Phosphatic Metabolites of the Intact Cornea by Phosphorus-31 Nuclear Magnetic Resonance

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The principal low molecular weight phosphatic metabolites of the intact cornea were identified and quantitated nondestructively by phosphorus-31 nuclear magnetic resonance (P-31 NMR) spectroscopy. As part of this analytical procedure, the intracorneal pH was approximated from the resonance shift position of inorganic orthophosphate. In addition the metabolic and pH stability of incubated corneas at 37 C in MK medium was evaluated during an 8-hr time course and compared to similar dynamic analyses performed on corneas with denuded endothelium. Perchloric acid extracts prepared from these same corneas were analyzed by P-31 NMR to verify the metabolite peak assignments and to quantitate the concentrations of minor corneal metabolites. The concentrations of phosphatic metabolites of the cornea, including three previously unidentified phosphorus-containing substances, were determined for freshly excised corneas. The initial corneal spectroscopic profile was not altered by removal of the endothelium. At 37 C the MK media-incubated intact whole corneas experienced a time-dependent decline in ATP levels with a concomitant rise in inorganic orthophosphate; however, the tissue levels of the other principal phosphatic metabolites were not altered by prolonged incubation. In contrast, removal of the endothelial layer of the cornea-induced progressive metabolic deterioration of intact corneas characterized, most prominantly, by time-dependent declines in ATP and glycerol 3-phosphorylcholine levels and concomitant increases in ADP and inorganic orthophosphate levels relative to intact whole corneas. This study has established the feasibility of monitoring the metabolic status of intact rabbit corneas nondestructively and noninvasively. As such, P-31 NMR spectroscopy offers a promising method that may enable analysis of the metabolic viability of intact human donor corneas to provide a basis for selecting donor corneas for transplantation. Invest Ophthalmol Vis Sci 24:535–542, 1983

Lack of a suitable analytical technique has precluded assessment of the dynamic metabolic status of the intact cornea during laboratory experimentation or clinical (ie, preservation and transplantation) procedures. Phosphorus-31 nuclear magnetic resonance (P-31 NMR) spectroscopy has been utilized extensively as a nondestructive, noninvasive method for analyzing dynamic metabolic events in other intact, functioning tissues through measurement of phosphatic metabolite levels and intracellular pH.[1–7] Generally, the major phosphatic metabolites that can be readily measured in most intact tissues include adenosine triphosphate, adenosine diphosphate, phosphocreatine, inorganic orthophosphate, phosphorylated hexoses and trioses, the dinucleotides, and nucleoside diphosphosugars. Various *in vitro* tissue incubation and perfusion techniques coupled with P-31 NMR have been used to enable continuous quantitation of time-dependent metabolic changes in intact tissues for correlation with functional and anatomical disturbances in the same specimen.[8–10]

Until recently, tissues (ie, skeletal[3,8] and cardiac muscle[9]) containing a relatively high concentration of phosphorus-containing metabolites were preferred for intact tissue P-31 NMR experimentation principally because the high tissue phosphorus concentration minimized the signal-averaging time required to perform an analysis. As demonstrated during the past few years, however, tissues with relatively low phosphorus concentrations (ie, crystalline lens[7]), as compared to muscle, can be effectively analyzed by this technique, despite the necessity for an order-of-magnitude greater signal averaging time. Since the feasibility and the unique metabolic perspectives obtained by this nondestructive analytical approach have been demonstrated in studies concerned with

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cataractogenic processes of the crystalline lens, an obvious extension of this program was to investigate the possibility that this analytical technique could provide dynamic metabolic information about the intact cornea. As such, this report constitutes the first dynamic study of intact corneal metabolism by P-31 NMR. Metabolite levels were quantitated and intracorneal pH determined during control and experimental conditions, ie, corneas denuded of endothelium. (Endothelial damage is a major complication of corneal transplant surgery.) Further, we describe the significance of these findings and the potential application of this analytical approach to cornea studies.

Materials and Methods

Surgical

Albino rabbits, weighing 2–3 kg each, were killed with sodium pentobarbital injections and enucleated. Using a razor blade knife an incision was made 2 mm from the limbus of each eye. The incision was extended circumferentially with curved scissors, and corneal buttons with a 2-mm scleral rim were placed in MK medium[11] at 37 C. During the excision of the cornea from the globe, a 2-mm scleral border was necessary to provide structural rigidity adequate to maintain corneal morphology and to minimize and avoid stress that may exert influence on the cornea's metabolic status. Subsequently, corneas were manipulated with forceps by grasping the scleral rim. While corneas remained immersed in MK medium, fine curved scissors were used to excise the scleral rims in final preparation of the corneas for P-31 NMR analysis. In experiments involving evaluation of corneas without endothelium, corneas were denuded of endothelium by scraping a cotton-tipped swab over the posterior corneal surface. Anatomic evaluation of randomly selected specimens following NMR analyses was conducted to insure that the endothelium had been removed. Samples were collected in serial order from the limbus to the apex of the cornea and were prepared for microscopic examination according to previously described procedures.[12]

