Nuclear Magnetic Resonance Study of Free and Bound Water Fractions in Normal Lenses

Paul J. Stankeiwicz, † Kenneth R. Metz, † Joseph W. Sassoni,* and Richard W. Briggs§

Proton NMR relaxation times $T_1$ and $T_2$ were determined for normal lenses excised from sexually mature animals from seven different species. Lenses were immersed in physiological buffer during measurements, and suppression methods were employed to null the buffer signal. This enabled selective analysis of lenticular water. Observed relaxation times were correlated with protein and water content. At 37°C and 1.89 Tesla, single-exponential spin–lattice relaxation was observed, but spin–spin relaxation was found to be double-exponential. It was shown that the short-$T_2$ fraction is proportional to protein concentration; this fraction was attributed to water bound to protein. The long-$T_2$ fraction was attributed to free lenticular water. The amounts of free and bound water thus obtained were used in the spin–lattice relaxation rate equation for rapid exchange in a two-component system to calculate the magnitudes of the two corresponding $T_1$ relaxation components. Invest Ophthalmol Vis Sci 30:2361–2369, 1989

Nuclear magnetic resonance (NMR) spectroscopy is potentially a sensitive tool for noninvasive monitoring of the physiological state of the lens and other biologic materials. Information such as pH, levels of organophosphate metabolites, the state of water, and intracellular sodium content can be obtained using NMR. We are seeking sensitive $^1$H NMR parameters that will enable normal and pathological states of a lens to be distinguished. Studies of in vitro lenses are being conducted in order to develop techniques useful for evaluating the factors in the development and prevention of cataract.

Lenses typically contain approximately 33% protein by weight, more than any other tissue. Therefore, a large fraction of lenticular water should be involved in protein hydration as bound water. NMR relaxation times of this water are expected to be sensitive to changes which are likely to accompany cataract formation, such as aggregation and structural alteration of the crystallin proteins.

According to the osmotic theory of cataractogenesis, elevated extracellular glucose concentrations (as in diabetes) can lead to intracellular hyperosmolality due to the accumulation of sorbitol. Osmotic swelling increases the intracellular water content and may change the ratio of free to bound water. NMR relaxation time analysis should be useful in detecting alterations in the ratio of free to bound water occurring during the cataractogenic process and in testing the effectiveness of medications in preventing cataractogenesis.

Initial attempts to measure proton relaxation times of excised lenses under physiological conditions were hindered by a large interfering proton signal from the physiological bathing solution. Therefore, methods were developed to null magnetically the buffer water signal on the basis of the 4-fold difference between the spin–lattice relaxation time of the bathing solution water (4.0 sec) and water in a typical lens (approximately 1 sec). Using these techniques, it is possible to analyze directly lenticular water spin–lattice and spin–spin relaxation times under physiological conditions and to establish normal ranges of these parameters in healthy lenses. This information could serve as a reference for diagnosing pathologic changes in the state of lenticular water.

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Materials and Methods

All animals except chickens were young adult males. Chickens (*Gallus domesticus*), 1.19 ± 0.19 kg (n = 7), were purchased from a local farm. Guinea pigs (*Cavia porcellus*), 710 ± 150 g (n = 6), and New Zealand white rabbits (*Oryctolagus cuniculus*), 3.92 ± 0.46 kg (n = 5), were purchased from Hazelton Research Animals (Denver, PA). Ferrets (*Mustela putorius furo*), 1.53 ± 0.15 kg (n = 5), were obtained from Marshall Research Animals (North Rose, NY). Rats (*Rattus norvegicus*), 422 ± 23 g (n = 7), were purchased from Charles River Laboratories (Wilmington, MA). Frogs (*Rana catesbeiana*), 284 ± 57 g (n = 12), were obtained from the William A. Lemberger Company (Oshkosh, WI) and were in the fourth week of hibernation when used. Rainbow trout (*Olmus gairdneri*), 525 ± 64 g (n = 4), were donated by the Pennsylvania Fish Commission (Huntsdale Fish Culture Station, Huntsdale, PA).

