Organophosphates of the crystalline lens: a nuclear magnetic resonance spectroscopic study

Jack V. Greiner, Stephen J. Kopp, Donald R. Sanders, and Thomas Glonek

We quantitated the concentrations of the principal organophosphate metabolites present in the intact crystalline rabbit lens, measured the intralens pH, and evaluated dynamic changes during 24 hr incubations, using phosphorus-31 nuclear magnetic resonance (P-31 NMR) spectroscopy. Tissue perchloric acid extracts prepared from these same lenses were analyzed by this technique to verify metabolite identifications and to quantitate the concentrations of the minor lens metabolites. Values for lens organophosphate concentrations, including three groups of previously unidentified phosphorus-containing substances, were established for freshly excised lenses, 24 hr incubated lenses, and lenses incubated in glucose-deficient media. Lens metabolite levels were not adversely affected by incubation in a medium previously shown to maintain lens clarity and ion transport capabilities. Conversely, lens incubation in glucose-deficient media induced significant metabolic changes characterized by a time-dependent decline in ATP, corresponding increases in ADP, inorganic phosphate, and phosphorylated hexoses. Cataract formation was noted after incubation in this medium. These findings support the hypothesis that alterations in the organophosphate levels of the lens actually precede changes in the Na^+ and K^+ concentrations and therefore may be the "initiating factor" in formation of lens cataracts. (INVEST OPHTHALMOL VIS SCI 21:700-713, 1981.)

Key words: crystalline lens, organophosphates, phosphorus-31 nuclear magnetic resonance spectroscopy, tissue pH

The crystalline lens is not a tissue with high metabolic activity, such as muscle or liver, but it does require chemical energy in the form of adenosine triphosphate for active transport processes. Moreover, lens ATP is utilized in synthetic and degradative pathways that are required to maintain tissue growth, structure, and function. Despite this descriptive understanding of lens metabolic activity, little is known about the actual dynamic energetics of intact crystalline lens metabolism, particularly the overall metabolic events that precede, and therefore predispose, the lens to initiate cataract formation.

Technical limitations have previously precluded measurement of dynamic metabolic activity in intact tissues. Recent advances in phosphorus-31 nuclear magnetic resonance (P-31 NMR) technology, however, have provided the necessary bioinstrumentation to analyze dynamic metabolic events nondes-

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tructively and quantitatively as they occur in intact, functioning mammalian tissues.[1-6] P-31 NMR spectroscopy permits rapid, accurate quantitative and qualitative determinations of the principal organophosphates present in the intact tissue.[3, 6] Adenosine triphosphate, adenosine diphosphate, creatine phosphate, and the phosphorylated hexoses and trioses are among the phosphatic metabolites that can be accurately quantitated in the intact tissue. With in vitro tissue incubation techniques, quantitative time-dependent metabolic changes can be monitored continuously for correlation with functional disturbances during defined increments in time in the same tissue.[7, 8] We successfully studied the dynamic measurements of organophosphate metabolite levels in the intact mammalian crystalline lens during control and experimental incubations. We describe the significance of these findings and the potential use of this analytical technique in the study of cataractogenesis.

Methods

Surgery. Albino rabbits, weighing 2 to 3 kg each, were killed with sodium pentobarbital injections, and both eyes were enucleated immediately. The lenses were obtained by opening the eyes at the posterior pole, gently separating the vitreous humor to the side, separating the zonules with curved blunt scissors, and removing the lens with a Parafilm-coated wire lens loupe.

In vitro incubation. Lenses for incubation were freshly excised, isolated, weighed, and placed together in a tared 12 mm NMR tube containing a volume of physiologic Earle's buffer (Difco Laboratories, Detroit, Mich.) at 37° C, pH 7.4. This buffer [NaCl 6.800 gm/L (116.4 mmol); dextrose 1.000 (5.6); KCl 0.400 (5.4); $CaCl_2$, anhydrous 0.200 (1.8); $MgSO_4$ 0.200 (1.4); $NaH_2PO_4 \cdot H_2O$, 0.125 (0.9); $NaHCO_3$, 2.2200 (26.4)], with an osmolarity of 295 mOsm, has maintained lens clarity and ion transport capabilities for at least 24 hr of in vitro incubation.[9] All incubated lenses were equilibrated in Earle's buffer for 2 hr at 37° C prior to initiation of each experiment. At each 1 hr point during the experimental incubation periods, used buffer was aspirated from the base of the NMR tube, the lenses were washed three times with fresh buffer, and fresh buffer was added to a constant volume. This procedure was necessary to prevent glucose depletion of the buffer bathing the lenses in the NMR tube. For the temporal control phase, 10 lenses were incubated for 24 hr in physiologic Earle's buffer. Individual P-31 NMR profiles were acquired during consecutive 1 hr intervals for the duration of the entire incubation period. Glucose-deficient incubations were conducted with glucose-deficient Earle's buffer, according to the same analytical protocol. The lenses in this phase of the study were incubated for a 12 hr period. After the experimental incubations, the lenses were weighed and frozen in liquid nitrogen and prepared for perchloric acid (PCA) extraction.[10] Ten freshly excised lenses, which served as nonincubated controls, were also prepared for PCA extraction by freezing in liquid nitrogen immediately after excision and weighing.

