Nuclear magnetic resonance analyses of the cold cataract: whole lens studies

Sidney Lerman, David L. Ashley, Robert C. Long, Jr., Jacob H. Goldstein, Judith M. Megaw, and Karen Gardner

Pulse nuclear magnetic resonance (NMR) magnetization decay studies were performed on normal and cold cataractous rat lenses. Computer analyses of the decay curves have been interpreted to reflect two types of water within the lens that do not exchange rapidly. Upon lowering the temperature in the presence and absence of 5% acrylamide (a known agent that prevents the cold cataract phenomenon), significant differences in the relaxation rates of one water fraction were noted. $^1$H (NMR) spectra on young rabbit and human lenses showed temperature-related linewidth changes, which are significantly diminished in lenses incubated in acrylamide. $^{31}$P spectra also showed similar inorganic phosphate linewidth changes and also reflected progressive alterations in the metabolic state of these lenses. These studies demonstrate the potential of NMR methods for monitoring physicochemical parameters in the normal and cataractous mammalian lens. (INVEST OPHTHALMOL VIS SCI 23:218-226, 1982.)

Key words: opacification, transverse relaxation, acrylamide, temperature transition

The normal mammalian lens is a precisely formed structure containing about 65% water and 35% organic material, chiefly the structural proteins. The water-soluble lens proteins consist of the $\alpha$, $\beta$, and $\gamma$-crystallins, and at birth they constitute over 95% of the total lens proteins. Since the structural proteins of the lens constitute most of the dry weight of this organ, they must play a major role in the transmission, absorption, and scattering of light by this organ. The constituent lens proteins are densely packed and arranged in a high degree of spatial order within the intact fiber. There is little change in the density of scattering particles from point to point within the normal lens. Localized alterations in the density of packing of lens proteins, because of aggregation and/or conformational changes developing in various portions of the lens, will lead to changes in transparency, i.e., the development of opacities due to localized changes in refractive index. This is well exemplified by the cold cataract phenomenon, which can be demonstrated in lenses from young animals (rat, rabbit, calf, and human) when they are exposed to temperatures below their ambient levels and which can be reversed by warming. 1-9

This phenomenon enables us to study a controlled opacification in vitro and to investigate in more detail the physicochemical events associated with a reversible form of cataract. It has been produced in the rat lens without disruption of cell size, shape, structure, or cellular organization, demonstrating that cell destruction is not necessarily the only mechanism involved in the generation of
NMR analyses of the cold cataract

lens opacification, although extensive cellular damage frequently accompanies advanced and irreversible cataracts.\textsuperscript{7, 10, 11} The lens changes produced prior to the development of extensive cellular damage could result from microstructural changes occurring in the subcellular or molecular level. The precise identification of these changes would be significantly advanced by an understanding of the physicochemical nature of the scattering elements that develop during the formation of the cold cataract. That is, when a lens from a young rat, rabbit, calf, or human is removed and cooled, the reversible cold cataract phenomenon is the result of light scattering caused by spatial fluctuations of the refractive index due to a phase-separation phenomenon of the protein-water mixture within the fiber cell cytoplasm.\textsuperscript{4, 6} When the phase-separation temperature becomes higher than the ambient temperature of the lens, the nuclear opacity appears in vivo and is probably the result of a similar mechanism.\textsuperscript{12} Recent studies indicate that a series of reagents can prevent the cold cataract phenomenon.\textsuperscript{6, 8, 13} These include glycerol, ethylene glycol, glutaraldehyde, acetaldehyde, formaldehyde, and acrylamide. The reversible clarifying reagents (acrylamides and glycols) interact noncovalently with lens constituents to change the position of the coexistence curve in the phase diagram so that the phase separation in the lens cytoplasm occurs at reduced temperatures.\textsuperscript{6, 8} The precise position of the coexistence curve is determined by protein-solvent and solvent-solvent interactions. By changing the nature of these interactions, Clark and Benedek\textsuperscript{8} have demonstrated a reversible shift in the position of the coexistence curve, thereby lowering the temperature at which the cold cataract develops. In previous studies, nuclear magnetic resonance (NMR) methods have been found to be useful in probing microstructural changes in tissues. This article describes our preliminary results in the application of selected NMR techniques to follow the physicochemical response of mammalian lenses to selected perturbants such as temperature and chemical reagents.