The techniques employed conform to the ARVO Resolution on the Use of Animals in Research. Chickens, guinea pigs, ferrets, and rats were euthanized with a lethal dose (540 mg/kg) of sodium pentobarbital. Frogs were killed by decapitation. Trout were euthanized by oxygen deprivation. Lenses were removed from the eye surgically with the posterior pole approach and were freed of surrounding tissue while bathing in Dulbecco’s phosphate-buffered saline containing 0.90 mM CaCl₂ and supplemented with 8.0 mM glucose, 0.3 mg/ml penicillin, and 0.3 mg/ml streptomycin. Lenses were incubated at 37°C in TC-199 medium 13 for 2–4 hours before NMR analysis.

Proton NMR measurements were performed at 80.285 MHz (1.89 Tesla) using an Oxford Instruments (Oxford, UK) magnet with 26-cm bore diameter. Nicolet (Madison, WI) radio-frequency (rf) hardware, 1280 computer, and 293C pulse programmer were employed. The coil, constructed in the laboratory, was a 4-turn 15- or 20-mm diameter solenoid constructed from 14AWG copper magnet wire. A 90° pulse width of 12 μsec was obtained using an Amplifier Research (Souderton, PA) 200L rf power amplifier.

For NMR studies each lens was immersed in a minimal volume of TC-199 normal medium contained inside a Pyrex tube. All lens relaxation measurements were performed at 37 ± 0.5°C using pulse sequences that efficiently null the buffer signal. 12 In order to avoid undue acidification of the buffer medium, T₁ and T₂ determinations were completed within 20 min and 6 min, respectively.

Spin–lattice relaxation times were determined using the following sequence\(^{12}\):

\[
180° - \tau_{\text{null}} - D1 - 90° - (+) \text{ Acq 1} - (H.S. - 90°) - D1 - 90° - (-) \text{ Acq 2 - RD}
\]

The initial 180° pulse inverts both lens and buffer magnetizations. After a period \(\tau_{\text{null}} \approx \ln 2 \times T_{1 \text{ buffer}}\), the lens signal is largely relaxed while the buffer has a net zero magnetization. The D1 – 90° – acquisition series then produces a saturation recovery relaxation curve for the buffer with very little contribution from the lens. In the second half of the experiment the lens and buffer signals are both saturated using a train of rf and homospoil pulses (typically, \(n = 8\)). The second acquisition then generates a saturation recovery curve for both the lens and the buffer. The buffer contribution disappears when the two sets of acquisitions are subtracted, leaving a lens recovery curve of the form:

\[
M_{\text{obs}} = M_0 \times K \exp(-D1/T_{1 \text{ lens}})
\]

The entire sequence may be repeated following a relaxation delay (RD) of \(5 \times T_{1 \text{ buffer}}\).

Spin–spin relaxation was measured with the following pulse sequence:\(^{12,14}\)

\[
180°_s - \tau_{\text{null}} - 90°_s - \left(\frac{\tau_{\text{echo}}}{2} - 180°_s - \tau_{\text{echo}} - 180°_s - \frac{\tau_{\text{echo}}}{2} - A\right)_n - \text{RD}
\]

The initial 180° pulse inverts both lens and buffer magnetizations. After a period \(\tau_{\text{null}} \approx \ln 2 \times T_{1 \text{ buffer}}\), the lens signal is largely relaxed while the buffer has a net zero magnetization. The recovered lens signal is nutated into the rotating frame \(xy'z'\) plane where it undergoes spin–spin relaxation. A series of \(n\) single data points are acquired (A) from the tops of the even numbered echoes formed using 180° s refocussing pulses to remove the effect of field inhomogeneities. A relaxation delay (RD) of \(5 \times T_{1 \text{ buffer}}\) then occurs before the sequence is repeated. For this work 16 acquisitions were summed, producing a very high signal-to-noise ratio. The correct value of \(\tau_{\text{null}}\) was determined empirically from a pure buffer sample before the analysis of each set of lenses. Values were consistently found to be near the theoretical \(\tau_{\text{null}}\) value of 2.8 sec based on the measured buffer \(T_1\) of 4.0 sec at 37°C.

Spin–lattice relaxation data were analyzed using a three-parameter single-exponential least squares fit.\(^{15}\) Spin–spin relaxation curves were analyzed using a five-parameter double-exponential least squares fit.

