Nature and localization of avian lens glycogen by electron microscopy and Raman spectroscopy

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ABSTRACT Electron microscopy confirms the presence of a high concentration of glycogen particles in the lens nuclear region of birds of flying habit such as the ring-neck dove and pigeon. This observation is consistent with Raman spectroscopy. The glycogen particles in the dove lens, which are ~35 nm in diameter, are classified as beta type particles. Although this type has been previously characterized by high rates of glycogen turnover in other tissues, its localization in the lens nucleus indicates that it may serve a structural function rather than as a storage depot of carbohydrate in the lens. In a comparative electron microscopy study, glycogen particles were not observed in the chicken lens.

INTRODUCTION

The occurrence of large amounts of glycogen in the lenses of most birds is a unique phenomenon (1–4) because all other species of vertebrate lenses contain only traces. Furthermore, the glycogen is concentrated in the lens nucleus of birds of flying habit. This histological and species distribution has not been satisfactorily explained. As Rabaey (1) has pointed out, the absence of glycogen in reptile lenses suggests that its presence in birds is a result of evolutionary changes subsequent to the separation of birds and reptiles from the ancient ancestor from which they have supposedly descended. It is worth noting that the changes evolved during this separation did not involve δ-crystallin which remains to this day an apparent substitute for the γ-crystallin found in all other vertebrate lenses except those of birds and reptiles.

Rabaey (1) believed that glycogen served to increase the refractive index of the bird lens and thereby improved accommodative capacity beyond the level found in ground-running birds and reptiles. Another advantage of glycogen is that it is not subject to the degradative changes characteristic of proteins and would not accumulate pigments and molecular aggregates causing impairment of vision. This chemical stability is less important in reptiles which are often nocturnal and do not need the visual acuity possessed by diurnal birds.

Hockwin, on the other hand, has presented evidence for an active metabolic function for lens glycogen (5). His studies, however, concerned the metabolism of only the bovine lens which has the lowest glycogen concentration of several species of vertebrate lenses. Although the glycogen concentration is highest in the nucleus, the nature of the deposit is quite unlike that in the pigeon lens which is typical of lenses of flying birds. Here the deposit is nearly spherical but slightly elongated along the visual axis. This positioning of the glycogen deposit would enhance any optical effect along the visual axis and would make it less accessible to the metabolically active cortex. The purpose of this investigation was to acquire further evidence of the distribution and function of glycogen in the bird lens as revealed by electron microscopy and Raman spectroscopy.

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MATERIALS AND METHODS

Materials

Living animals (chickens, pigeons, and ring-neck doves) were acquired from various local sources. The animals were decapitated in the laboratory, eyes enucleated at once and lenses kept in TC199 medium (for electron microscopy), and a modified T-K medium (for Raman spectroscopy) (6). All animals were killed by methods which conform to the "ARVO Resolution on the Use of Animals in Research" (7).

Raman spectroscopy

The laser Raman system used in this work consisted of a Spex 1401 double monochromator, RCA C-31034 type photomultiplier, Coherent radiation model CR-5 argon-ion laser (Coherent, Inc., Palo Alto, CA), and Spex DC-1 photon-counting electronics (SPEX Inds., Inc., Edison, NJ). All of the spectra were obtained with the 514.5-nm excitation-line.

To record the Raman spectra of the intact lens, the specimen was immersed in culture medium in a glass tube. A Raman spectrum from a selected small zone (a column of 50-μm diameter × 0.6-mm length) of intact lens was obtained by focusing the laser beam on that zone and by admitting Raman emission from only that zone to the spectrometer.

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Electron microscopy

Within three minutes of removal, the lenses were fixed in a mixture consisting of 2.5% in glutaraldehyde, 0.1 M cacodylate buffer (pH 7.3), 50 mM L-lysine, and 1% tannic acid (8). After three hours of fixation, a whole lens was mounted on the specimen holder with superglue and cut into 200 μm slices with a Vibratome (EM Corp, Chestnut Hill, MA). Lens tissues were oriented so that the cross or longitudinal sections containing cortical and nuclear regions could be obtained. Lens slices were fixed for an additional hour, post-fixed in 1% aqueous OsO_4 or in 1% OsO_4-1.8% $K_4Fe(CN)_6$ (9) for 1 h at room temperature, and en bloc stained with 0.5% uranyl acetate in 0.15 M NaCl overnight at 4°C. All tissues were dehydrated in a series of ethyl alcohols (30% through absolute), propylene oxide and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Thin sections were cut with an LKB Ultramicrotome, stained in 5% uranyl acetate, followed by Reynold's lead citrate, and examined with a JEOL 1200EX electron microscope. As far as we are aware, this work reports the first application of electron microscopy to glycogen in the bird lens.