In Vitro Incubation

Four corneas for each incubation were placed together in a tared 12-mm NMR tube containing a volume of MK medium at 37 C, pH 7.4. All experiments were run in quadruplicate. All incubated corneas were equilibrated in MK medium for 2 hr at 37 C prior to initiation of each experiment, these yielded zero time points on time-course plots. At ½-hr intervals over an 8-hr experimental incubation period, the used medium was aspirated from the base of the NMR tube, corneas were washed three times with fresh medium, and fresh medium was added to a constant volume. This procedure was instituted to prevent metabolic artifacts caused by depletion of any of the medium's components. Individual phosphorus-31 magnetic resonance profiles were acquired during consecutive 2-hr intervals for the duration of the experimental incubation period. Corneas denuded of endothelium were incubated for 8-hrs, and phosphorus magnetic resonance profiles were generated according to the same analytical protocol described above. Twenty freshly excised corneas (ten intact whole corneas and ten cornea denuded of endothelium) were prepared for perchloric acid (PCA) extraction by freezing in liquid nitrogen immediately after excision. Incubated corneas from which PCA extracts were prepared were immersed in physiologic saline prior to liquid nitrogen freezing to rinse MK media from the corneal surface.

Corneal Perchloric Acid (PCA) Extracts

Frozen corneas were pulverized to a fine powder using a liquid nitrogen chilled stainless steel mortar and pestle maintained in a liquid-nitrogen bath. The tissue powder was added to a polyallomer centrifuge tube containing 0.1 v/w 60% PCA prefrozen in liquid nitrogen. The powder was stirred continuously, while warming until a paste consistency could first be discerned. This procedure facilitates complete coating of the tissue powder particles with PCA and subsequent extraction of the water soluble metabolites contained in the tissue powder at a temperature (−20 C) below which hydrolysis of acid labile compounds occurs. The sample was then immediately centrifuged at 43,500 g for 15 min at −5 C. Following centrifugation, the supernatant was transferred immediately to an equivalent volume of 10 N KOH, and the pH was adjusted to 10. The sample was centrifuged to remove precipitated $KClO_4$, and the final supernatant was passed through a potassium Chelex-100 column to remove polyvalent cations. the column effluent was lyophilized and then redissolved in 0.8 ml 20% D_2O and filtered through glass wool into an NMR microcell assembly for NMR analysis. This procedure minimizes the dilution of the tissue extract and enhances signal intensities as much as possible for quantitation of minor corneal metabolites. The preparation of the extracts and the P-31 NMR calibrations and analysis were performed according to accepted and well-validated procedures previously described for phosphorus NMR intact tissue and tissue extract analysis.[3,6,7] Subsequent to the NMR analysis, total phosphate determination were performed on the tissue extracts

and referenced to the extracted protein concentration to enable expression of the metabolite levels as concentrations in micromole per gram of corneal noncollagenous protein.

Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

The NMR spectrometer used in this investigation was a Nicolet NT-200 system equipped with deuterium stabilization, variable temperature, and Fourier-transform capabilities operating at 80.987663 MHz for P-31. This system is interfaced to a wide-bore (89 mm) Oxford superconducting magnet (4.7 Tesla). Intact corneas were analyzed under nonspinning, proton-decoupled conditions. Extracts prepared from these corneas were analyzed with and without proton decoupling, while spinning the sample to enhance signal resolution. Twelve-millimeter sample tubes were used in the analysis of the intact corneas. In this manner usable signal averaged data could be acquired within 30 to 60 min; however, the phosphorus-31 NMR data presented in the time-course plots were obtained from at least 2-hr intervals of signal averaging.

Chemical shift data are reported relative to the usual standard of 85% inorganic orthophosphate[13]; however, the primary internal standard was the natural glycerol 3-phosphorylcholine (GPC) resonance of the cornea. This compound has a relatively constant chemical shift for a phosphate (−0.13 δ) and is not influenced by variable physiologic pH, ionic strength, or countercation conditions.[14] The procedures for corneal incubations and P-31 NMR spectroscopic analysis are similar to those previously described for the crystalline lens.[7] Since the analytic spectrometer conditions used in this study varied somewhat depending upon whether intact corneas or cornea PCA extracts were being analyzed, the parameters common to both are listed, as follows, for the intact corneal analyses and, when different, the actual parameters used during the PCA extract analyses are presented as parentheticals: pulse sequence, one pulse; pulse width, 9 microsec, 45° flip angle; acquisition delay, 200 microsec; cycling delay, 200 microsec; number of scans 8,400 (144,100); number of data points per free-induction-decay, 8,192 (16,384); acquisition time, 0.819 (1.64) sec; sweep width +/− 2,500 Hz. In addition, a computer-generated filter time constant introducing 10 (3.0) Hz line broadening was applied. Data reductions, including peak area and chemical shift measurements were calculated using 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 δ (parts/million).