Lens PCA extracts. The frozen lenses were pulverized to a fine powder with a stainless steel mortar and pestle chilled with liquid nitrogen. The mortar and pestle were maintained in a liquid nitrogen bath during the pulverization procedure. The tissue powder was added to a centrifuge tube containing 0.1 w/v 60% PCA prefrozen in liquid nitrogen. The powder was stirred continuously while it was warmed to a paste consistency. The sample was then immediately centrifuged at 43,500 × g for 15 min at −5° C. This procedure made possible complete coating of the tissue particles with PCA and subsequent extraction of the powder at a temperature of about −20° C. After centrifugation, the supernatant was immediately transferred to an equivalent volume of 10N KOH, and the pH was adjusted to 10. The sample was centrifuged to remove precipitated $KClO_4$. The final supernatant was passed through a potassium Chelex-100 column to remove alkaline earth and transition metal cations. The column effluent was lyophilized and redissolved in 1.0 ml of 20% D_2O for NMR analysis. The preparation of the extracts and the P-31 NMR calibrations and analyses were performed according to accepted and well-validated procedures previously described for P-31 NMR intact tissue and tissue extract analysis.[3, 6] Subsequent to the sample NMR analysis, total phosphate determinations were performed on the tissue extracts and referenced to the extracted protein concentrations to enable expression of the metabolite levels as concentrations in micromole per gram of lens protein.

P-31 NMR spectroscopy. The NMR spectrometer used in this investigation was a Nicolet NT-200 system equipped with deuterium stabilization, variable temperature, and Fourier-transform cap-

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abilities operating at 80.987663 MHz for P-31. This system is interfaced to a wide-bore Oxford superconducting magnet (4.7 T). Intact lenses were analyzed under nonspinning, proton-coupled conditions. Extracts prepared from these lenses were analyzed with and without proton decoupling, while the sample was being spun to enhance signal resolution. Twelve-millimeter sample tubes were used for the analysis of the intact lenses. In this manner usable data were obtained in as little as 5 min, although 1 hr was needed to gather the bulk of the P-31 NMR data presented here. For the analysis of the PCA extracts, the 1.0 ml samples were placed in NMR microcell assemblies so that signal intensities could be enhanced as much as possible for the quantitation of minor lens metabolites. The NMR analyses were conducted at 37° C.

Chemical shift data are reported relative to the usual standard of 85% inorganic orthophosphate (Pi)[11]; however, the primary internal standard was the natural glycerol 3-phosphorylcholine (GPC) resonance of the lens. This compound has a relatively constant chemical shift for a phosphate (−0.13 ppm), which is not influenced by variable physiologic pH, ionic strength, or countercation conditions.[12]

A variety of spectrometer conditions were used in this study; however, those described below, which were used in the microcell analysis of lens PCA extracts, are typical: pulse sequence 1 pulse; pulse width 9 μsec (45 degree flip angle); acquisition delay 200 μsec; cycling delay 200 μsec; number of scans 144,100; number of data points per free-induction-decay 16,384; acquisition time 1.64 sec; sweep width ± 2500 Hz. In addition, a computer-generated filter time constant introducing 0.6 Hz line broadening was applied. Data reductions, including peak area and chemical shift measurements, were effected with the spectrometer's computer. The chemical shifts reported here follow the International Union for Pure and Applied Chemistry convention and are reported in the field-independent units of parts per million (ppm); coupling constants (J values) are reported in units of hertz (Hz).

Chemical identification of intact tissue resonance peaks. P-31 NMR spectroscopic analysis performed on the intact rabbit lens yields a spectrum in which each peak corresponds to a single phosphorus-containing functional group having a discrete resonance shift position. The precise resonance shift position of each peak is a characteristic physicochemical marker for individual organophosphate metabolites present in the tissue. The shift position is determined by the molecular and macromolecular environment of the phosphorus atom contained in each different metabolite; however, the macromolecular environment to which the phosphorus atom of various metabolites is exposed cannot be assumed to be uniform throughout the cell. Ionic strength, pH, and cationic and protein concentration differences in cellular microcompartments of the lens may complicate the chemical identification of a resonance peak recorded from intact tissue. For this reason, the chemical identity of each resonance peak recorded from the intact lens is based on several physical and chemical criteria: (1) magnetic resonance analysis of intact lenses and subsequent analysis of PCA extracts prepared from these same lenses to demonstrate a corresponding pattern of resonance peaks; (2) analysis of the tissue extract under proton-coupled conditions to define peak multiplicities of proton-decoupled resonance peaks and to determine the corresponding peak J values (spacing intervals between multiplets of the same decoupled peak, in Hz); (3) variable pH titration of the tissue extract using P-31 NMR analysis to define the pH-dependent resonance shift curve for each peak (the shift curve measures the mole ratio of an acidic anion and its corresponding conjugate base involved in protonation-deprotonation reactions); (4) magnetic resonance analysis of the extract under proton-coupled and proton-decoupled conditions at various sample pH values after addition of known phosphate compounds to the sample to demonstrate that a specific resonance peak enhances, completely superimposes, and migrates in the pH spectrum as the added (known) compound; (5) P-31 NMR pH titration analysis of aqueous mixtures of known organophosphate compounds under identical extract conditions (countercation, ionic strength, and temperature) to demonstrate that the pH titration-resonance-shift-position curve derived from peaks contained in the extract superimpose with corresponding known compounds present in the aqueous mixtures; and (6) confirmation of peak assignments based on the known theoretical molecular chemistry that defines the magnetic resonance patterns of specific phosphate molecules.