Immediately following NMR analysis, the water content of some lenses was measured gravimetrically by heating at 105°C to constant weight.\(^{16}\) This procedure precluded biochemical assays on the same lens, so different lenses were employed for the two types of measurements.
Prior to biochemical assays, lenses were preserved at -20°C. Lenses were then extracted into 1% Triton X-100 in deionized water. All but a small residue of lens material was found to be soluble. Extracts were subjected to atomic absorption analysis using a Perkin-Elmer model 360 atomic absorption spectrophotometer. Sodium and potassium levels were determined against known standards using 589-nm and 766-nm absorption lines, respectively. Background corrections for Triton X-100 were performed.

All other biochemical assays were conducted with Triton X-100 lens extracts which had been deproteinized with perchloric acid and neutralized with KOH. Glucose and fructose were quantitated by enzymatic methods. Aliquots of lens extract containing 0.01 to 0.1 μmol of glucose plus fructose were diluted to 1.0 ml (pH 7.5) and final concentrations of 50 mM glycyglycine, 0.5 mM ATP, 5.0 mM MgSO₄, and 1.0 mM NADP⁺. The reaction was initiated by the addition of 0.4 U hexokinase (Sigma, St. Louis, MO; C-130, from bakers' yeast) and 0.4 U glucose-6-phosphate dehydrogenase (Sigma; type VII, from bakers' yeast). Glucose was quantitated spectrophotometrically at 340 nm after 30 min. To the same set of solutions, 0.4 U glucose-6-phosphate isomerase (Sigma, type III, from bakers' yeast) was added. Fructose was quantitated by the absorbance change after 30 min.

Sorbitol was determined by a modification of the method of Williams-Ashman. Aliquots of lens extract containing 0.01 to 0.1 μmol of sorbitol were diluted to 1.0 ml (pH 9.0) and final concentrations of 100 mM tris-glycine, 5.0 mM reduced glutathione, 0.005% sodium azide, and 1.0 mM NAD⁺. The reaction was initiated by the addition of 0.2 U sorbitol dehydrogenase (Sigma, lyophilized powder from sheep liver). The increase in absorbance at 340 nm was measured after 24 hr at room temperature.

Lenticular reduced glutathione was measured by the method of Beutler et al. Aliquots of lens extract containing 0.01 to 0.05 μmol of reduced glutathione were diluted to 1.0 ml (pH 7.7) and a final concentration of 0.24 M sodium monohydrogen phosphate. The reaction was initiated by adding DTNB reagent, 0.4 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma) in 1% sodium citrate. Color development was instantaneous, and absorbance at 412 nm was read immediately.

Results

Biochemical Analysis

Lens biochemical analyses were performed in order to verify that the excision and incubation procedures did not produce abnormalities in composition. Table 1 summarizes biochemical data for each species.

### Table 1. Lens biochemical data

<table>
<thead>
<tr>
<th>Species</th>
<th>Total lens weight (mg)</th>
<th>Reduced glutathione (μmol/g lens)</th>
<th>Glucose (μmol/g lens)</th>
<th>Sorbitol (μmol/g lens)</th>
<th>Fructose (μmol/g lens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>115 ± 13 [11]</td>
<td>0.33 ± 0.22 [7]</td>
<td>0.53 ± 0.22 [7]</td>
<td>0.31 ± 0.29 [7]</td>
<td>0.31 ± 0.29 [7]</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>669 ± 5.8 [11]</td>
<td>0.38 ± 0.13 [6]</td>
<td>0.67 ± 0.13 [6]</td>
<td>0.31 ± 0.15 [6]</td>
<td>0.39 ± 0.15 [6]</td>
</tr>
<tr>
<td>Ferret</td>
<td>588 ± 12.8 [10]</td>
<td>0.50 ± 0.17 [12]</td>
<td>0.69 ± 0.17 [12]</td>
<td>0.30 ± 0.09 [5]</td>
<td>0.50 ± 0.09 [5]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>428 ± 1.4 [10]</td>
<td>0.50 ± 0.17 [12]</td>
<td>0.69 ± 0.17 [12]</td>
<td>0.30 ± 0.09 [5]</td>
<td>0.50 ± 0.09 [5]</td>
</tr>
<tr>
<td>Frog</td>
<td>409 ± 3.1 [20]</td>
<td>0.50 ± 0.17 [12]</td>
<td>0.69 ± 0.17 [12]</td>
<td>0.30 ± 0.09 [5]</td>
<td>0.50 ± 0.09 [5]</td>
</tr>
<tr>
<td>Rat</td>
<td>406 ± 3.2 [12]</td>
<td>0.50 ± 0.17 [12]</td>
<td>0.69 ± 0.17 [12]</td>
<td>0.30 ± 0.09 [5]</td>
<td>0.50 ± 0.09 [5]</td>
</tr>
<tr>
<td>Trout</td>
<td>116 ± 7 [7]</td>
<td>0.50 ± 0.09 [4]</td>
<td>0.50 ± 0.09 [4]</td>
<td>0.40 ± 0.03 [3]</td>
<td>0.50 ± 0.09 [4]</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± standard deviation.