Materials and methods

Weanling Sprague-Dawley rats from our own laboratory strain and 3-week-old albino rabbits were studied. The eyes were removed immediately after sacrificing the animals, and the lenses were carefully excised, blotted on Parafilm, and immediately placed in the appropriate sample tubes for NMR analyses, except for the incubation studies. Human lenses were derived from donor eyes (within 24 hr after death) provided by the Georgia Lions Eye Bank. For the incubation experiments the media consisted of a balanced salt solution (bicarbonate buffer) containing 7 mM glucose, 0.75 mM L-glutamine, 2 mg/dl potassium penicillin G, and streptomycin sulfate. This solution was gassed with 95% oxygen-5% CO\textsubscript{2} to maintain oxygen tension and to regulate pH. The incubations were maintained at controlled temperatures in a circulating-water bath. To incorporate acrylamide (and prevent the cold cataract), 5% acrylamide was added to the media. For the deuteration experiments, the components of the incubation media were added to D\textsubscript{2}O (Aldrich, >99% pure) to make up 100% or 5% D\textsubscript{2}O solutions, and the individual lenses were incubated in tightly sealed vials. The pH was maintained at preincubation levels (±0.1 pH units) in the 2 hr incubation experiments and at ±0.2 to 0.3 in the extended incubations (up to 16 hr) in these sealed vials. These pH measurements were performed in rabbit lens incubates treated in an identical fashion and performed as soon as the seal was broken. Lenses from these incubations were not used for NMR analyses.

Water-proton transverse relaxation rates were measured on lenses to which 1.3 mM dextran-magnetite (Meito-Sangyo, Nagoya, Japan) had been added in the solution outside the lens. The transverse magnetization decay was obtained by signal averaging the output from a Spin-Lock CPS-2 pulsed spectrometer (Spin-Lock, Ltd., Port Credit, Ontario, Canada) employing the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence\textsuperscript{14, 15} and sampling echo maxima.\textsuperscript{16} Data analysis was performed on a DECsystem-10 computer with DISCRETE, a program based on the Fourier convolution theorem.\textsuperscript{17} This program separated the different exponential contributions to the spectra. Temperature was controlled to ±1.0°C by a YSI Model 72 Proportional Temperature Controller (Yellow Springs Instrument Co., Yellow Springs, Wyo.) employing thermally regulated nitrogen gas.
Deuterium, phosphorus, and carbon-13 studies were obtained on a Bruker CXP-300 superconducting spectrometer at 46, 121, and 75 MHz, respectively. Spectra were acquired in the Fourier transform mode by means of quadrature detection with a standard phase alteration and routing scheme to minimize image frequencies. Data tables of up to 32K were employed. Sample temperature was monitored by a probe thermocouple and regulated by the instrument temperature controller (BVT-1000). The amount of acrylamide in the lenses was estimated with $^{13}$C NMR to measure the loss of acrylamide from the incubating solution with dioxane as a reference and measur-
Table I

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Temperature (°C)</th>
<th>$T_{2a}$ (msec)*</th>
<th>$T_{2d}$ (msec)*</th>
<th>$T_{2b}$ (msec)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh lenses</td>
<td>7</td>
<td>109.9 ± 10.8</td>
<td>22.3 ± 2.9</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>71.8 ± 9.9</td>
<td>16.9 ± 2.7</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>Lenses incubated without acrylamide</td>
<td>2</td>
<td>112.7 ± 7.7</td>
<td>19.8 ± 1.3</td>
<td>6.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>79.2 ± 4.9</td>
<td>19.8 ± 2.2</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>Lenses incubated with acrylamide</td>
<td>2</td>
<td>65.0 ± 4.0</td>
<td>16.1 ± 1.1</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>54.2 ± 5.1</td>
<td>17.4 ± 0.4</td>
<td>5.7 ± 0.6</td>
</tr>
</tbody>
</table>

*Values shown are mean ± S.D.

ing area ratios. In these experiments the nuclear Overhauser effect was removed by a gated de-coupling scheme. $^2$H and $^{31}$P spectra on whole lenses were obtained in the unlocked mode.