Enzyme treatment

Once the 200-μm vibratome sections of the lens cortical and nuclear regions were cut, they were fixed for an additional hour, and immersed in 0.1 M cacodylate buffer (pH 7.3) for 24–36 h at 4°C to remove the remaining glutaraldehyde. The lens slices were then treated with amylo-1,6-glucosidase (4.5 mg/ml; Sigma Chemical Co., St. Louis, MO) (10) in 0.01 M sodium acetate buffer (11) (pH 4.6) for 1 or 2 h at 37°C. They were rinsed in 0.1 M cacodylate buffer for 30 min, post-fixed in 1% aqueous OsO_4 or in 1% OsO_4-1.8% $K_4Fe(CN)_6$ (9) for 1 h at room temperature, and en bloc stained overnight in 0.5% uranyl acetate in 0.15 M NaCl at 4°C. The lens tissues were then prepared for thin-section electron microscopy as described above.

Histochemical demonstration of glycogen phosphorylase

The lenses and the gastrocnemius muscles from eight-week-old ring-neck doves were frozen in liquid nitrogen and cut into approximately 20-μm thick sections with a Reichert cryostat (Reichert Scientific Instruments, Buffalo, NY). The sections were then mounted on slides and immersed in a reaction mixture containing 25 mM glucose 1-phosphate, 2 mM AMP, 50 mM sodium fluoride, 12 mM EDTA, 0.4 mg/ml glycogen, 90 mg/ml polyvinylpyrrolidone MW 10,000, 10% ethanol, at pH 6.0 for 30 min at 37°C (12). They were then washed with water, fixed in 40% ethanol for 1 min, covered with Gram's iodine solution (2 min), and examined with a light microscope (12, 13).

RESULTS

Raman spectra

Fig. 1 shows Raman spectra (400–1,800 cm^{-1}) from the nucleus, intermediate zone, and cortex region of a ring-neck dove lens; a background spectrum is not included because it is featureless. The peaks at 484, 861, and 944 cm^{-1} in the nucleus spectrum (Fig. 1 *a*) are due to glycogen vibrations (14). These Raman lines were not detectable in the spectra of 10-d-old chick or 10-yr-old hen's lens (14), indicating that the glycogen concentration is below the Raman detection limit (i.e., ~1 mM).

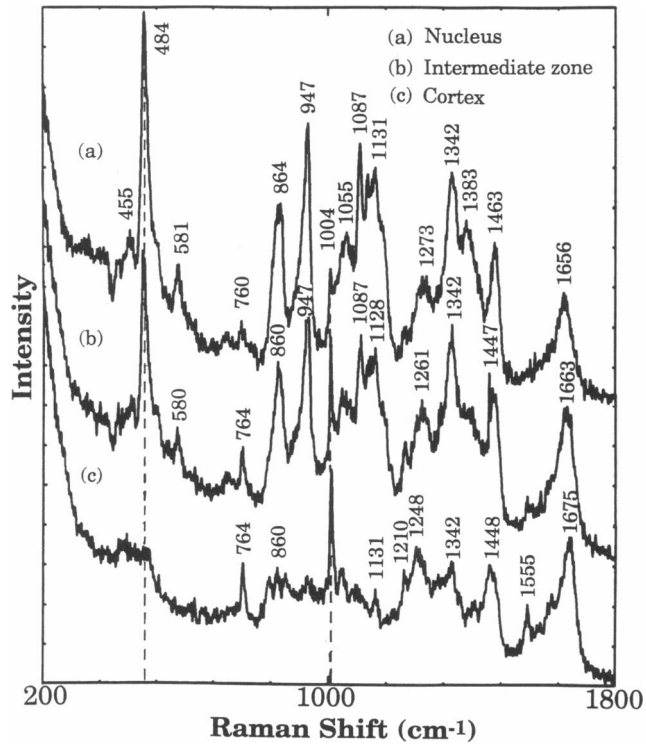


FIGURE 1 Raman spectra of ring-neck dove lens at three different locations along the visual axis. The instrumental conditions for the three spectra are identical so that Raman intensities at 484 cm^{-1} are indicators of relative glycogen concentrations. Laser power = 100 mW; rate of scan = 1 cm^{-1}/s; slit width = 450 μm.