Number of lenses shown in brackets.

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species. The concentrations of glucose, sorbitol, and fructose are all within normal ranges for excised lenses of these species. The concentrations of reduced glutathione are consistent with the values reported for normal rats and other species. The values for frogs differ somewhat from those reported previously, probably as a result of the hibernating state of these animals. The lenticular sodium and potassium contents are consistent with values reported for rabbit, rat, and frog lenses. The species variations in water content are as expected. Fish generally have the lowest water content, ranging from 50% in dogfish and trout to 60% in carp. Mammalian lenses typically contain 60–70% water.

Avian lenses have the highest water content, with reported values of 74% in chickens, 77% in buzzards, and 80–84% in pigeons. The unusually high water content in birds may be associated with the annular pad, a unique anatomical structure found in all avian species.

The relative standard deviation of lenticular water content is below 2% in all cases in Table 1, showing that the lenses had reached a steady state during the 2–4 hr incubation period prior to analysis. In addition, these experimental values for water content agree with published data for normal lenses, as described above. Finally, metabolite and cation concentrations are in the expected ranges. Overall, the analytical results of Table 1 demonstrate the absence of temporal variations or other abnormalities in lens composition during these experiments.

Relaxation Data

Figure 1 shows a typical lenticular spin–lattice relaxation curve obtained using the buffer suppression pulse sequence described above. T1 values of lenses immersed in buffer were similar to those measured from "dry" lenses (not immersed in buffer), demonstrating the absence of a long T1 component which might have been nulled by the water suppression technique. Within experimental uncertainty, spin–lattice relaxation appears to be a single exponential process for all of the lenses in this study.

The spin–spin relaxation curve for a guinea pig lens is shown in Figure 2. Attempts to fit the data to a single-exponential equation produced poor results, but a five-parameter double-exponential fit produced a very small residual standard deviation. From least-squares analysis, values for both the relaxation times and the relative percentage of the two water components were obtained with good precision (Table 2). These values were found to be quite species-dependent.

Discussion

Water proton relaxation times for excised lenses have been reported by other investigators and are summarized in Table 3. These data represent a wide range of magnetic field strengths and several different temperatures. Since lenticular water relaxation times are expected to depend on these experimental conditions, it is not possible to quantitatively compare our results with those in the literature for similar species. However, general agreement is evident. Previous studies of lens and cornea water relaxation have revealed single-exponential spin–lattice and double-exponential spin–spin decays. The single-exponential behavior has been explained using the two-compartment rapid-exchange model. The observation of double-exponential spin–spin relaxation is consistent with the existence of two motionally distinct water fractions. A major fraction of lenticular...
Table 2. NMR relaxation parameters for lenticular water at 1.89 Tesla and 37°C