Results

**Pulse NMR magnetization decay studies.**

The results from a typical experiment on young rat lenses with dextran-magnetite relaxant added to the bathing solution are shown in Figs. 1A to 1D. In addition to the experimental data, the best-fit double-exponential curve is shown in Figs. 1A and 1B and the best-fit triple-exponential curve in Figs. 1C and 1D. Figs. 1B and 1D are y-direction expansions of the lower half of curves in Figs. 1A and 1C. It is apparent in Fig. 1B that the double-exponential fit does not completely characterize the data, but the triple-exponential shown in Fig. 1D does. Although the difference between the data and best-fit double-exponential curve in Fig. 1B is not enormous, the finding was clearly reproducible in all lenses tested. Computer fitting of the data indicated that a triple-exponential fit is necessary to characterize the relaxation curve. The standard deviation and signal-to-noise ratio of the fits also showed that the data are best characterized with a triple exponential. The corresponding $T_2$ values were 110, 22, and 5 msec at 33°C, as shown in Table I. These values correspond to at least three states of water between which the exchange rates are not fast compared with the relaxation rates. Experiments done with and without dextran-magnetite have shown that the third fraction ($T_2c = 5$ msec) is due to water outside the lens, in the solution bathing it. The paramagnetic properties of dextran-magnetite serve to lower the relaxation-rate constant of water outside the lens to a value distinctly lower than that of water within the lens, thus more readily distinguishing the exterior component. In addition to identifying the water outside the lens, these experiments demonstrated that the rate of water exchange between the lens and the bathing solution is slow compared with the relaxation rates. The presence or absence of dextran-magnetite in the external medium had no noticeable effect on the measured relaxation rates of water inside the lens.

Our studies (on rat lenses) were performed at temperatures ranging from 33°C to 7°C. The studies on fresh lenses and lenses incubated without acrylamide demonstrated a significant decrease in the longest $T_2$ value as the temperature decreased, from $T_{2a} = 110$ msec at 33°C to $T_{2a} = 72$ msec at 7°C, with recovery when the temperature returned to 33°C. The $T_{2b} = 22$ msec value showed no significant change with temperature. Both of these findings are summarized in Table I.

After incubation of weanling rat lenses in 5% acrylamide, $T_{2a}$ (at 33°C) was significantly lower (≈65 msec) than the $T_{2a}$ value of ≈113 msec at 33°C obtained on untreated lenses (incubated in media without acrylamide) or fresh lenses ($T_{2a} = 110$ msec). By contrast with the untreated lens, the temperature effect in this case was smaller (about 16% vs. 30% in the untreated lens). $T_{2b}$ showed no significant temperature change between 33°C and 7°C even though this value was also slightly depressed in the acrylamide-treated case. These results are also summarized in Table I.

**$^3$H studies.** Natural-abundance $^3$H signals
due to D₂O (46 MHz) were detectable (in a Bruker Model CCXP 300 superconducting NMR spectrometer) at room temperature (298° K) in less than 2000 scans (Fig. 2A), and broad ²H resonances could be obtained with time (62,179 scans) as shown in Fig. 2B. The spectra obtained at room temperature demonstrate a single resonance with a linewidth at half height of 68.4 ± 1.0 Hz (Fig. 2A), which represents mobile water, and the broad ²H resonances (Fig. 2B) can be assigned to relatively immobile deuterons.
Fig. 3A. $^2$H (46 MHz) NMR spectrum of a 3-week-old human lens enriched with D$_2$O (un-treated) at 297° K.