By a comparison of the pH titration curves derived from standard phosphates with those obtained from the lens PCA extracts, the common energy metabolites involved in intermediate metabolism were identified with a high degree of certainty. These peak assignments were further reinforced by the demonstration that, under expanded

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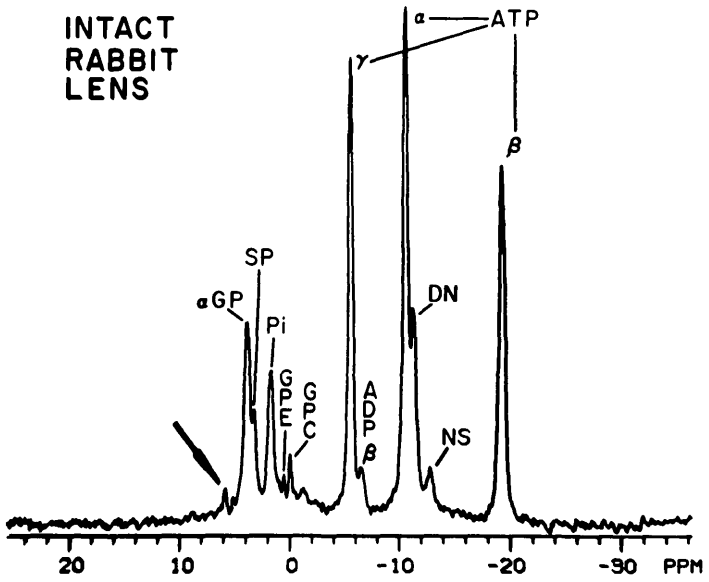

Fig. 1. P-31 NMR spectrum of three rabbit lenses accumulated over a period of 6 hr to reveal the fine structure of the spectrum. The lenses were maintained during the experiment by Earle's buffer (with glucose) at pH 7.4 at 37°. Arrow indicates a new phosphorus metabolite at 6 ppm; *SP*, other sugar phosphates; ADP and ATP are shown with respective γ-, α-, and β-resonance signals; *DN*, dinucleotides; *NS*, nucleoside diphosphosugars and related species. The chemical shift scale follows the shift convention of the International Union for Pure and Applied Chemistry and is given relative to the resonance position of 85% phosphoric acid.

spectral conditions, known phosphates superimposed with specific extract resonance peaks throughout the pH titration range. In regions where a number of closely spaced resonances were found, however (e.g., the hexose region), the peak assignments were reinforced by selectively shifting the resonance of interest with respect to the others by judiciously adjusting ionic strength[13] or by changing the countercation[14] in solution and subsequently adding known compounds[3] to the sample.

Mathematical analysis of dynamic lens changes in glucose-depleted medium. The time-course data obtained in this study were reduced by a least-squares regression analysis using a polynomial expression of the form $y = Ax^3 + Bx^2 + Cx + D$. The regression analysis was carried out until the chi-square was reduced to 0.1%. The best fit values of the constants are presented in tabular form along with their standard deviations. The third-degree expression was necessary to adequately approximate the real time-course data obtained from the intact lens tissue. The location of maxima and minima in the time course of changing rate expressions was obtained by differentiation of the locus determined from the regression analysis.

Results

The P-31 NMR spectrum obtained over a period of 6 hr from intact rabbit lenses incubated in control Earle's buffer is shown in Fig. 1. The 6 hr measuring time was necessary to easily observe the minor resonances above the background noise. Metabolite concentrations derived from lens spectra are summarized in Tables I and II. The signal indicated by the arrow at 5.7 ppm arose from a phosphorus-containing molecule of unknown origin and chemical nature. The large signal immediately up-field arose from α-glycerophosphate (αGP). This compound is much more abundant in lens than in any other intact tissue thus far examined by P-31 NMR.[15] The sugar phosphate band *(SP)* was composed of two closely spaced groups of resonance signals arising from other triose

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Table I. Lens P-31 NMR profiles

Phosphatic compound	*Chemical shift (ppm[A])*		*Amount (as % of the total P in the profile)*			
				PCA extract		
				Control		
	Intact lens	*PCA extract*	*Intact lens*	*Freshly excised*	*24 hr incubation*	*Glucose-depleted*
Unknown[B]	[C]	18.02-17.81[D]	[C]	0.5	0.5	0.9
Unknown[B]	[C]	10.80, 10.72, 10.57[E]	[C]	0.3	0.5	0.3
Unknown[B]	5.70	6.00[F]	1.2	1.1	1.1	1.9
Trioses	3.67	4.30[G]	8.7	7.3	8.8	13.0
Hexoses	3.19	3.78[H]	7.2	6.1	5.1	13.9
PE	[C]	3.31	[C]	0.3	0.2	0.5
PC	[I]	3.33[J]	[I]	1.5	1.5	2.4
Pi	1.63	2.75	11.1	8.1	3.4	30.4
GPE	0.40	0.92	1.3	0.9	1.2	0.5
GPC	−0.13	−0.13	2.1	2.0	1.9	0.9
PCr	[C]	−3.10	[C]	0.05	0.1	—
Unknown[B]	[C]	−5.36, −5.50, −5.56[E]	[C]	0.05	0.1	—
ATP	α, −10.65; β, −19.24; γ, −5.62	α, −10.92; β, −21.45; γ, −5.80[K]	46.8	52.2	50.3	6.1
ADP	α, −10.65; β, −6.66	α, −10.61; β, −6.11[L]	5.8	5.4	4.7	15.0
Dinucleotides	−11.34	−11.37[M]	13.6	12.5	18.8	11.7
Nucleoside diphosphosugars	−12.89	−12.90[N]	2.2	1.7	1.8	2.0
Unknown[B]	[C]	−28.03	[C]	—	—	0.5

[A] Field-independent nuclear magnetic resonance units relative to the shift position of the 85% inorganic orthophosphoric acid reference phosphate at 25°.
[B] Compound, as of this writing, is not identified with any known phosphorus-containing biomolecule.
[C] Minor component not detectable in the intact tissue.
[D] Resonance band, comprising a group of separate resonance signals, is present.
[E] Three separate resonance signals are detected.
[F] J(P-H[phosphorus-hydrogen]) = 10.69 Hz.
[G] Complex resonance band, the principal resonance signals of which come from the αGP triplet; J(POCH) = 6.68 Hz.
[H] Hexose and pentose phosphates, principally the resonance triplet of inosine monophosphate; J(POCH) = 3.81 Hz.
[I] Cannot be determined in the intact tissue as a separate resonance band but is combined with the Pi signal.
[J] J(POCH) for the triplet = 5.63 Hz.
[K] J(POP, αβ) = 19.44 Hz; J(POP), βγ) = 19.44 Hz.
[L] J(POP, αβ) = 22.44 Hz.
[M] Principal resonance signals of this band arise from the P,P′-diesterified pyrophosphate residues of NAD and NADH.
[N] Complex resonance band composed of three sets of overlapping, ^{31}P-^{31}P, ab NMR multiplets.