<table>
<thead>
<tr>
<th>Species</th>
<th>( T_1 ) (sec)</th>
<th>( T_1^b ) (msec)</th>
<th>( F^b )</th>
<th>( T_2^f ) (msec)</th>
<th>( F^f )</th>
<th>% ( H_2O ) (weight/weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>1.48 ± 0.21 [11]</td>
<td>30.0 ± 1.5 [11]</td>
<td>0.401 ± 0.024</td>
<td>92.5 ± 11.3 [11]</td>
<td>0.599 ± 0.024</td>
<td>77.4 ± 1.1 [4]</td>
</tr>
<tr>
<td>Ferret</td>
<td>1.01 ± 0.12 [11]</td>
<td>12.2 ± 1.0 [11]</td>
<td>0.513 ± 0.014</td>
<td>72.1 ± 8.3 [11]</td>
<td>0.487 ± 0.014</td>
<td>64.5 ± 0.6 [4]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.10 ± 0.07 [9]</td>
<td>10.4 ± 0.5 [9]</td>
<td>0.556 ± 0.005</td>
<td>74.7 ± 2.8 [9]</td>
<td>0.444 ± 0.005</td>
<td>63.5 ± 0.4 [4]</td>
</tr>
<tr>
<td>Frog</td>
<td>0.86 ± 0.03 [10]</td>
<td>13.5 ± 1.7 [10]</td>
<td>0.603 ± 0.086</td>
<td>79.3 ± 8.7 [10]</td>
<td>0.397 ± 0.086</td>
<td>61.2 ± 1.1 [4]</td>
</tr>
<tr>
<td>Rat</td>
<td>0.81 ± 0.11 [15]</td>
<td>11.5 ± 1.7 [9]</td>
<td>0.623 ± 0.040</td>
<td>54.6 ± 8.3 [15]</td>
<td>0.378 ± 0.040</td>
<td>60.4 ± 0.8 [15]</td>
</tr>
<tr>
<td>Trout</td>
<td>0.76 ± 0.04 [4]</td>
<td>8.9 ± 0.3 [4]</td>
<td>0.697 ± 0.009</td>
<td>60.9 ± 2.2 [9]</td>
<td>0.303 ± 0.009</td>
<td>54.2 ± 0.9 [4]</td>
</tr>
</tbody>
</table>

\( F^b \) and \( F^f \) are relative fractions of signal intensity for the fast-relaxing (bound) and slowly relaxing (free) components, respectively.

Table 3. Water proton \( T_1 \) and \( T_2 \) values reported for normal whole lenses

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature (°C)</th>
<th>Field (Tesla)</th>
<th>( T_1 ) (msec)</th>
<th>( T_2 ) (msec)</th>
<th>Data from (reference no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>—</td>
<td>—</td>
<td>1060</td>
<td>22-62</td>
<td>35</td>
</tr>
<tr>
<td>Human</td>
<td>25</td>
<td>2.114</td>
<td>790</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Human</td>
<td>32</td>
<td>2.114</td>
<td>900</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Human</td>
<td>33</td>
<td>0.799</td>
<td>600</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Human</td>
<td>37</td>
<td>2.114</td>
<td>800</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td>Human</td>
<td>37</td>
<td>1.409</td>
<td>840</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Buzzard</td>
<td>25</td>
<td>2.114</td>
<td>890</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td>Pig</td>
<td>37</td>
<td>1.409</td>
<td>960</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>36.1</td>
<td>0.587</td>
<td>490</td>
<td>29.3</td>
<td>6</td>
</tr>
<tr>
<td>Frog</td>
<td>—</td>
<td>0.235</td>
<td>—</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Frog</td>
<td>—</td>
<td>0.7</td>
<td>425</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Rat</td>
<td>37</td>
<td>7.05</td>
<td>—</td>
<td>22110</td>
<td>7</td>
</tr>
<tr>
<td>Carp</td>
<td>25</td>
<td>2.114</td>
<td>730</td>
<td>13-55</td>
<td>4</td>
</tr>
</tbody>
</table>

Values reported are mean ± standard deviation. Number of lenses is in brackets.
tabulated in Table 4. Interspecies differences in the ratio can be attributed to variations in the molecular weight and oligomeric structures of the lens proteins and to differences in the relative amounts of the predominant proteins, the \( \alpha \) (high MW), \( \beta \) (medium MW), and \( \gamma \) (low MW) crystallins. The proteins of chicken lenses appear to be the most hydrated, and those of trout the least.