When the lenses were deuterated (by incubation in fully deuterated media) the single narrow resonance (linewidth = 68.4 Hz at 298° K) is obtained rapidly (50 scans or less). Enhancement of the broad component by $^2$H exchange was not possible because of slow exchange. With the lenses in place in the sample chamber, the temperature was decreased (278° to 283° K, depending on the species examined), and there was a significant increase in linewidth of the total signal to 82.8 Hz or greater. The significant increase in linewidth observed at the lower temperatures could be reversed (by warming...
to 298° K) to a signal closely approximating the initial 2H resonance seen in such lenses at the start of the experiment. By contrast, rabbit lenses incubated in media containing 5% acrylamide (for 2 hr or more) did not show this temperature-related increase in linewidth. The acrylamide-treated lens has a linewidth at half height of 54.7 ± 1.3 Hz at 298° K, which increases only slightly to 57.9 ± 0.6 Hz at 283° or 278° K.

Similar experiments on one set of 3-week-old human lenses incubated for 2 hr in 5% deuterated media again demonstrated an acrylamide effect correlating well with the rabbit lens data. That is, there was little, if any, change in the 3H signal linewidth in the acrylamide-treated lenses as a function of temperature, whereas the untreated lens showed an increase in linewidth from 44.0 Hz (297° K) to 61.1 Hz (275° K) (Figs. 3A and 3B) with return to normal values upon warming.

31P studies. Natural-abundance 31P studies were performed on normal lenses derived from 3-week-old rabbits. Fig. 4, A, which represents 1000 scans (5 Hz line broadening) performed on four fresh lenses at 297° K, demonstrates spectra that can be assigned to ATP, ATP + ADP, inorganic phosphate, and sugar phosphate moieties, corresponding to the results of Glonek et al.18 and Weidman et al.19 The inorganic phosphate linewidth (provided build-up of inorganic phosphate to significant levels had occurred) at 297° K measured 119.6 Hz and increased to 275.9 Hz when the temperature was lowered to 278° K (Figs. 4, B and C, respectively). These studies were repeated on a different set of lenses 24 hr after sacrifice and again demonstrated a temperature-related effect with respect to the linewidth of the inorganic phosphate peak (96 Hz at 296° K and 168 Hz at 278° K), with return to normal levels upon warming. The marked decrease in ATP and increase in inorganic phosphate in the incubation experiments (Fig. 4, B) is not unusual, since the initial spectra were obtained on much fresher lenses. In contrast with the spectra by Weidman et al.,19 our 31P spectra on fresh lenses taken within 30 min after sacrifice show very low levels of inorganic

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Fig. 4. A, $^{31}$P (121.4 MHz) spectrum of four fresh rabbit lenses shows ATP$\delta$ (18.45 ppm), ATP$\delta$ + ADP$\delta$ (9.71 ppm), ATP$\gamma$ + ADP$\gamma$ (4.76 ppm), inorganic phosphate (P$_1$) (-2.68 ppm), and sugar phosphates (SP) (-4.50 ppm); 1000 scans, repetition rate 3 sec, 297° K, 46.2 ppm displayed. Chemical shifts are measured with respect to 85% phosphoric acid. B, $^{31}$P (121.4 MHz) spectrum of four incubated rabbit lenses shows ATP$\delta$ (18.45 ppm), ATP$\delta$ + ADP$\delta$ (9.81 ppm), ADP$\delta$ (5.57 ppm), ATP$\gamma$ (4.89 ppm), P$_1$ (-2.61 ppm), SP (-4.23 ppm); 1000 scans, repetition rate 3 sec, 297° K, 46.2 ppm displayed. C, Same sample as shown in B obtained at 278° K.
phosphate and relatively high ATP levels (Fig. 4, A). These discrepancies may be due to the different incubation media employed by Weidman et al. and to the increased sensitivity of our spectrometer. When the experiment was repeated on lenses after the 2 hr incubation, inorganic phosphate had accumulated and there was a significant increase in inorganic phosphate, a corresponding decrease in ATP and a slight increase in ADP (Fig. 4, B).