Chemical Identification of Intact Tissue Resonance Peaks

Phosphorus-31 NMR spectroscopic analysis performed on intact rabbit corneas yields a spectrum in which each resonance peak corresponds to a single phosphorus-containing functional group having a unique spectroscopic resonance window. The precise resonance shift position of each signal is a characteristic physiochemical marker for individual phosphatic 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, cationic, and protein matrix differences in cellular microcompartments of the cornea 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 cornea is based on the physical and chemical criteria previously described for lens[7] and numerous other intact tissues.[15]

Briefly, comparing the pH titration curves derived from standard phosphates with those obtained from the corneal PCA extracts, the common energy metabolites involved in intermediary metabolism of the cornea were identified with a high degree of certainty. These peak assignments were reinforced further by the demonstration that, under expanded 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 (eg, the sugar phosphate region), the peak assignments were reinforced by selectively shifting the resonance of interest with respect to the others by judiciously adjusting ionic strength[16] or by changing the countercation[17] in solution and subsequently adding known compounds[3] to the sample.

Mathematical Analysis of Dynamic Corneal Changes

The staggered-point time-course data of each set of four time-course runs obtained in this study were pooled and fitted to a least-squares regression analysis using primarily a linear expression. The best fit values of the coefficients are presented in the legends to Figures 1 and 2. A quadratic expression was necessary to approximate adequately the real time-course data

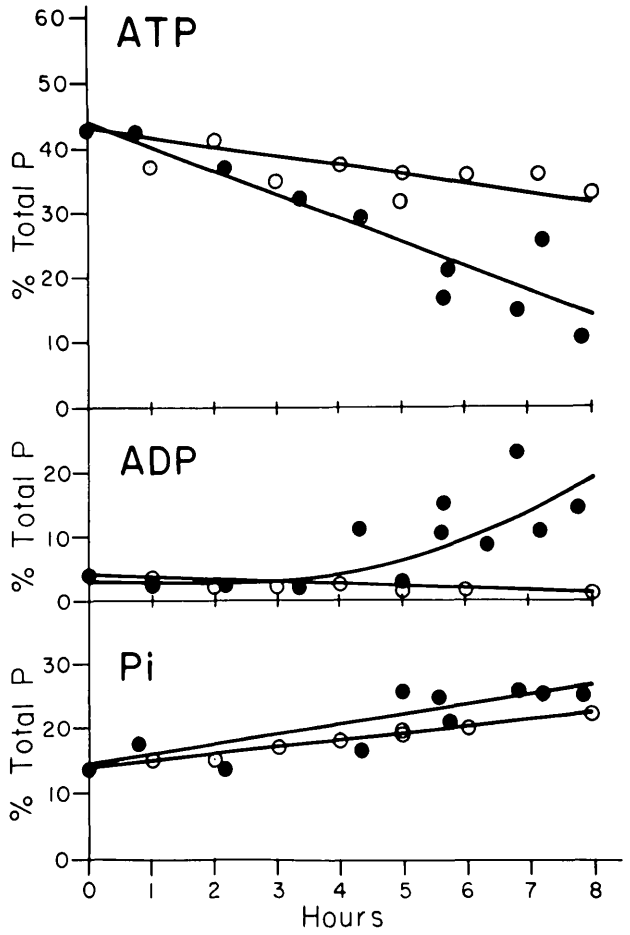

Fig. 1. Time-course denoting the time-dependent changes in rabbit cornea ATP, ADP and Pi: control cornea (open circles), cornea minus endothelium (filled circles). The data points were experimentally determined; the smooth curves were derived from a least squares analysis of the data from four kinetic runs. Coefficients for the expression $y = Ax + B$ obtained for the linear regression of the above data; control ATP time-course, $A = -1.477 \pm 0.205$, $B = 40.63 \pm 0.652$; ATP minus endothelium, $A = -3.725 \pm 0.268$, $B = 44.24 \pm 1.231$; ADP control, $A = -0.296 \pm 0.041$, $B = 3.56 \pm 0.133$; Pi control, $A = 1.049 \pm 0.016$, $B = 13.96 \pm 0.056$; Pi minus endothelium, $A = 1.553 \pm 0.212$, $B = 14.36 \pm 0.970$. For ADP minus endothelium an expression $y = Ax^2 + Bx + C$ was used; $A = 0.225 \pm 0.442$, $B = -0.003 \pm 0.284$, $C = 2.33 \pm 1.34$.

obtained from the intact endothelium-denuded corneal adenosine diphosphate (ADP) rate data. Data from four separate time-course experiments were used to calculate the rate curves drawn in Figures 1 and 2.

