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# Effect of catalytically active recruited crystallins on lens metabolism

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The impact on duck lens metabolism of recruited ε-crystallin/LDH-B4 and τ-crystallin/enolase was investigated by NMR spectroscopy. A comparison of the duck lens metabolite profile with that of the calf in which recruited crystallins are absent revealed significant increases in ATP, α-glycerophosphate (α-GP) and pyridine dinucleotide concentrations. The alterations in the concentrations of ATP and α-GP appear to be related to both the high concentration of NAD and the elevated reduced to oxidized NADH/NAD$^+$ ratio.

ε-Crystallin; τ-Crystallin; Duck lens; LDH-B4 isozyme; Glycolysis

## 1. INTRODUCTION

Transparency and refractive properties of the lens are dependent upon the concentration and organization of its major structural proteins, the crystallins. During the evolution of the complement of crystallins, preexisting enzymes have been recruited to serve a structural role in lenses of numerous and diverse species [1,2]. Many of the recruited enzyme/crystallins are derived from dehydrogenases and oxidoreductases which require pyridine dinucleotides, NAD or NADP as coenzymes [3]. Several of the recruited crystallins maintain a high level of catalytic activity while others have a more limited sequence homology with the parent enzyme and have little or no activity [4]. To date, the majority of investigations have focussed on mechanisms for gene overexpression to explain the natural selection of enzyme/crystallins [5]. However, there are few studies to assess their selection with respect to modification of lens metabolism as it relates to the maintenance of lens transparency. In the present study, NMR spectroscopy was employed to compare metabolite concentrations in both duck and calf lenses which depend almost entirely on anaerobic glycolysis for the generation of high energy phosphates. The duck lens contains enzymatically active recruited ε- [6,7] and τ- [8] crystallins while there are no reports of recruited crystallins in the calf lens.

ε-Crystallin is derived from lactate dehydrogenase (LDH) and comprises about 2–23% of lens proteins of numerous avian and reptilian species [9]. In the duck, 8% of lens protein is ε-crystallin and is accompanied by an elevated NAD concentration [3,10]. The catalytic activity of ε-crystallin/LDH is approximately 500 times that reported in six mammalian species whereas τ-crystallin/enolase has an activity only 2–3 times that in four of the six species [7]. Therefore, it is expected that the presence of high concentrations of catalytically active ε-crystallin/LDH will dominate metabolic effects in the duck.

Our comparison of $^{31}$P NMR spectra of duck and calf lenses revealed that significant alterations in concentrations of phosphorus-containing metabolites including the dinucleotides and their ratios of reduced to oxidized forms appear to be related to the presence of recruited duck lens crystallins. Since LDH controls the interconversion of pyruvate and lactate, $^1$H NMR spectroscopy was also used to determine the concentration of lactate. A comparison of the spectra revealed that lactate concentrations are marginally elevated in the duck. The latter observation may be related to efflux of membrane-permeable lactate and/or to the fact that LDH is the B4 isozyme that favors the production of pyruvate [6].

## 2. MATERIALS AND METHODS

### 2.1. *Lens perchloric acid extract (PCA)*

Calf eyes (3–4 months) were enucleated from freshly slaughtered animals and immediately immersed in liquid nitrogen at the local abattoir. Ducks were anesthetized by an injection of sodium pentobarbital (i.m.); the eyes were enucleated and frozen in liquid nitrogen. Lenses from frozen calf and duck eyes were decapsulated, weighed and re-immersed in liquid nitrogen. The PCA extracts in our studies are that of the decapsulated lens which removes the epithelium as well. Winkler and Riley [11] have shown in the rabbit lens under both aerobic and anaerobic conditions that the concentration of ATP in the epithelial layer is inconsequential in comparison to fiber cells that comprise the bulk of the lens. PCA extracts [12] were prepared by pulverizing pairs of decapsulated lenses to a fine powder with a mortar and pestle chilled with liquid nitrogen. After addition of 8% PCA, the powder was stirred continuously to a paste consistency. This procedure extracted the acid soluble cellular metabolites. After centrifugation at 41,000 × *g* for 20 min at 0°C, the supernatant was neutralized with 10 N KOH and adjusted to pH 9.0. The resultant perchlorate

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precipitate was removed by an additional centrifugation and the supernatant was lyophilized and redissolved in 0.5 ml of 20 mM EDTA for NMR analysis.