Discussion

In the earliest reported relaxation study of lens water, Neville et al. recognized the multieponential character of the relaxation curve but reported only a single "relaxation time," $T_2^*$, as the time for the signal to decay to $1/e$ (37%) of its initial value. Subsequently, by graphic methods, Racz et al. were able to obtain two relaxation times for lenses from three different species. In the present study, we have also obtained two relaxation rates for water inside the lens, $T_{2a}$ and $T_{2b}$, but by detailed fitting of the relaxation curve over its entire course. These two parameters and their changes are the basis for measuring the response of the lens system to external perturbations, such as temperature and acrylamide. If it can be further assumed that the exchange rate between the water fractions is slow, $T_{2a} \approx T_{2b}$, would also be the true relaxation rates of their corresponding fractions. The values obtained by Racz et al. (62 and 22 msec on human lenses) are in reasonable agreement with our data. The present results show two $T_2$ values ($\approx 110$ and 22 msec) in the very young rat lens. It is entirely possible that the 110 msec value changes with age and species.

The results shown in Table I demonstrate that incubation with acrylamide affected both the ambient temperature $T_{2a}$ value and its temperature response. The possibility that the acrylamide-induced change in the $T_{2a}$ value is simply due to viscosity effects has been investigated. Experiments done on pure water, a 0.5% aqueous solution of acrylamide, and a 1% acrylamide solution gave $T_2$ values of 2.1, 1.7, and 1.6 msec, respectively. When lenses are incubated in a 5% acrylamide solution, the maximum concentration of acrylamide within the lens does not exceed 1% according to our $^{13}$C data. Correcting the lens results for the small $T_2$ changes noted in 1% aqueous acrylamide vs. pure H$_2$O would only result in a decrease of the $T_{2a}$ value from 113 msec to approximately 108 msec. Thus small changes in viscosity would not account for the significant change in $T_{2a}$ in the acrylamide-treated lens. Furthermore, when we examined extracted lens proteins ($\alpha$-, $\beta$-, and $\gamma$-crystallin) incubated with 1% acrylamide, there was no difference in the $T_2$ values for the $\alpha$- and $\beta$-crystallin fractions with and without acrylamide. Only the $\gamma$-crystallin fraction showed a significant $T_2$ response when 1% acrylamide was added to this protein solution. This indicates that the differential acrylamide effect is real and should not be attributed to simple viscosity changes within the whole lens.

The transverse relaxation times of water in biologic systems are greatly reduced compared with their values in pure water. Current findings suggest that this change is the result of the interaction of water molecules with macromolecular entities. The variation of the value of $T_{2a}$ after acrylamide treatment suggests some change in the state of the lens protein that would affect the protein-water interactions. The acrylamide effect is consistent with the observation that acrylamide acts as a noncovalent crosslinking agent with one or more of the lens proteins.

Our $^2$H studies on the young rabbit and human lenses have demonstrated a significant increase in linewidth as the temperature is lowered and cold cataract formation is manifest with return to normal values upon warming. The $^{31}$P studies also showed a marked temperature-related effect on the inorganic phosphate linewidth, which increased from 119.6 Hz at room temperature to 275.9 Hz when the temperature was lowered to 278° K, with return to normal values upon warming (Fig. 4, B and C). Treating such lenses with acrylamide virtually abolished this effect. These data are consistent with the pulsed NMR magnetization studies on the rat...
lens. In both the rat and rabbit lenses the temperature effect could be significantly diminished by previously incubating these lenses in media containing 5% acrylamide. A temperature effect consistent with a phase separation of at least one relatively mobile lens-protein water component could be demonstrated in the untreated lenses utilizing two NMR approaches. An acrylamide effect can be demonstrated in the rat, rabbit, and human lenses by these studies. In these species, incubating the lenses in 5% acrylamide prevented the cold cataract, decreased the temperature-related change in the more mobile H2O phase in the rat lens (T2a value), and prevented the increase in 2H and 31P linewidth signals in the rabbit and human lenses when the temperature was lowered. These studies demonstrate that the foregoing NMR experiments can be utilized to study at least one physicochemical parameter in the genesis of cold opacification and could also prove valuable in studying the development of light scattering areas in other forms of experimental cataractogenesis.

REFERENCES