Results

The phosphorus-31 NMR spectrum obtained from four intact rabbit corneas incubated in MK medium for 2 hr is shown in Figure 3. Resonance signals observed include phosphorylated sugars, buffer inorganic orthophosphate, corneal inorganic phosphate, glycerol 3-phosphorylethanolamine, glycerol 3-phosphorylcholine, α, β, and γ phosphates of ATP, the dinucleotides, and nucleoside diphosphosugars. The majority of the resonance signals arise from the low molecular weight phosphatic metabolites associated with intermediary metabolism. Figure 4 illustrates a corresponding spectrum obtained from a rabbit cornea PCA extract. All of the major signals present in the extract spectrum correspond to those observed in the intact cornea spectrum of Fig. 3; however, as a result of improved signal-to-noise ratios and narrower line-widths, the extract spectrum exhibits signals from the minor cornea phosphates, α-glycerophosphate, ribose 5-phosphate, choline phosphate, phosphocreatine (PCr), unidentified phosphorus containing molecules of unknown origin and chemical nature (4.85δ, 4.64δ, and 1.85δ), and the α- and β-phosphates of

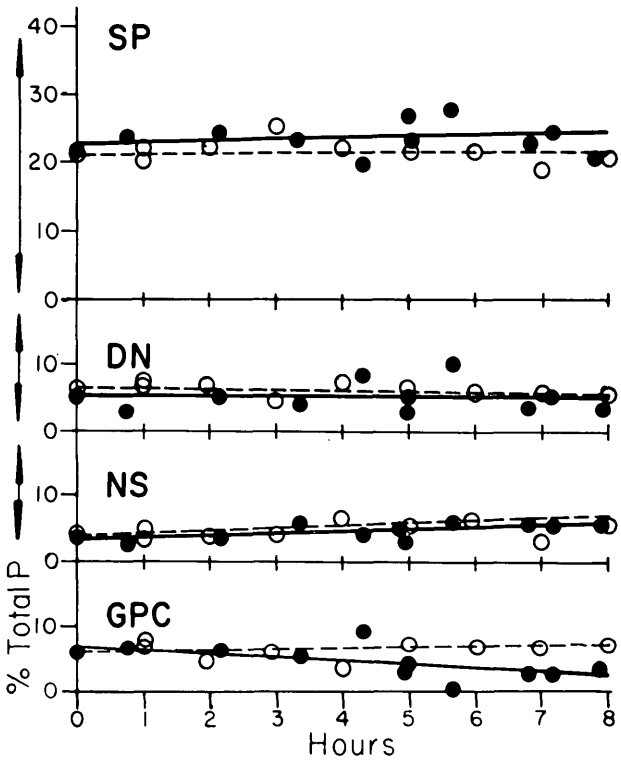

Fig. 2. Time-course for the changes in rabbit cornea phosphates (SP; sugar phosphates; DN, dinucleotides; NS, nucleoside diphosphosugars; GPC, glycerol 3-phosphorylcholine): control cornea (open circles, dashed lines), after removal of the corneal endothelium (filled circles, solid lines). The data points were experimentally determined; the smooth curves were derived from a least-squares analysis of the data from four kinetic runs. Coefficients for expression $y = Ax + B$ obtained for the linear regression of the above data; SP control time-course, $A = 0.149 \pm 0.201$, $B = 21.23 \pm 0.688$; SP minus endothelium time-course, $A = 0.180 \pm 0.154$, $B = 22.80 \pm 0.706$; DN -0.142 ± 0.097, 6.35 ± 0.331; -0.066 ± 0.000, 5.46 ± 0.000: NS, 0.219 ± 0.040, 4.36 ± 0.158; 0.252 ± 0.058, 3.81 ± 0.268: GPC, 0.189 ± 0.075, 6.16 ± 0.257; -0.551 ± 0.085, 6.96 ± 0.139: PCr (control only, not illustrated), 0.039 ± 0.016, 1.26 ± 0.055.

ADP. The total recovered phosphate content of the whole cornea extract per gram of extracted noncollagenous (NC) protein was 85 μmol. The individual tissue metabolite levels are reported as percent of the total P detected. These values can be converted to absolute levels (μmol/g NC protein) by multiplying the mole fraction of each metabolite by the total extractable phosphate concentration of the tissue. The mole fraction of any given metabolite is calculated by dividing the percent P value by the sum of the phosphorus atoms contained in the molecule, eg, the reported value of ATP is divided by 3 to calculate the ATP mole fraction.