2.2. *NMR measurements*

$^{31}P$ NMR quantitative calibrations and analyses of the PCA extracts were performed according to standard procedures [13,14]. Earlier it has been reported that concentration values determined by NMR were in good agreement with those given by HPLC measurements of perchloric acid extracts [15]. $^{31}P$ NMR measurements were conducted using a Varian VXR 500 spectrometer operated at 202.3 MHz with gated proton decoupled conditions. Samples of 0.5 ml were run in 10 mm NMR tubes equipped with Wilmad micro cell assembly (Buena, New Jersey). The outer chamber contained $D_2O$ for field frequency lock measurements. The typical spectrometer conditions for $^{31}P$ NMR experiments were as follows for 1 pulse: 90° pulse width, 44 $\mu$sec; acquisition delay, 30.0 s; sweep width, 10 KHz. $T_1$ values of the most intense resonances, ATP, GPC and inorganic phosphate of freshly prepared lens PCA extract, were determined using the inversion recovery method [16]. Inorganic phosphate is known to have the highest $T_1$ value among lens phosphorus metabolites due to $^{31}P$ chemical shift anisotropy [17]. Therefore to obtain fully relaxed spectra, the $T_1$ value for inorganic phosphate, 6.8 ± 0.08, was used to set an interpulse delay of 30.0 s which is greater than four times $T_1$ [17]. To evaluate the amount of saturation for each resonance, the routine spectra which is recorded using a 0.5 s delay were compared to the fully relaxed spectra. The saturation factor was the ratio of peak areas measured under non-saturating conditions to the area measured under experimental conditions [13]. Each spectrum was an average of 1,800 scans accumulated over a period of 16 h. A line broadening of 2 Hz was applied to decrease spectral noise. Each measurement was repeated three times on a freshly prepared lens PCA extract of 10–12 lenses and with known concentrations of phosphorus metabolites obtained from Sigma Chemical Company (USA). Measurements of the peak area were performed using a sub-routine of the spectrometer programme. Different expanded regions of the NMR spectrum were individually integrated and summed. The resonance peak areas were used to evaluate absolute concentrations of the various metabolites by comparing the integral areas obtained with known concentrations recorded under identical conditions. Values obtained for each metabolite are provided in Table I. The chemical shifts were reported with internal reference to glycerol 3-phosphorylcholine (GPC, −0.130 $\delta$ ppm).

Lactate concentration in lens PCA extracts was determined by $^1H$ NMR spectroscopy using a Varian 200 MHz FT-NMR spectrometer. The $^1H$ NMR spectrum of lactate was recorded using a pre sat. pulse sequence with low r.f power for removing water from $D_2O$. The $T_1$ value for methyl resonance in lactate, 1.53 ± 0.14, was determined using the inversion recovery sequence [16]. The fully relaxed $^1H$ NMR spectrum of lactate in PCA is recorded using a long repetition delay that is greater than 4–5 times $T_1$. The $^1H$ NMR spectrum of a known concentration of lactate dissolved in PCA is recorded under identical conditions. The absolute concentration of lactate in the extract is determined by comparing the integral areas of the methyl signal in the lens PCA with the integral area of the methyl signal in the known concentration of lactate after applying a correction for saturation. Spectral parameters were as follows: 90° pulse angle, 20.0 $\mu$s; sweep width, 3 KHz; delay between pulses, 10 s; acquisition time, 1.6 s; and number of scans, 100.