phosphate sugars in one group and hexose and pentose phosphate sugars in the other. The next up-field resonances were the signals from Pi and two small signals arising from the phosphodiesters, glycerol 3-phosphorylethanolamine (GPE) and GPC. These were followed by the ionized γ chain-terminal phosphate of ATP and the β chain-terminal phosphate of ADP; the alpha-esterified phosphates of ATP and ADP; the P,P′-diesterified pyrophosphate residues of the dinucleotides, principally nicotinamide adenine dinucleotide (NAD) *(DN);* a low-amplitude broad resonance of nucleoside diphosphosugars *(NS);* and finally the β chain-middle phosphorus of ATP. When the control Earle's buffer that contains glucose was changed at regular 1 hr intervals, the intact lens NMR profile did not change adversely at 37° C for at least 26 hr. The chemical shift position of each peak as well as their respective amplitudes remained identical within the precision of the NMR measurement, which in this example was ±0.015 ppm.

The stability of these intact lens preparations permitted quantitative data of the major lens metabolites to be obtained from a single lens. Four-hour signal averaging was required for single lens analysis.

The peak assignments given for the intact lens spectrum corresponded to assignments reported for a large variety of mammalian

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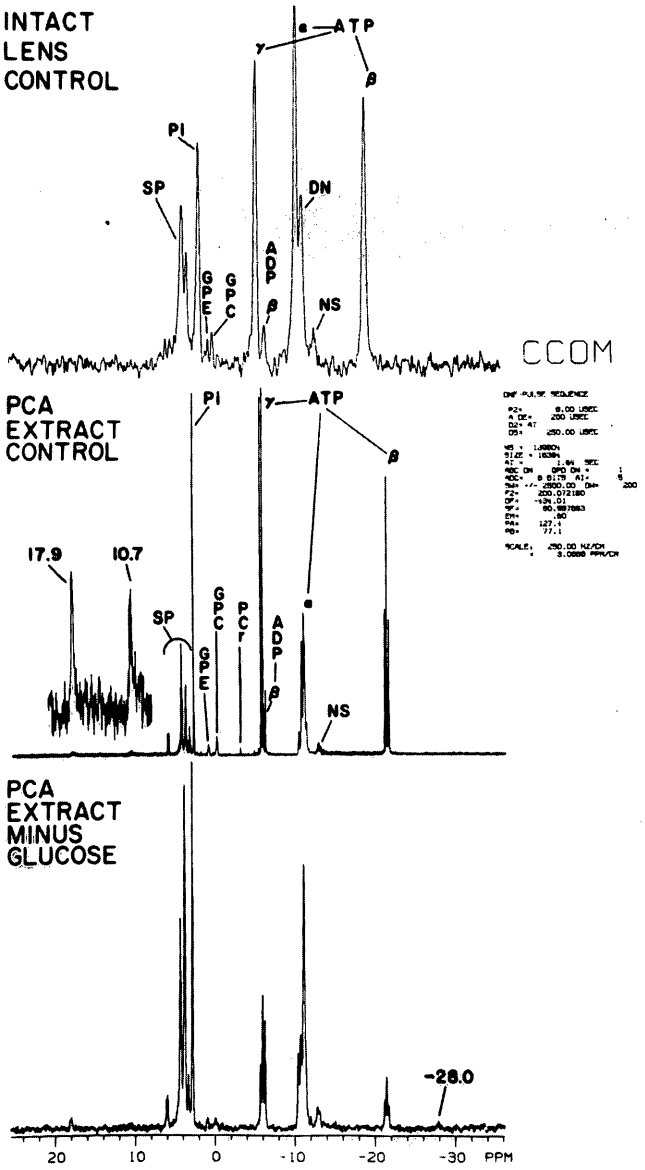

Fig. 2. P-31 NMR spectra from intact rabbit lens and its corresponding PCA extract and from an extract of lens incubated in a glucose-deficient medium. Some of the signals are multiplets. *DN*, Dinucleotides; *SP*, sugar phosphate resonance group; *NS*, nucleoside diphosphosugars; *PCr*, phosphocreatine. Signals at 17.9, 10.7, and −28.0 ppm represent phosphatic metabolites of unknown chemical nature.

and other living tissues studied by P-31 NMR.[2, 3, 10] The resonance from inorganic orthophosphate was readily identified as a result of its accumulation to high concentrations after death of the tissue. The other resonance signals were characterized by comparing the intact tissue spectra to similar spectra obtained from classic PCA extracts of the *same* tissue. This is illustrated in Fig. 2, in which an intact rabbit lens spectrum accumulated over a period of 1 hr is compared to corresponding PCA extract spectra.