Data such as those illustrated in Figure 3 may be plotted as a function of time to obtain detailed kinetic plots of metabolic processes. Such time-dependent changes are illustrated in Figures 1 and 2, wherein the concentration of seven intracorneal metabolites are plotted as a function of time for intact whole rabbit corneas and for rabbit corneas that have had their endothelia removed surgically. Incubation of intact whole corneas in MK medium at 37 C shows a decline in ATP of approximately 1.5%/hr during the 8-hr incubation. This decline is complimented by a corresponding rise in Pi. The other metabolites measured do not change. As illustrated in Figures 1 and 2, the overall metabolism of incubated intact corneas following removal of the endothelum is altered relative to that of incubated whole intact healthy corneas. The adenosine triphosphate (ATP) level declines progressively and more rapidly (approximately 2.5-fold) with time; ADP increases, while the inorganic orthophosphate changes little relative to intact whole corneas. Although corneal GPC content decreased with time in corneas denuded of endothelium (Fig. 2), other metabolites measured were not significantly altered during the incubation period represented in the figures. Thus, in a time period of hours, removal of the endothelium results, first, in the loss of high energy phosphate as ATP, followed by a subsequent compensation by the tissue to restore these levels through phospholipid fragment catabolism (decreased GPC). Processes associated with other biochemical pathways, such as glycolysis monitored through the sugar phosphate (SP) reasonance level, were essentially unaffected by endothelial removal within this time-course.

Perchloric acid extracts of corneal tissues yield static information exhibiting considerable detail in terms of the number of quantifiable corneal phosphates. Table 1 presents a comparison of the phosphate profiles obtained from nonincubated control rabbit corneas and nonincubated rabbit corneas minus endothelium. Nineteen phosphatic metabolites involved in corneal intermediary metabolism are

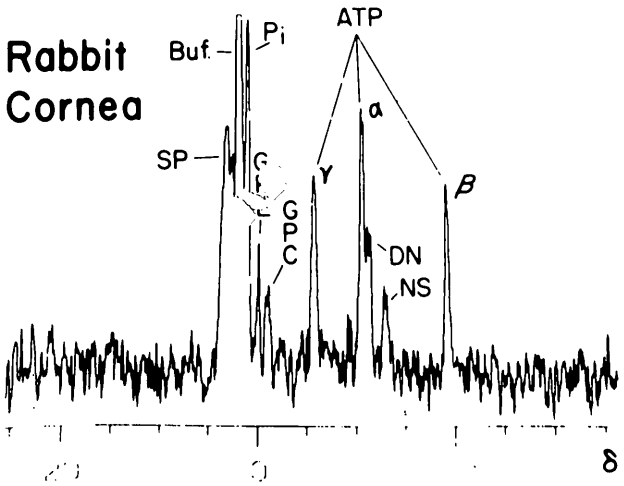

Fig. 3. Phosphorus-31 nuclear magnetic resonance spectrum from four rabbit corneas accumulated over a period of 2 hrs. The corneas were maintained during the experiment by MK medium at pH 7.4 at 37 C, Sp indicates the sugar phosphates; Pi, inorganic orthophosphate; GPE and GPC, glycerol 3-phosphoryl-ethanolmine and -choline, respectively; ATP, adenosine triphosphate with respective, α, β, and γ-resonance signals; DN, the dinucleotides; and NS, nucleoside diphosphosugars and related species. Buf. identifies the Pi resonance signal from the supporting medium. The chemical shift scale follows the International Union for Purified and Applied Chemistry shift convention and is given relative to the resonance position of 85% phosphoric acid.

present in quantifiable levels in the PCA extracts prepared from rabbit corneas. A comparison of the metabolite levels present in the cornea revealed that the ATP and inorganic orthophosphate contents of the

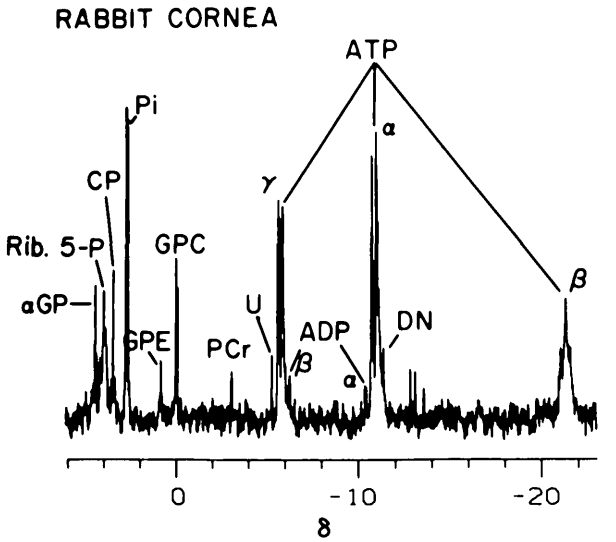

Fig. 4. Phosphorus-31 nuclear magnetic resonance spectrum from a perchloric acid extract of five control rabbit corneas. Minor resonances, some of which are multiplets, arise from: α-GP, α-glycerophosphate; CP, choline phosphate, Rib.5-P, ribose 5-phosphate; PCr, phosphocreatine; and U, an unidentified end-group-region phosphate. The corneas were rinsed in physiologic saline immediately prior to freezing in N_2, this eliminates any buffer contribution to the Pi resonance.