## 3. RESULTS

Typical $^{31}P$ NMR spectra in our study of calf and duck lenses are illustrated in Fig. 1A and B, respectively. The concentrations in $\mu$mol/g lens wet weight and mean relative mol percentage of phosphorus metabolites determined by $^{31}P$ NMR as well as lactate concentrations determined by $^1H$ NMR are provided in Table I. NMR analyses performed prior to this study were not useful for data comparison because of lack of attention to spin-lattice relaxation, $T_1$ attenuation [17], and the absence of metabolite concentrations. In our study, the major differences between the $^{31}P$ spectra for the duck and calf lenses involve ATP and α-glycerophosphate (α-GP). In the duck lens, ATP concentration is 9.55 and α-GP is 2.0 $\mu$mol/g. The respective concentrations are 3 and 5 times greater than in the calf. Our $^{31}P$ NMR data of calf lens ATP are in accord with the traditional biochemical assay of ATP concentrations reported by Lou and Kinoshita of 3.1 and 2.4 $\mu$mol/g for rabbit and calf lenses, respectively [18]. These comparisons support the assumption that $^{31}P$ NMR spectra faithfully reflect the bulk acid extractable phosphate composition of lens tissue. The high ATP value reported

Table I

Bovine and duck lens $^{31}P$ and $^1H$ NMR data

| Metabolites | Chemical shifts* ($\delta$ ppm) | Calf lens | | Duck lens | |
|---|---|---|---|---|---|
| | | $\mu$mol/g ± S.E.M. | (%) | $\mu$mol/g ± S.E.M. | (%) |
| Adenosine triphosphate | α −10.92<br>β −21.45<br>γ −5.80 | 3.01 ± 0.03 | 29.39 | 9.55 ± 0.80 | 61.08 |
| Adenosine diphosphate | α −10.61<br>β −6.11 | 1.23 ± 0.03 | 8.02 | 1.42 ± 0.04 | 6.05 |
| Adenosine monophosphate | 3.73 | 0.37 ± 0.04 | 1.20 | 0.16 ± 0.05 | 0.34 |
| α-Glycerophosphate | 4.30 | 0.41 ± 0.05 | 1.41 | 2.00 ± 0.05 | 4.25 |
| Inorganic phosphate | 2.60 | 12.71 ± 1.39 | 41.38 | 11.14 ± 1.55 | 23.74 |
| Pyridine dinucleotides | −11.37 | 0.67 ± 0.02 | 3.04 | 1.73 ± 0.04 | 6.23 |
| Glycerophosphorylcholine | −0.13 | 2.02 ± 0.05 | 6.60 | 0.10 ± 0.02 | 0.19 |
| Glycerophosphorylethanolamine | 0.80 | 0.43 ± 0.03 | 1.41 | 0.04 ± 0.01 | 0.08 |
| Phosphocreatine | −3.10 | 0.28 ± 0.02 | 0.91 | 0.13 ± 0.02 | 0.27 |
| Phosphorylcholine | 3.33 | 1.71 ± 0.03 | 5.60 | 0.50 ± 0.04 | 1.06 |
| Lactate | | 58 ± 1.70 | | 73 ± 2.30 | |

*Chemical shifts are expressed with respect to 85% phosphoric acid.