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Table II. Concentrations of acid-extractable phosphates in rabbit lens (μmol/100 gm of fresh tissue)

Reference	*Total phosphorus*	*ATP*	*ADP*	*Pi*	*PCr*	*αGP*	*Hexoses*	*Trioses*	*NS*
Present paper	1851	323	50	150	1	127	113	8	31
van Heyningen and Pirie[34]	1660	494[C]		355	0	146	30[D]		234
LePage[27]	1534	663[E]		171	86	377	402[D]		
Hauschildt et al.[35]	1813	506[C]		300					
Frohman and Kinsey[28]		700[C]		1290	195		1955		
Pirie et al.[36]		253[C]		253	5		32[D]		
Klethi and Mandel[37]		245[F]	30						26
Nordmann and Mandel[38]				171					
Palm[39]				278					
van Heyningen[40]						940	12	5	
Pirie[41]						140			

[A]Summed concentrations of all unidentified species.
[B]AMP resonance not identified as such in the phosphorus resonance spectrum.
[C]Pooled adenyl nucleotide fraction.
[D]Pooled phosphorylated sugar fraction.
[E]Pooled adenyl diphosphate and triphosphate fractions.
[F]Combined nucleoside triphosphates.

The signals from the extracts, which because of the favorable physical properties of dilute aqueous solutions were highly resolved, were identified through their chemical shift position and other spectral properties as detailed in Methods. The favorable signal-to-noise ratio of the extract spectrum yielded usable quantitative data on several minor phosphorus resonances as well as on the prominent resonances. The extract spectrum showed two additional signal groupings at 17.9 and 10.7 ppm arising from compounds that have not been identified as to the nature of their source phosphatic compound. The chemical shift position of these two signals suggest that the source materials were not phosphates or phosphoroamidates common to biologic systems, since such compounds do not generate resonance signals at the location observed for the unknowns. The 17.9 ppm resonance was in a characteristic position of phosphonates, i.e., compounds containing the C — P bond.[16] The 10.7 ppm resonance could not be assigned with any degree of certainty because it lay in a region of the spectrum where only a few model compounds are known, including anhydrides of phosphonic acids and their derivatives.[17] Between the 10.7 ppm resonance and the sugar phosphate grouping was a doublet corresponding to the unidentified resonance observed in Fig. 1. The down-field resonance position of this compound is unique,[18] and it is unlikely that the substance is a rare sugar phosphate. The substances giving rise to the 17.9 and 10.7 ppm signals were only sparingly soluble in the PCA extraction solvent, suggesting that the source molecules may be of relatively high molecular weight similar to the "presumptive phosphopeptides" isolated from bovine lens by Bettelheim[19] and Bettelheim and Wang.[20] These low field signals, however, cannot arise from phosphoserine or phosphothreonine residues in peptides or proteins, since their shifts were 3.7[21, 22] and 4.6[21] ppm, respectively, and varied only a few tenths of a ppm with the nature of the protein.

The sugar phosphate resonance band in almost all tissues examined exhibited three characteristic groups of signals: a band corresponding to triose phosphates at 4.4 ppm, a band corresponding to hexose or pentose phosphates at 3.8 ppm, and a band corresponding to phosphocholine (PC) and phosphoethanolamine (PE) at 3.3 ppm. The principal triose phosphate in the lens is αGP, which exhibits a characteristic triplet due to the coupling to the neighboring CH_2 group. The hexose and pentose resonance band shows a complex system of overlapping resonance multiplets that, depending on con-

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DN	*AMP*	*GPC*	*GPE*	*PC*	*PE*	*Unknown*[A]
115	—[B]	38	17	26	7	34
		77	29	55	13	
	115	0	0	0	0	
31	22					

ditions, may show prominent signals from molecules such as fructose-1, 6-diphosphate, inosine monophosphate, or adenosine monophosphate.

Although the resonance from phosphocreatine was of very low intensity in the lens, it could be identified at −3.1 ppm. Ionized polyphosphate end group resonances from other nucleoside triphosphates, principally guanosine triphosphate, could also be identified at −6 ppm. The ionized end groups of the nucleoside triphosphates were split into doublets though their interaction with the middle group phosphate at −21.5 ppm; this phenomenon is well resolved in the middle spectrum of Fig. 2. Like the end group of ATP, the end group of ADP was also a doublet that, under the scan conditions employed, lay close to the ATP end group doublet, so that the high-field arm of the ATP doublet and the low-field arm of the ADP doublet nearly overlapped.

The esterified phosphate end group region at −11 ppm was complicated by the presence of numerous signals arising from the dinucleotide cofactors of intermediate metabolism. In this region lay the resonances of NAD, nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide, and coenzyme A, as well as resonances from other monoesterified pyrophosphate groups. Up-field of this group at −13 ppm were a group of multiplets corresponding almost exclusively to nucleoside diphosphosugar resonances.[23]

The bottom spectrum of Fig. 2 shows a

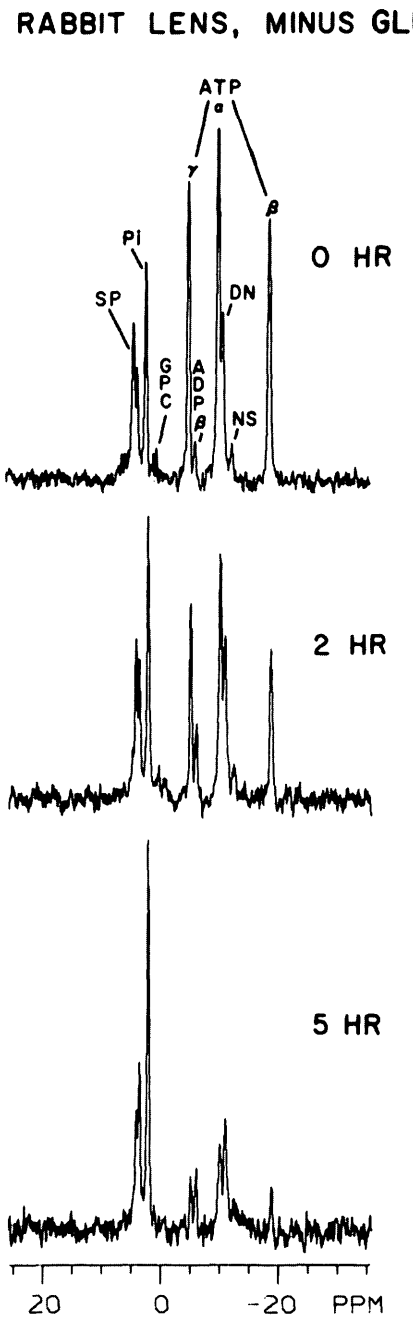

Fig. 3. P-31 NMR spectra from three time points during an incubation of lens in a glucose-deficient medium.