In the foregoing discussion, the \( T_2 \) values of Table 2 were analyzed with a two-compartment model. However, for each species only a single lenticular \( T_2 \) value has been observed, and it may be the weighted average of values for the two compartments suggested by the \( T_2 \) data. We can attempt to resolve the two putative lenticular \( T_2 \) values by applying the spin-lattice relaxation rate equation for a two-compartment system in rapid exchange:

\[
\frac{1}{T_1^{obs}} = \frac{R_1^{obs}}{R_1^b} = F^b \times R_1^b + F^f \times R_1^f \]

This equation enables the calculation of observed spin-lattice relaxation rates \( R_1^{obs} \) from changes in the fraction of bound water \( (F^b) \) when relaxation rates in the bound and free environments \( (R_1^b \) and \( R_1^f \)) are known. The data of Table 2 show that the amount of bound water is species-dependent. On the other hand, the free-water \( T_1 \) values should be relatively insensitive to the details of the lenticular protein structure and might be more uniform from one species to another. In order to take into account the unique characteristics of each case, we reformulate equation (7) in terms of the species-dependent parameters \( g_{\text{water}} / g_{\text{protein}} \) and \% water. First, equations (2) and (5) are combined to yield

\[
F^b = \frac{g_{\text{water}}}{g_{\text{protein}}} \times \left( \frac{1 - \% \text{ water}}{100} \right)
\]

which may be substituted into equation (7) to form

\[
R_1^{obs} = g_{\text{water}} \times (R_1^b - R_1^f) \times \left( \frac{100}{100 - \% \text{ water}} - 1 \right) + R_1^f
\]

This equation permits the evaluation of both \( R_1^b \) and \( R_1^f \) from a linear plot of \( R_1^{obs} \) against \( [(100/\% \text{ water}) - 1] \), as shown in Figure 4. The intercept provides an average value of \( R_1^b \) for all species. Given this quantity and the known values of \( g_{\text{water}} / g_{\text{protein}} \), values of \( T_1^b \) \((= 1/R_1^b)\) may be calculated for each species (Table 4). The mean \( T_1^b \) obtained from the intercept is 3.15 sec, only moderately smaller than the experimental spin-lattice relaxation time of buffer water under these conditions (4.0 sec). The difference might be attributable to paramagnetics or other solutes in the intracellular environment which interact with water.

Although the fraction of bound water varied among the lenses in this study, the relaxation time \( T_2^b \) remained relatively constant at 10.9 ± 1.9 msec except in chicken lenses, which had 3-fold higher values. The relaxation time of the bound water should be sensitive to protein molecular weight and flexibility changes. Chicken lenses have been reported to contain less of the high molecular weight \( \alpha \)-crystallin protein fraction than frog, rabbit, rat, or human lenses. \(^38\)\(^-40\) The molecular weight of chicken lens \( \alpha \)-crystallin is also significantly lower than that of rabbit, rat, frog, or fish. \(^41\)\(^-43\) These molecular weights are consistent with the longer \( T_2^b \) in chicken compared to other species.

Another important factor contributing to the observed \( T_2^b \) may be that chicken lenses contain 40 to

Table 4. Index of lenticular protein hydration and calculated \( T_2 \) values for bound water in lenses

<table>
<thead>
<tr>
<th>Species</th>
<th>( g_{\text{water}} / g_{\text{protein}} )× 100</th>
<th>( T_2^b ) (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>1.37</td>
<td>0.84</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.93</td>
<td>0.62</td>
</tr>
<tr>
<td>Ferret</td>
<td>0.96</td>
<td>0.64</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.95</td>
<td>0.63</td>
</tr>
<tr>
<td>Frog</td>
<td>0.95</td>
<td>0.63</td>
</tr>
<tr>
<td>Rat</td>
<td>0.83</td>
<td>0.56</td>
</tr>
<tr>
<td>Trout</td>
<td>0.82</td>
<td>0.56</td>
</tr>
</tbody>
</table>

\* \( g_{\text{water}} / g_{\text{protein}} \) values are calculated using equation (6) and the data of Table 2.
The analysis of the frozen lens is based on the disappearance of free water signal upon freezing at $-9^\circ$C. Protein hydration values ($\frac{g_{\text{water}}}{g_{\text{protein}}}$) determined in this manner have been reported to be 0.53 in normal human lenses, 0.50 in fish lenses, and 0.82 in buzzard lenses.\(^4\) Model systems of pure protein solutions\(^49\) yielded values of 0.33 for ovalbumin and 0.40 for serum albumin.