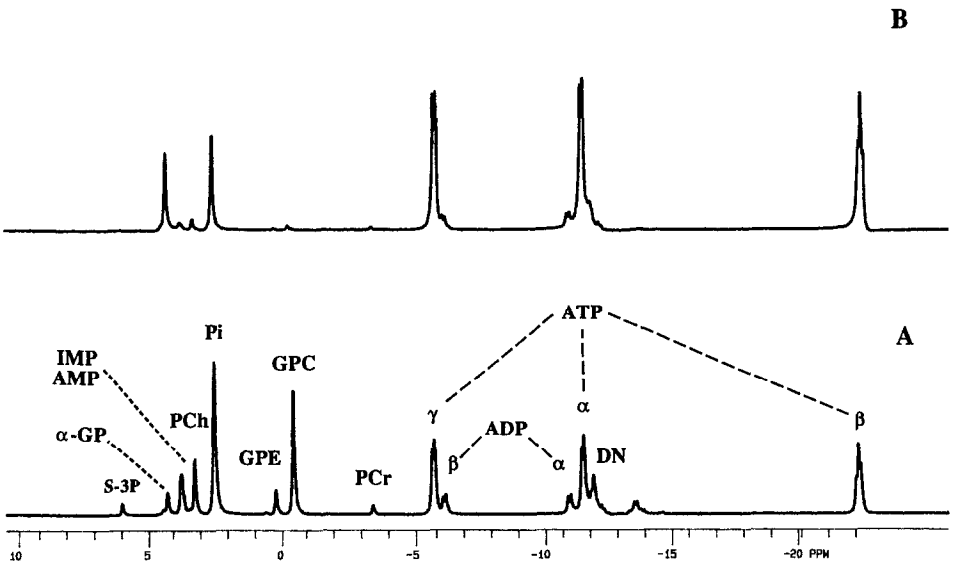


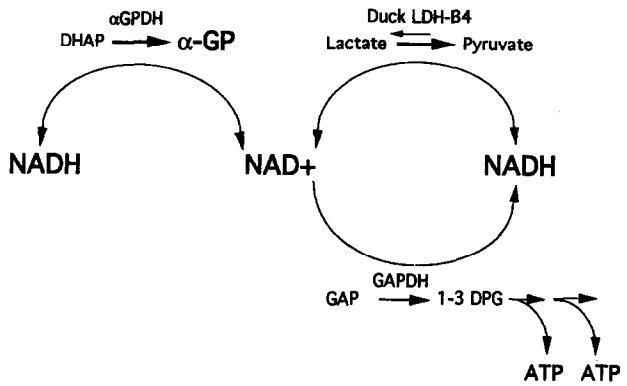

Fig. 1. $^{31}P$ NMR spectra of phosphorus metabolites in the calf (A) and duck (B) lens. Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DN, dinucleotide; PCr, Phosphocreatine; GPC, glycerophosphorylcholine; GPE, glycerophosphoryl ethanolamine; $P_i$, inorganic phosphate; PCh, Phosphorylcholine; α-GP, alphaglycerophosphate; AMP, adenosine monophosphate; IMP, inosine monophosphate; S-3P, sorbitol-3-phosphate.

by the same investigators for the rat lens at 30 days of age (25 mg wet weight) is probably related to its stage of lens maturity. It is well established that ATP concentration in the lens is age dependent; for example, the mean relative mol percent of ATP in juvenile and adult rabbit lenses in organ culture is 19.6 and 9.7, respectively [19]. Additionally, using biochemical assays, Klethi and Mandel [20] showed that the levels of ATP in pigeon and chicken lenses are twice that found in a variety of mammalian lenses. Despite an absence of ε-crystallin in both of these avian species, the activity of LDH in the chicken is about 7–30 times that reported in 6 mammalian species [7].

ADP concentrations and percent composition in the duck and calf are comparable. The AMP and IMP concentrations in both species are relatively low; however, in each case the concentrations tend to be higher in the calf (Table I).

The high concentration of α-GP in duck and rabbit lenses [21] appears to correlate with increased concentrations of NADH [3]. The reduced dinucleotide serves as a coenzyme for the conversion of dihydroxyacetone phosphate (DHAP) to α-GP by α-glycerophosphate dehydrogenase (Fig. 2). In the lens, this serves as the major pathway for the generation of α-GP [22]. The concentrations of α-GP and NAD in the calf lens are 0.41 and 0.67 μmol/g, while in the duck lens they are 2.0 and 1.73, respectively (Table I). Our NMR data are in reasonable agreement with NAD concentrations in the duck lens of 1.4 [3] and 1.9 [10] using biochemical assays. Similarly, in the rabbit lens, a high concentration of α-GP (0.94 μmol/g) [21] accompanies a high concentration of NAD (1.04 μmol/g) [3].