PCA extract of lenses deprived of glucose for 5 hr at 37° C. Signals from the same phosphorus groups could be detected, although their relative proportions were markedly altered. In addition, a new resonance appeared at

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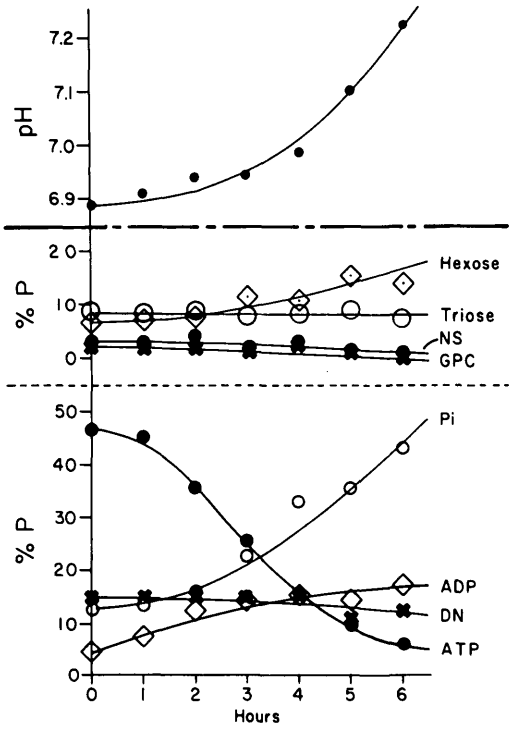

Fig. 4. Time course for the changes in the lens phosphate profile when the lens is incubated in a glucose-deficient medium. The pH curve is the intralens pH determined from the resonance shift of the Pi signal.

−28.0 ppm. This resonance has not been identified, although its chemical shift suggests that it may be a reduced phosphorus grouping, such as a phosphine,[24] and not a phosphate.

Intact lens spectra taken at various times while the lenses were being incubated in a glucose-free buffer are shown in Fig. 3. Under these conditions the phosphate profile showed the loss of ATP with the concomitant rise of Pi, so that after 5 hr the major resonance signal was from Pi and the resonance signals from ATP had virtually disappeared into the spectral noise. A time course plot corresponding to such spectra is shown in Fig. 4. When glucose was removed from the buffering medium, the concentration of ATP fell to values corresponding to less than 10% of the total phosphorus while the concentration of Pi increased correspondingly. ADP also increased, approximately doubling its value at the end of 6 hr, and the hexose-phosphate band also increased after about 4 hr. Examination of the PCA extract shows that the principal phosphate contribution to the hexose band increase was adenosine monophosphate. The levels of the triose sugars, the nucleoside diphosphosugars, the phosphodiesters such as GPC, and the unknown phosphorus-containing molecules were unchanged during this time course.

Lens pH. Phosphate chemical groups bearing a dissociable proton underwent chemical shift changes as a function of pH in the range corresponding to the pKa of the dissociable proton. This is illustrated in Fig. 5, which shows the P-31 spectroscopic pH titration curves at 37° of several phosphates of biologic origin whose resonance signals have been detected in lens extracts. This property renders the chemical shift behavior of molecules such as Pi or αGP useful in determining the pH value of media in which the ion is a solute. Note that in Fig. 5 the phosphorus resonance signals may be up-field or down-field of each other, depending on the solvent pH; therefore the control of pH is absolutely essential for the proper interpretation of phosphate chemical shifts.

We and a number of other investigative groups[2–6] have used the chemical shift of Pi to measure intracellular pH values in a number of intact cellular systems. Applying these principles to the lens and using the shift of both Pi and αGP, we have determined that the average pH of the interior of the lens is 6.9. This value is surprisingly low for mammalian tissues; it is 0.5 pH unit lower than the average value of 7.4, which has been reported as the probable pH of the lens.[25, 26]

When glucose was removed from the buffering medium (pH 7.4), the intralenticular pH, as measured by the chemical shift of both phosphates, began to drift toward higher values as the ATP content of the lens was depleted (Fig. 4). At the end of 6 hr the pH had increased more than 0.3 units to a pH of 7.2. This behavior is atypical for mammalian cells, which usually become acidic on loss of ATP, and it is direct evidence in the lens for an active hydronium ion pump.

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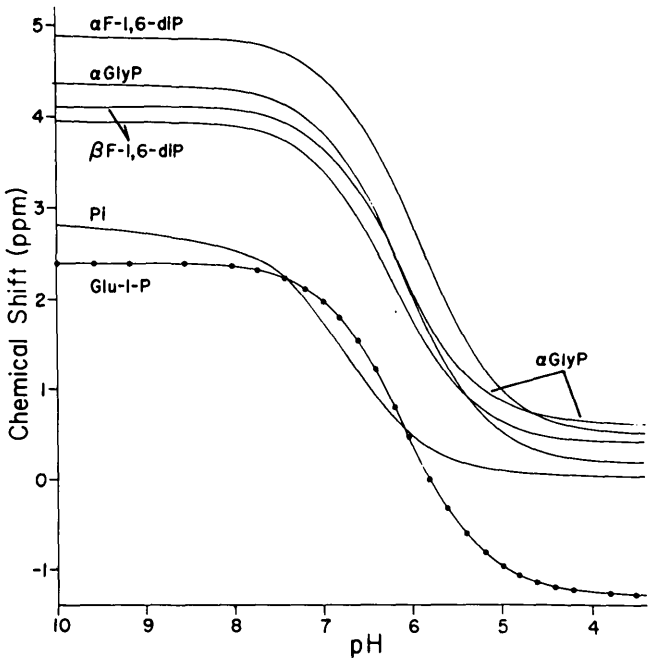

