the number of gap junction channels.¹⁴ This observation is inconsistent with feedback regulation. It is more likely that lenses from different species are genetically programmed to express different amounts of Na^+ -leak channels.

Why Is Central Hydrostatic Pressure in Lenses from Different Species the Same?

Our observation is that all lenses studied have an intracellular hydrostatic pressure, suggesting they all have an internal circulation of fluid. Our standing hypothesis has been that the circulation of fluid is a microcirculatory system for the avascular lens. If so, then one would think that, in larger lenses, it would be advantageous to have a faster rate of flow to carry nutrients over longer distances. In fact, however, data presented here show the rate of water flow decreases in larger lenses as $1/a$. This brings into question our standing hypothesis. However, in the presence of water flow, advection of nutrients in the aqueous and vitreous humors into extracellular spaces of the lens is inescapable, so rather than discarding this hypothesis, we looked to expand upon it. Moreover, the following caveat applies when comparing the microcirculatory system in different species.

There is a general rule in biology that larger animals tend to have lower metabolic rates and longer lifespans, such that over the lifespan of animals of differing species, the total metabolic energy expenditure per unit volume is approximately constant.²⁴ The correlation is not perfect and there are exceptions; but, if we adopt this view at face value, then the larger the lens, the lower the basal metabolic rate and the less the need for speed in delivering nutrients to central fiber cells. This would explain why decreasing water flow velocity in larger lenses is not deleterious, but it does not explain why the central hydrostatic pressure is so precisely regulated at 335 mm Hg.

The most important physiological property of the lens is optics, and there are some reasons to think pressure and refractive index are related. All lenses have a radial gradient in refractive index, which is created by a radial gradient in protein concentration.²⁵ In mice, rats, and rabbits, the refractive index varies from approximately 1.5 in central fiber cells to 1.35 in outer cortical fiber cells,^{26–28} whereas in the larger lenses of humans and cows, it varies from approximately 1.4 in central fiber cells to 1.35 in outer cortical fiber cells.^{29–31} Unfortunately, we could find no information on the refractive index of dog lenses. However, in the lenses we have studied here, the refractive index varied parabolically from center to surface, like

the pressure gradients shown in Figure 2. And in mouse, rat, and rabbit lenses, the center-to-surface refractive index change was independent of species, like the hydrostatic pressure. While these similarities alone mean little, they are not inconsistent with a relationship. Moreover there are other lines of evidence that suggest a relationship. The first line of evidence is from mathematical modeling of the relationship between hydrostatic and osmotic pressures in fluid transporting epithelia.³² The second line of evidence is based on some recent experiments.³³

Based on model calculations,³² in fluid transporting epithelia there is approximate balance between osmotic and hydrostatic pressures. To provide an intuitive understanding of why this is so, consider the factors generating transmembrane water flow:

$$u_m = L_m(RT\Delta c_m - \Delta p_m),$$

where u_m (cm/s) is the transmembrane water flow velocity, L_m (cm/s/mm Hg) is the membrane hydraulic conductivity, Δc_m (moles/cm³) is the transmembrane osmotic difference, and Δp_m (mm Hg) is the transmembrane hydrostatic pressure difference. In the absence of a hydrostatic pressure, if L_m becomes very large, water flow velocity increases but not proportionally, because increases in u_m wash away solute, reducing Δc_m . The theoretical maximum limit for u_m is termed isotonic transport,³⁴ and it occurs when the transported solution has the same osmolarity as that of the bathing medium (c_0 moles/cm³). The osmolarity of the transported solution is given by j_m/u_m (moles/cm³), where j_m (moles/cm³) is transmembrane salt flux. Hence the transmembrane water flow velocity is limited by:

$$u_m < \frac{j_m}{c_0}$$

Combining the above two equations gives:

$$\Delta c_m - \Delta p_m/RT < \varepsilon c_0$$

The small parameter $\varepsilon = (j_m/c_0)/(RTc_0L_m)$ can be thought of as the ratio of membrane salt to water permeability. Where experimental data are available (reviewed in Mathias and Wang³⁴), $\varepsilon \approx 10^{-3}$. Assume $c_0 = 300$ mM and $\Delta p_m = 0$, then a transmembrane osmotic gradient of a little less than $\Delta c_m = 0.3$ mM would generate near isotonic transport. However, if a pressure of 335 mm Hg is present in the cell, then $\Delta p_m/RT = 16.75$ mM and for the above inequality to hold, Δc_m would have to be close to 17 mM. Thus, the experimental observation that membrane water permeability is much larger than salt permeability implies hydrostatic pressure will be approximately balanced by osmotic pressure and transmembrane water flow velocity will be approximately independent of pressure, insofar as transmembrane salt transport is independent of pressure. Transmembrane salt transport depends on Nernst potentials, intracellular voltage and membrane conductances, and should not appreciably change when pressure develops.