Table 1. Rabbit cornea phosphorus-31 nuclear magnetic resonance data

	Chemical shift† (δ)		*Amount (as % of the total P detected)*		
				In perchloric acid extract	
*Phosphate**	*In intact cornea*	*In perchloric acid extract*	*In intact cornea*	*Nonincubated intact cornea*	*Nonincubated cornea minus endothelium*
Unknown‡		4.85		1.2	1.3
Unknown‡		4.64		0.4	0.4
Glu 6-P		4.47		1.0	0.7
α-GP		4.28		4.4	4.5
Fruc 1,6-DiP		4.08		2.2	2.3
Rib 5-P	3.50§	3.84§	23.1‖	5.9	4.5
IMP		3.77		0.3	—
AMP		3.74		2.7	2.3
NADP 2'-P		3.54		1.0	1.0
Choline P		3.31		3.1	3.6
Pi	1.43	2.61	14.3	13.9	13.5
Unknown‡		1.85		0.1	0.1
GPE	0.42	0.75	2.7	2.5	2.2
GPC	−0.13	−0.13	4.1	3.8	3.3
PCr	−3.11	−3.13	1.6	1.0	—
ATP	, −10.65	, −10.8	41.9	43.0	48.4
	−19.24	, −21.3			
	, −5.62	, −5.7			
ADP	, −10.65	, −10.4	2.3	4.0	3.2
	, −6.66	−6.13			
DN	−11.84**	−11.34**	4.9	5.7	4.3
NS	−12.89††	−12.89††	4.9	4.1	4.5

* Glu 6-P, glucose 6-phosphate; α-GP, α-glycerophosphate; F 1,6-DiP, fructose 1,6-diphosphate; Rib 5-P, ribose 5-phosphate; IMP and AMP; inosine and adenosine monophosphate; NADP 2'-P; the 2' phosphate of nicotinamide adenine dinucleotide phosphate; Choline-P; choline phosphate; Pi, inorganic orthophosphate; GPE and GPC, glycerol 3-phosphorylethanolamine and 3-phosphorylcholine; PCr; phosphocreatine; ATP and ADP, adenosine tri- and diphosphate; DN, the dinucleotides NAD and NADP; and NS, the nucleoside diphosphosugars, uridine diphospho-galactose, -glucose, and -mannose.

† In the field-independent nuclear magnetic resonance units of, δ parts/million relative to the shift position of the 85% inorganic orthophosphoric acid reference phosphate at 25°.

‡ Compound, as of this writing, is not identified with any known phosphorus-containing biomolecule.

§ The chemical shift center of the sugar phosphate resonance band is given.

‖ The relative area of the sugar phosphate resonance band.

** The principal resonance signals of this band, arise from the P,P'-diesterfied pyrophosphate residues of NAD and NADP. The signals comprise two NMR ab multiplets.

†† A complex resonance band composed of the 3 sets of overlapping, ^{31}P-^{31}P, ab NMR multiplets from uridine diphosphogalactose, -glucose, and -mannose. The chemical shift indicated corresponds to the sugar phosphate portion of the ab multiplets.

rabbit cornea exceeded those of the other water-soluble phosphatic compounds extracted by PCA. Other relatively abundant corneal phosphates detected were α-glycerophosphate (α-GP), ribose 5-phosphate (Rib 5-P), choline phosphate (CP), GPC, ADP, and the dinucleotides, principally nicotinamide adenine dinucleotide (NAD). The relative levels of corneal metabolites quantified from intact cornea phosphate profiles are consistent with the findings obtained from the extract analyses with the exception that PCr is not readily detected in the intact cornea. Except for the somewhat elevated ATP levels detected in the nonincubated corneas denuded of endothelium, these corneas have the same relative distribution of phosphatic metabolites as that detected in whole intact corneas.

The chemical shift of inorganic orthophosphate in conjunction with a calibrated spectroscopic pH titration curve,[18] was used to compute the intracorneal pH environment of inorganic orthophosphate within the intact cornea with certainty of ±0.1 pH unit. Note in Figure 3, that the inorganic orthophosphate signal from rabbit corneas is distinctly shifted from that of the buffer inorganic orthophosphate (pH 7.4). Using the chemical shift characteristic for inorganic orthophosphate, we determined that the average pH of the interior of the cornea is 6.7. In spite of the dramatic alteration in the phosphatic metabolite profile following removal of the endothelium from the cornea there was no apparent change in corneal transparency following the 8-hr time-course.