Fig. 5. Control rabbit lens PCA extract pH titration data. P-31 NMR–pH titration curves in water (5% D_2O) at 37° C with potassium as the countercation, and a total salt concentration approximately 100 mM. *F-1,6-diP*, Fructose-1,6-diphosphate; *Glu-1-P*, glucose-1-phosphate.

Table III. Coefficients for the expression, $y = Ax^3 + Bx^2 + Cx + D$, obtained in the linear regression analysis of lens time-course data*

Component of time course	*Value of coefficient*			
	A	*B*	*C*	*D*
Adenosine triphosphate	−0.4300 ± 0.0059	3.7613 ± 0.0321	−0.3951 ± 0.0607	−0.2070 ± 0.0795
Adenosine diphosphate	0.1194 ± 0.0062	−1.3226 ± 0.0337	5.8318 ± 0.1689	−0.2426 ± 0.1090
Pi	−0.2029 ± 0.0071	2.2979 ± 0.0391	−1.3481 ± 0.1955	−0.5142 ± 0.0951
Hexose phosphate	−0.1078 ± 0.0051	0.9773 ± 0.0278	−0.8419 ± 0.0392	−0.1416 ± 0.0520
pH	0.0025 ± 0.0001	−0.0095 ± 0.0005	0.0204 ± 0.0025	0.0069 ± 0.0092

*The ordinate is the percent of total phosphorus detected and the abscissa is time in hours.

Other intact tissues such as chicken pectoralis muscle[3, 15] frequently show more than one intracellular pool of Pi; thus far, we have not observed this property in the lens.

The data of Fig. 4 were subjected to a linear-regression analysis, so that the rate of change of any component could be determined at any time. It was found that a third-degree equation of the form $y = Ax^3 + Bx^2 + Cx + D$ was necessary to fit the time course data adequately within the 6 hr period examined. Only ATP, ADP, Pi, the hexose phosphates, and the pH curves were analyzed, since the other curves varied little over this period of time. The coefficients of the above equation, which were determined from the data of Fig. 4, are given in Table III along with their standard deviations. The fit to the above polynomial was reasonably precise, as indicated from the standard deviations in the value of the coefficients and the fit of the regression-analysis–derived curve drawn among the data points in Fig. 4.

The ATP curve exhibited a sigmoid shape,

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with a maximum rate of consumption somewhere near 3 hr. The derivative of the fitted ATP polynomial was used to calculate the precise position of the maximum rate of ATP utilization. This was found to occur at 2.9 ± 0.8 hr. Further analysis indicated that ADP accumulation reached a minimum value at 3.7 ± 0.3 hr, Pi accumulation reached a maximum at 3.8 ± 0.2 hr, and hexose accumulation obtained a maximum at 3.0 ± 0.2 hr. The minimum change in the intralens pH occurred at the beginning of the time course.

Discussion

The mammalian lens, like numerous other intact tissues, produces a highly resolved P-31 NMR spectrum that exhibits resonance signals from the common low-molecular-weight metabolites of intermediate metabolism. Compared to profiles of a variety of mammalian tissues,[3, 4] that of the intact rabbit lens exhibits several distinctive features. The tissue is rich in glycolytic sugar phosphates and in the dinucleoside cofactors required for the metabolism of these phosphates. The tissue also shows a surprising amount of low-molecular-weight phosphodiester and nucleoside diphosphosugars.

Readily detected were the resonances from ATP, αGP, Pi, various phosphorylated sugars, and several nucleotides. In addition, these metabolites could be quantitated in reasonably precise agreement with chemical analyses performed through classic biochemical procedures (Table II). P-31 NMR, however, made possible the simultaneous determination of phosphorus-containing compounds on a single intact lens without the extensive precautions that are necessary to avoid breakdown of delicate high energy phosphates in destructive procedures such as homogenization. The simplicity, accuracy, and comprehensiveness of this method are extremely attractive.

The intact lens phosphorus spectrum exhibits the highest resolution that has yet been obtained from an intact tissue. In addition to ATP and prominent sugar phosphates, the phosphodiesters GPE and GPC are readily observed above the background noise and quantitated; similarly, ADP, the dinucleotide signal, and the nucleoside diphosphosugar resonance can be precisely determined. With such detailed spectroscopic data, we believe that we have, for the first time, detected a resonance signal from an unknown phosphatic molecule in the intact tissue, thus precluding the possibility that the new substance, also observed in the PCA extract, was generated by the PCA preparative procedure.

The ATP/inorganic phosphate ratio at 2.15 is similar to that exhibited by other tissues, whereas the ATP/ADP ratio at 6.44 is quite high. The lens does not contain appreciable quantities of phosphocreatine (chemical shift −3.1 ppm), although this may be a species variation. This finding is in contrast to those of other reports, in which the occurrence of phosphocreatine in appreciable quantities in rabbit lens has been reported.[27, 28] From the phosphorus profile, the phosphorylation potential[29] of the lens, ATP/ADP · Pi, is calculated to be 0.043 per mole.