Instead, Raman signals characteristic of δ-crystallin (with predominantly α-helical conformation) are very strong in the spectra of the hen's nucleus and chick lens. On the other hand, the dove lens exhibits only weak Raman signals characteristic of δ-crystallin (Fig. 1 *a*: 1,656 cm^{-1}). Thus, it appears that the glycogen in the dove lens is in place of δ-crystallin in the hen/chick lens. In comparing the three spectra (from nucleus to cortex) shown in Fig. 1, one notices the intensity decreases at 484, 861, and 944 cm^{-1}, indicative of decreasing glycogen concentration. Because the number of phenylalanine residues per monomeric unit of lens proteins (~20,000 daltons) is approximately the same, the Raman signal of Phe at 1,004 cm^{-1} is a good indicator of lens protein concentration. In fact, experimentally, Barron (15) and Bergbauer (16) found that the 622 cm^{-1} Raman signal (also corresponding to Phe) is indeed a reliable indicator of lens protein. Thus, the increase of the Raman signal at 1,004 cm^{-1} (Fig. 1) reflects an increasing protein concentration (relative to glycogen) in going from nucleus to cortex. In other words, Raman spectroscopy indicates glycogen to be present not only in the lens nucleus but also in the intermediate and cortical regions

of the dove lens with the highest concentration in the nucleus. In addition to glycogen, δ-crystallin is present in the nucleus of the dove lens, as evidenced by the Raman amide I band at 1,656 cm^{-1}. However, some crystallins with antiparallel β-pleated sheet conformation (amide I at 1,667 cm^{-1}) are also present in the cortical region of dove and pigeon lenses; these would be α and β crystallins.

As expected, the lack of detectable Raman S-S or S-H stretching vibrations at 505 or 2,580 cm^{-1}, respectively, is characteristic of a lack of γ-crystallin. The best indicator for glycogen is the Raman line at 484 cm^{-1}. Other glycogen Raman lines shown in Fig. 1 are 1,087, 1,131, 1,342, 1,383, and 1,463 cm^{-1}. Fig. 2 shows the relative concentration of glycogen throughout the visual and equatorial axes of the lens. The relative concentration was taken as the ratio of the glycogen Raman line at 484 cm^{-1} and the phenylalanine line at 1,004 cm^{-1}. The typical error in the measurement of the 484/1,004 intensity ratios is ~3%. Thus, our data confirms that glycogen is present throughout the dove lens with a central maximum at the lens' nucleus.

Electron microscopy

Electron microscopy showed that glycogen was present in the nuclear fiber cells of the ring-neck dove lens (Fig. 3). Individual deposits appeared as electron dense round particles with homogeneous content, approximately 35 nm in diameter (Fig. 3). These particles were scattered throughout the cytoplasm. The lack of organelles in this region of the lens avoided any possible confusion between ribosomes and the glycogen particles.

To ascertain that these particles were, in fact, glycogen, the lens tissue was treated with amylo-1,6-glucosidase which specifically digested the glycogen. After a one-hour treatment with the enzyme, a reduction in the number and size of glycogen particles occurred (data not shown). After a two-hour treatment, the glycogen was almost completely digested (Fig. 4).

In a comparative EM study, glycogen particles were not observed in the nuclear fiber cells of the chicken lens (Fig. 5).

Histochemistry

The lens metabolic activity was further studied by conducting a histochemical test for the presence of glycogen phosphorylase in the lens tissue, using muscle tissue as a control. Glycogen phosphorylase in the tissues was first activated with adenylic acid, primed by glycogen, and stained with Gram's iodine, which colors the newly formed (unbranched) glycogen dark blue (positive reaction) and the native glycogen brown or red brown (negative reaction) (12, 13). Microscopic examination of the sections revealed a lack of glycogen phosphorylase activity in the lens tissue (red-brown color) and a strongly positive (dark blue) glycogen phosphorylase activity in the muscle tissue (data not shown).