Discussion

The cornea, like the crystalline lens,[7] produces highly resolved P-31 NMR spectrum that exhibits resonance signals from the low molecular weight metabolites of intermediary metabolism. The P-31 NMR technique permits measurement of the metabolic health of whole *intact* corneas, nondestructively and noninvasively, and avoids breakdown of the labile high energy phosphates common to destructive

procedures such as homogenization. The relationship between the specific metabolic changes that occur with loss of corneal transparency and their actual involvement in the process of opacification has not been clearly elucidated. Previous investigations have reported corneal metabolite concentrations for selected phosphatic compounds in isolated epithelium or stroma in normal and experimentally damaged corneas[19-31]; however, quantitative data in the intact cornea and data regarding sequential dynamic changes in corneal metabolite concentrations during incubation or corneal damage are absent. Our results demonstrate, for the first time, the time sequences of metabolic changes that occur with in vitro 37 C MKmedium incubation of intact whole cornea and cornea denuded of endothelium. The latter condition was examined since it is accepted that corneal tissue viability for penetrating keratoplasty depends almost exclusively on the survival of corneal endothelial cells. The loss of ATP and subsequent rise in ADP are anticipated features of a metabolically deteriorating tissue. The small change in intracorneal inorganic orthophosphate levels of corneas minus-endothelium relative to control coupled with the absence of any substantial rise in any other spectral component indicates that most of the inorganic orthophosphate resulting from ATP hydrolysis may be lost to the medium. Since the formation of a rigid macromolecular complex involving ATP or inorganic orthophosphate during prolonged incubation would result in an apparent loss of these compounds from the intact cornea as measured by NMR, this possible explanation for the observed findings cannot be discounted. An interesting feature of this time course is the declining level of the phosphodiesters, glycerol 3-phosphorylethanolamine (GPE) and GPC. The relatively small change in the phosphodiester levels precludes a definitive assessment of their fate. It is possible that they are being catabolized. Metabolic turnover of GPE and GPC compounds in all tissues in which they have been measured is low, however, and it is not likely to be elevated in the cornea. An additional and plausible explanation for their decline would be to suggest that they may be lost to the medium. GPE and GPC are readily soluble in both lipid and aqueous solvents and could easily diffuse from damaged cornea with impaired translocation activity. Although corneal viability is dependent on the metabolic contribution of the endothelium, the layer of endothelial cells constitutes less than 1% of the tissue mass.[32] This anatomic observation explains in part why no significant quantitative differences were detected between the relative levels of phosphatic metabolites detected in the intact cornea and the cornea minus endothelium prior to incubation.

The present findings demonstrate the feasibility and application of this nondestructive and noninvasive P-31 NMR technique to the study of the intact cornea and provide information on the metabolic status of corneal tissue maintained in culture for variable time periods. The viability of corneal donor material for penetrating keratoplasty depends on the metabolic health of the tissue. As a consequence the future application of the described nondestructive NMR technique to human donor tissue may provide information that would allow improved selection of donor material and perhaps enhance the postsurgical prognosis of the keratoplasty procedure. However, before P-31 NMR evaluation of human donor corneas can become a practical reality and provide meaningful predictive information regarding the probability of corneal survival following transplantation, correlative relationships need to be formulated based on experimental findings that define the irreversible changes in the metabolic status of the cornea that lead to corneal opacity. Furthermore, examination of the control data of Figure 1 reveals that at 37 C corneal tissue declines metabolically even though the tissue is incubated in the traditionally used MK medium. This observation suggests a necessity for additional studies designed to evaluate supporting buffers for their efficacy in preserving intact stored cornea preparations for transplantation.

Key words: intact cornea, endothelium, phosphatic metabolites, phosphorus-31 nuclear magnetic resonance, intracorneal pH, ATP, inorganic orthophosphate.

References

1. Moon RB and Richards JH: Determination of intracellular pH by ^{31}P magnetic resonance. J Biol Chem 248:7276, 1973.
2. Hoult DI, Busby SJW, Gadian DG, Radda GK, Richards RE, and Seeley PJ: Observation of tissue metabolites using ^{31}P nuclear magnetic resonance. Nature 252:285, 1974.
3. Burt CT, Glonek T, and Bárány M: Analysis of phosphate metabolites, the intracellular pH, and the state of adenosine triphosphate in intact muscle by phosphorus nuclear magnetic resonance. J Biol Chem 251:2584, 1976.
4. Glonek T: Applications of ^{31}P NMR to biological systems with emphasis on intact tissue determinations. *In* Phosphorus Chemistry Directed Toward Biology. Stec WJ, editor. New York, Oxford, Pergamon Press, 1980, pp. 157–174.
5. Navon G, Ogawa S. Shulman RG, and Yamane T: ^{31}P nuclear magnetic resonance studies of Ehrlich ascites tumor cells. Proc Natl Acad Sci USA 74:87, 1977.
6. Gadian DG, Radda GK, Richards RE, and Seeley PJ: ^{31}P NMR in living tissue. *In* Biological Applications of Magnetic Resonance, Shulman RG, editor. New York, Academic Press, 1978, pp. 463–535.
7. Greiner JV, Kopp SJ, Sanders DR, and Glonek T: Organophosphates of the crystalline lens: A nuclear magnetic resonance spectroscopic study. Invest Ophthalmol Vis Sci 21:700, 1981.
8. Dawson MJ, Gadian DG, and Wilkie DR: Contraction and