For high energy phosphates, the lens is virtually dependent upon the Embden–Meyerhof pathway; therefore, the oxidized form of the dinucleotide, $NAD^+$, generated by the conversion of pyruvate to lactate and DHAP to α-GP, is essential for the initiation of the second stage of glycolysis. In the duck, the conversion of lactate to pyruvate with generation of NADH is favored due to the presence of the LDH-B4 isozyme [6] (Fig. 2). This may explain in part the differences in the reduced to oxidized dinucleotide ratios encountered in various lenses. In the absence of enzyme/crystallins in rat, cat and calf lenses, the ratio of the concentrations of NADH/NAD are well below 0.5, i.e. 0.14, 0.31, and 0.15, respectively [3]. However, in duck and chicken lenses with elevated LDH activity (53,788 and 817.7




| | |
|---|---|
| 1-3 DPG | 1-3 diphosphoglycerate |
| GAP | glyceraldehyde phosphate |
| GAPDH | glyceraldehyde phosphate dehydrogenase |
| LDH-B4 | lactate dehydrogenase |
| α-GPDH | α-glycerophosphate dehydrogenase |

Fig. 2. Proposed metabolic scheme of the relationship of duck lens ε-crystallin/LDH-B4 and elevated levels of pyridine dinucleotides, ATP and α-GP.

$IU \cdot g^{-1}$ [7]), the ratios are elevated to 0.90 and 0.94, respectively [3]. Although the NADH/$NAD^+$ ratio in the rabbit lens is 0.34, the concentration of NADH is about 10 times that found in other lenses [3].

The $^{31}P$ NMR data analysis includes phosphocreatine (PCr), both inorganic and organic phosphates and phospholipid precursors. PCr is generally a minor lens component, but appears to be an alternative source of ATP. In calf and duck lenses, PCr values are 0.28 and 0.13 μmol/g, while ATP values are 3.01 and 9.55 μmol/g, respectively (Table I). The estimated content of both inorganic and organic phosphate in calf and duck lenses is approximately 30 and 50 μmol/g, respectively (Table I), while the respective values for inorganic phosphate are 12.71 and 11.14 μmol/g. In the calf lens this constitutes 41.38% of total phosphate whereas in the duck it is only 23.74%. This difference can be attributed to the high level of ATP in the duck. The concentrations of phospholipid precursors in the duck are a small fraction of that found in the calf (Table I). This is somewhat unexpected since the duck lens contains a high concentration of α-GP, which can serve as a precursor of phospholipid synthesis.

## 4. DISCUSSION

The results of the present study clearly demonstrate that the presence of enzyme/crystallins with catalytic activity has a significant impact on metabolite concentrations and, therefore, lens metabolism. Kopp and coinvestigators [23] suggested that interspecies differences in lens metabolites detected by $^{31}P$ NMR spectroscopy represent divergent phylogenic modifications in the control of enzymatic reactions that regulate intermediary metabolism. Our study indicates that the presence of ε-crystallin/LDH-B4 in the duck lens is related to high levels of ATP, α-GP and dinucleotides. The presence of the B4 isozyme favors the conversion of lactate to pyruvate with the generation of the reduced coenzyme, NADH. Therefore, compared to other lenses without recruited crystallins, the duck lens has a high NADH/$NAD^+$ ratio. However, the overall increased concentrations of the coenzyme, which accompany expression of the enzyme/crystallin, result in an elevated $NAD^+$ necessary for the second half of glycolysis and the generation of high levels of ATP. The elevated α-GP content in the duck lens suggests that a major source of the $NAD^+$ is conversion of DHAP to α-GP (Table I, Fig. 2). The increased duck lens ATP could also be related to the high concentrations of NAD compared to NADP [3]. This would tend to selectively funnel glucose through the Embden–Meyerhof pathway versus the NADP-dependent pentose shunt.

The selection of LDH-B4 to serve a structural role in the lens may also be related to its capacity to bind its coenzyme. The more lens-specific crystallins have also been shown to bind phosphorylated metabolites, for example, αGP to γ-crystallin [24] and ATP to α-crystallin [25]. In each example, protein stability is increased, the net charge and more importantly, the pattern of electrostatic potential are altered, thereby modifying their structural role in the maintenance of lens transparency [3,26].

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