When $\frac{g_{\text{water}}}{g_{\text{protein}}}$ ratios are calculated using DSC literature data, substantially larger values are usually found. For example, studies of normal human lenses yielded values of 1.14 and 0.80.\(^{47,48}\) Another investigation reported 0.99 in normal human eye bank lenses.\(^3\) A value of 0.51 was calculated for lenses from 180-day-old rats.\(^32\) Studies of pure protein solutions\(^30\) yielded values of 0.72 and 1.14 for ovalbumin and for crystallins, respectively, while aqueous gelatin solutions\(^51\) gave values ranging from 0.71 to 1.1. Several other techniques have been employed to obtain $\frac{g_{\text{water}}}{g_{\text{protein}}}$ values,\(^52\) including high-speed centrifugation, osmotic analysis, and studies of NMR $T_1$ values in partially dehydrated lenses. These methods yielded values even higher than those from DSC, ranging from 1.29 for bovine lens nucleus to 2.21 in pig whole lenses. These high $\frac{g_{\text{water}}}{g_{\text{protein}}}$ ratios suggest a one-component model for lenticular water, since they represent an approximate theoretical upper limit if all the water is bound.

Although some variability is evident, the overall protein hydration determined by calorimetric methods is about twice that determined by NMR analyses of frozen lenses. This reflects the larger freezable water fraction found using NMR versus the smaller fraction observed with DSC. It is possible that a part of the decrease in NMR signal intensity attributed to the freezing of free water actually results from partial immobilization of a third pool containing loosely bound water, which thus increases the apparent fraction of free versus bound species. A relatively modest increase in correlation time beyond about $10^{-7}$ sec could broaden the NMR signal without releasing a large latent heat of fusion in the DSC experiment.

At $37^\circ$C, a two-pool model appears to account for our experimental relaxation data. The mean value of 0.97 $\frac{g_{\text{water}}}{g_{\text{protein}}}$ for all seven species is in general agreement with the DSC results, suggesting that any loosely bound, motionally hindered water fraction which is not observable by NMR at $-9^\circ$C is visible at $37^\circ$C and is indistinguishable from tightly bound water. Evidence for this pool of loosely bound water, which thus increases the apparent fraction of free versus bound species. A relatively modest increase in correlation time beyond about $10^{-7}$ sec could broaden the NMR signal without releasing a large latent heat of fusion in the DSC experiment.

At $37^\circ$C, a two-pool model appears to account for our experimental relaxation data. The mean value of 0.97 $\frac{g_{\text{water}}}{g_{\text{protein}}}$ for all seven species is in general agreement with the DSC results, suggesting that any loosely bound, motionally hindered water fraction which is not observable by NMR at $-9^\circ$C is visible at $37^\circ$C and is indistinguishable from tightly bound water. Evidence for this pool of loosely bound lenticular water has accumulated as more studies using different techniques have been conducted.\(^48,52\) The comparatively small bound water values measured using NMR at $-9^\circ$C presumably exclude this loosely bound pool, while DSC and our NMR analysis in-
clude this pool with the tightly bound water. The relative magnitudes of the results suggest that the quantities of tightly and loosely bound water in the lens are roughly equal. In addition, we note that an increase in temperature is known to favor the binding of water to proteins, so techniques such as ours, which measure bound water at physiological temperatures, might be expected to yield somewhat higher values than methods which rely on freezing. However, since our values at 37°C generally agree with those from DSC near 0°C, this effect does not appear to fully account for the differences in reported results.

Alterations in the proportions of free and bound water are thought to accompany cataractogenesis through a syneresis mechanism. These changes have been observed with both frozen lens NMR analysis and calorimetry. For example, the bound water fraction was found to decrease from 24% in normal human lenses to 13% in cataractous human lenses by NMR analysis at -9°C, and from 57% (0.77 g water/g protein, normal human) to 37% (0.77 g water/g protein, cataractous human) by differential scanning calorimetry, using an average of nucleus and cortex values. This type of analysis can be expected to have diagnostic value. Of the experimental methods discussed here, only NMR relaxation measurements at 37°C are applicable to both in vivo and in vitro lenses. Therefore, the further development of these NMR methodologies for lens studies is recommended.

Key words: lens, nuclear magnetic resonance (NMR), relaxation rate, crystallins, hydration

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