In the intact lens, the chemical shift of the ADP β-group is 0.1 ppm higher than observed in other tissues, particularly muscle[3] and synthetic systems[3, 30] involving ATP, ADP, and PCA extracts. This chemical shift difference does not appear to be a function of pH involving separate pools for ADP and ATP, since the relative shift difference remains constant throughout the time course of the death of the lens when the pH, monitored by Pi and αGP is increasing. This chemical shift difference is unusual and undoubtedly reflects a specific property involving the microenvironment of the nucleotides in the lens. The chemical shift of ATP at −5.62 ppm is nearly identical to that observed in various muscles (−5.60 ppm),[3] which suggests that it is the microenvironment of ADP that has specific alterations in lens relative to that found in a number of other tissues.

The relative γ-group and β-group ATP chemical shifts in the intact tissue support the suggestion that ATP exists as its monomagnesium complex. This finding parallels that in intact muscle[3] and liver mitochondria.[31]

The pH of the intact lens measured spec-

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troscopically is 6.9, which is a full 0.5 pH unit lower than the commonly accepted value for lens pH.[25, 26] This marked discrepancy undoubtedly reflects the fact that previous determinations involved disruption of cellular integrity and not the inability of the NMR method to determine pH in lens. The inorganic phosphate signal in phosphorus spectra from intact tissues has been used by investigators as an intracellular indicator of intact tissue pH[32, 33]; however, our determination involved the shift position of two intracellular metabolites, Pi and αGP. The pH value given by each resonance shift was the same to within 0.02 pH unit. This agreement supports the hypothesis that the resonance shift of Pi reflects the intracellular pH and is not the result of nonspecific solvent effects. If solvent effects were operant, it would be necessary that they influence the alcohol ester to the same degree and in the same sense that they influence the inorganic anion; such a chemical anomaly is not likely.

The PCA extract spectra exhibit relatively intense signals from several phosphates of intermediate metabolism that have been identified through their characteristic spectroscopic properties and their phosphate titration data; these are αGP, PE and PC, GPE and GPC, phosphocreatine, ADP and ATP, and NAD. In addition, three heretofore unidentified groups of phosphate molecules have been detected. The resonance at 6 ppm is unique to lens tissue; those at 10 and 18 ppm have also been detected in other tissues, in particular, liver and kidney. The 18 ppm resonance is characteristic for phosphonic acids. The 10 ppm signal is unidentified except that it cannot arise from an orthophosphate or any of its ester or anhydride derivatives. None of these signals corresponds to known resonances from a variety of phosphorylated proteins.

Metabolic decay processes. When the glucose buffer is replaced with a glucose-deficient buffer, the tissue decays metabolically before evidence of gross physical changes, which involve formation of a cataract-like opacity. The metabolic decay involves the loss of ATP, with the concomitant increase of Pi, a sugar phosphate, and ADP. Other compounds are not mobilized to sustain ATP levels as they are, for example, in the muscle where energy source–deprivation results in the catabolism of glycogen with the subsequent production of triose phosphates detectable in the P-31 NMR spectrum.[3] The catastrophic loss of ATP begins about 1 hr after the removal of glucose-containing media, with the lag-time probably reflecting the size of the intralens glucose pool.[16, 17] The optimum rate of ATP consumption occurs at 2.9 hr, which coincides with the optimum rate of hexose phosphate accumulation. The optimum rates of ADP and Pi accumulation lag behind this value by approximately 0.8 hr.

The chemical destruction of ATP can be interpreted as a straightforward hydrolysis of ATP to ADP and Pi, with the adenylate kinase activity, $2ADP \rightleftharpoons ATP + AMP$, operating to generate ATP from ADP with the resultant production of AMP; AMP is the probable source of the increasing sugar phosphate resonance. The accumulated endproducts of the overall reaction are AMP and Pi. Thus interpreted, the first event that occurs after the loss of glucose from the sustaining buffer is the phosphorylation of intralens metabolites by ATP. Shortly thereafter, this pool of metabolites is exhausted, whereupon ATP cannot be regenerated from ADP, and ADP begins to accumulate.

The loss of energy occurs as ATP compromises the ability of the lens to pump hydronium ions (and, presumably, sodium ions as well). Under the conditions of our experiment, which is parallel to standard clinical practices, this loss results in an elevation of the intralens pH. The specific contribution of this more alkaline intracellular lens pH to cataractogenesis in laboratory models is uncertain.

The ATP concentration detected in the intact lens and lens extracts by P-31 NMR analysis was within the range of those values previously reported (Table II). The Pi content is the lowest value reported. This finding is consistent with the NMR method of analysis, which requires minimal chemical treat-

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ment of the sample, thereby decreasing the opportunity for hydrolytic breakdown of labile compounds. The phosphocreatine concentration is low, as is the αGP, but in accord with previously reported values.[28] The values obtained for the choline and ethanolamine derivatives are also in accord with the same general concentration range as observed previously.[34] The ratio of the choline to the ethanolamine derivatives agrees rather well.

The underlying metabolic changes that result in the functional and morphologic damage associated with cataract development are not well understood. Physiologic stressors have been implicated as etiologic factors that predispose the crystalline lens to cataract formation, and the functional[42] and morphological[43, 44] damage resulting from the effects of these stressors are described in animal models. The use of NMR spectroscopy on the intact crystalline lens allows comprehensive biochemical analysis and permits subsequent functional (measurement of Na and K levels) and morphological (examination by electron microscopy) analyses to be performed on the *exact* same lenses. Moreover, NMR spectroscopy is a totally nondestructive technique, which not only permits the measurement of metabolic changes in live tissue as they are occurring and permits examination of tissue morphology by electron microscopy in the *same tissue sample* but also allows for the monitoring of tissue recovery subsequent to experimental therapeutic intervention. A technique such as this can substantially enhance our understanding of lens cataractogenic mechanisms by providing a more comprehensive view of the multifactorial basis of this degenerative process.

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