recovery of living muscles studied by ^{31}P nuclear magnetic resonance. J Physiol 267:703, 1977.
9. Hollis DP, Nunnally RL, Jacobus WE, and Taylor GJ IV: Detection of regional ischemia in perfused beating hearts by phosphorus nuclear magnetic resonance. Biochem Biophys Res Comm 75:1086, 1977.
10. Greiner JV, Kopp SJ, Weidman TA, Sanders DR, and Glonek T: Anatomical pathology of cataract formation correlated with biochemical changes in organophosphates: An electron microscopic and P-31 nuclear magnetic resonance spectroscopic study. Anat Rec 199:100A, 1981.
11. McCarey BE, Meyer RF, and Kaufman HE: Improved corneal storage for penetrating keratoplasties in humans. Ann Ophthalmol 8:1488, 1976.
12. Greiner JV and Chylack LT Jr: Posterior subcapsular cataracts. Histopathologic study of steriod-associated cataracts. Arch Ophthalmol 97:135, 1979.
13. Glonek T and Van Wazer JR: Aqueous tetrahydroxphosphonium perchlorate as a narrow-line phosphorus-31 NMR reference substance. J Magnetic Reson 13:390, 1974.
14. Burt CT, Glonek T, and Bárány M: Phosphorus-31 nuclear magnetic resonance detection of unexpected phosphodiesters in muscle. Biochemistry 15:4850, 1976.
15. Bárány M and Glonek T: Phosphorus-31 nuclear magnetic resonance of contractile systems. Methods Enzymol 85(Pt. B):624, 1982.
16. Costello AJR, Glonek T, and Van Wazer JR: Phosphorus-31 chemical shift variations with countercation and ionic strength for the various ethyl phosphates. Inorg Chem 15:972, 1976.
17. Glonek T, Kleps RA, Griffith EJ, and Myers TC: Phosphorus-31 nuclear magnetic resonance studies on condensed phosphates. I. Factors influencing the phosphate middle group chemical shift. Phosphorus 5:157, 1975.
18. Greiner JV, Kopp SJ, and Glonek T: Dynamic changes in the organophosphate profile upon treatment of the crystalline lens with dexamethasone. Invest Ophthalmol Vis Sci 23:14, 1982.
19. Herrmann H and Hickman FH: Exploratory studies on corneal metabolism. Bull Johns Hopkins Hosp 82:225, 1948.
20. Herrmann H and Hickman FH: The utilization of ribose and other pentoses by the cornea. Bull Johns Hopkins Hosp 82:287, 1948.
21. Langham M: Utilization of oxygen by the component layers of the living cornea. J Physiol 117:461, 1952.
22. Morley NH and Toth A: Oxidized pyridine nucleotides and lactic acid in the corneal tissue of rabbits. Can J Biochem Physiol 39:477, 1961.
23. Kinoshita JH: Some aspects of the carbohydrate metabolism of the cornea. Invest Ophthalmol 1:178, 1962.
24. Herrmann H and Lebeau PL: ATP level, cell injury, and apparent epithelium-stroma interaction in the cornea. J Cell Biol 13:465, 1962.
25. Reim M, Schmidt F, and Meyer D: Die Metabolite des Energie liefernden Stoffwechsels im Hornhautepithel verschiedener Säugetiere. Ber Deutsch Ophthalmol Ges 67:164, 1965, publ. 1966.
26. Reim M and Schmidt F: Biochemische Veränderungen bei der Vereisung der Hornhaut in vivo. Ein Beitrag zur Kältetherapie. Klin Monatsbl Augenheilkd 150:96, 1967.
27. Reim M, Meyer D, and Cattepoel H: Über die Bedeutung des Frierstopverfahrens für den *in-vivo* Status der Metabolite im Corneaepithel. Albrecht von Graefes Arch Klin Exp Ophthalmol 174:97, 1967.
28. Reim M and Meyer D: Über Veränderungen von Metabolitgehalten im Corneaepithel in Anaerobiose. Albrecht von Graefes Arch Klin Exp Ophthalmol 174:103, 1967.
29. Reim M, Meyer D, and Turss R: Some observations on the relation between the energy-producing metabolism and the transparency of the cornea. *In* Biochemistry of the Eye; Symposium at Tutzing Castle, August 10-13, 1966, Dardenne MU and Nordmann J, editors. Basel, Karger, 1968, pp. 68–73.
30. Turss R, Friend J, and Dohlman CH: Effect of a corneal fluid barrier on the nutrition of the epithelium. Exp Eye Res 9:254, 1970.
31. Reim M, Beermann HR, Luthe P, and Cattepoel H: The redox state of the glutathione in the bovine corneal epithelium. Albrecht von Graefes Arch Klin Exp Ophthalmol 201:143, 1976.
32. Maurice DM and Riley MV: The cornea. *In* Biochemistry of the Eye, Graymore CN, editor. New York and London, Academic Press, 1970, pp. 1–103.