The above quantitative analysis leads to some counter intuitive predictions for the lens. Fiber cell transmembrane entry of water depends on the transmembrane osmotic and

hydrostatic pressures, but water exits the fiber cells by flowing down the cell-to-cell hydrostatic pressure gradient to the surface, where once again osmotic gradients cause it to exit the lens. Consider a thought experiment in which fiber cell gap junctions have an infinite hydraulic conductivity so a circulation of fluid occurs due to transmembrane osmosis (approximately a 0.3-mM transmembrane osmotic gradient) without any hydrostatic pressure. Next, assume the hydraulic conductivity of fiber cell gap junctions is switched to a typical realistic value. There would be an immediate jump in cell-to-cell hydrostatic pressure due to cell to cell fluid outflow, but that would have two effects. First, fluid inflow would be reduced due to the transmembrane hydrostatic pressure, so the ratio j_m/u_m would become hypertonic and the cells would begin to accumulate salt. Second, fluid outflow would exceed fluid inflow so the fiber cells would lose water and salt through the gap junctions. But the crystallins are too large to pass through gap junctions so they would be concentrated by the loss of water. Eventually a steady state would be reached where the combined osmotic effect of intracellular salt and crystallins would balance the transmembrane effect of the hydrostatic pressure to within approximately 0.3 mM and fluid circulation would be restored. We are left with the conclusion that the positive intracellular pressure causes the cells to shrink. Though counterintuitive, this idea is consistent with experimental data.

Vaghefi et al.³³ use MRI to measure the protein to water ratio in bovine lenses. When the lenses were placed in low Na^+ , high K^+ external solution to block the lens circulation, the gradient in protein-to-water ratio collapsed to approximately 60% of normal in a period of 4 hours. This external solution is first used by Parmelee,⁴ who uses the vibrating probe to show that it blocks circulating currents in the frog lens. Gao et al.¹⁴ use this solution to block the circulation of Na^+ in mouse lenses and observe the effect on the central hydrostatic pressure. The central hydrostatic pressure goes to near zero in a period of approximately 2 hours. As the new external solution diffuses into the extracellular spaces within the lens, the transmembrane electrochemical gradient for Na^+ is reduced and then eventually eliminated and Na^+ influx ceases, leading to elimination of circulating currents, fluid flow, and intracellular hydrostatic pressure. If intracellular hydrostatic pressure causes fiber cells to lose water, then reduction in this pressure should cause water to enter the cells, as measured by Vaghefi et al.³³

Two hours is approximately what one would expect for the central pressure to go to zero in a mouse lens. Because the radius of a bovine lens is approximately 10 times larger than that of a mouse lens, 4 hours is unlikely to have been sufficiently long for the low Na^+ , high K^+ solution to have diffused into extracellular spaces at the center of the bovine lenses, so the central fiber cell intracellular hydrostatic pressure was unlikely to have gone to zero. Thus the 40% reduction in the central protein-to-water ratio seen in bovine lenses is probably not a steady state number, so we do not know just how much of the lens protein gradient is associated with the lens circulation. Nevertheless, their data clearly show that the protein concentration gradient is actively maintained, possibly through the link to hydrostatic pressure described above.

Summary

The results presented here have generated some new thoughts on homeostasis in the lens. The lens circulation may provide a microcirculatory system for the avascular lens, but it appears to have other roles as well. Our data suggest that one additional role is to generate intracellular hydrostatic pressure, which is regulated across the species we studied to be approximately

335 mm Hg at the lens center. Our data also suggest that regulation may be through species-dependent expression of the fiber cell Na^+ -leak channels. These results lead to the question: What is important about a central pressure of 335 mm Hg? We have discussed the possibility that the pressure gradient creates the refractive index gradient, which is essential for visual acuity. While this is a particularly intriguing answer to our question, there are many gaps in knowledge that will need to be filled before this can be considered a viable hypothesis.

One such gap concerns the model calculations. The predicted balance between hydrostatic and osmotic pressures was derived from structurally based models of fluid and salt transport into the lateral intercellular spaces of the proximal tubule epithelium.³² This is an extracellular compartment, so the pressure-osmolarity balance in the presence of gap junctions and intracellular crystallins has not been quantitatively modeled. More appropriate model calculations are needed.

The second gap is that experiments in the lens linking intracellular pressure to protein concentration were done in different species, with lenses of very different sizes, and at different timescales. Gao et al.¹⁴ show that a low Na^+ , high K^+ external solution causes the pressure at the center of a mouse lens to go to zero in approximately 2 hours, whereas Vaghefi et al.³³ show that the same external solution causes the protein-to-water gradient in a cow lens to drop to 60% normal in approximately 4 hours. A more direct experimental correlation between intracellular hydrostatic pressure and intracellular protein concentration is needed. Moreover, the refractive index gradient in the cow lens goes from approximately 1.4 at the center to 1.35 at the surface, whereas the gradient in the mouse lens goes from approximately 1.5 at the center to 1.35 at the surface. If refractive index and pressure are related, the central pressure in a cow lens might be significantly lower than in a mouse lens, although differences in crystalline subtypes with different refractive power might confound this prediction. Nevertheless, it would be interesting to measure hydrostatic pressure in cow lens.

Despite gaps, these ideas and recent experiments, suggesting that the refractive index gradient in the lens could be actively established by the lens circulation, are new, exciting, and important. They provide a new direction for investigation of the optical properties of the eye lens.

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