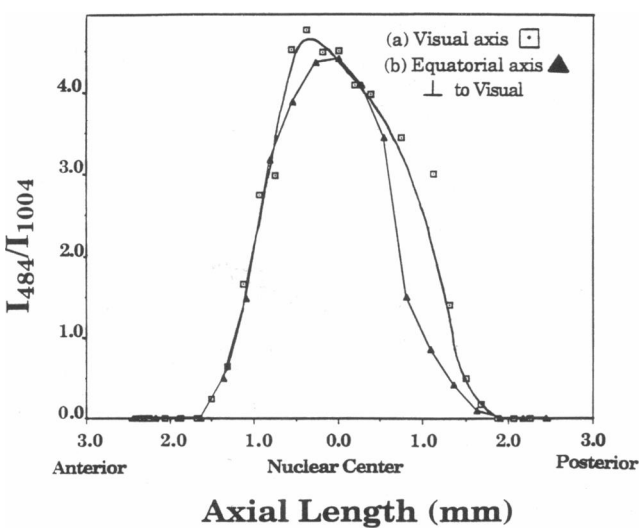
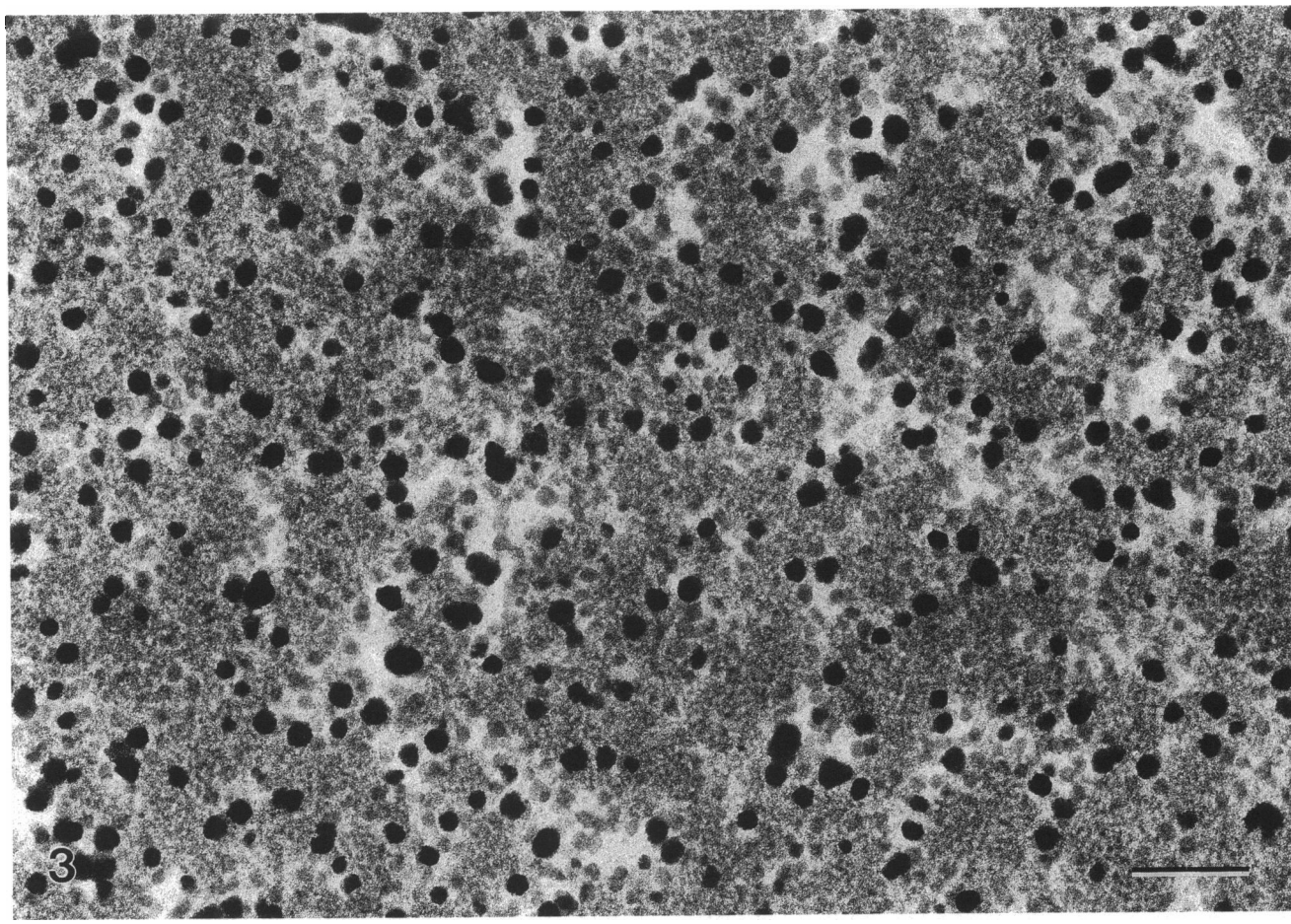


FIGURE 2 Relative concentration of glycogen in the visual and equatorial axis of the ring-neck dove lens. The distribution of glycogen was calculated as a ratio of the height 484 cm^{-1} glycogen line vs the 1,004 cm^{-1} phenylalanine line.

DISCUSSION

The high concentration of glycogen in the lens nucleus of birds of flying habit was discovered by Rabaey (1) who noted that it might serve as a substitute for γ-crystallin, the prominent nuclear protein in most vertebrate lenses. The advantage of this substitution is that a light and oxygen-sensitive protein is replaced by a much more stable carbohydrate not subject to the discoloration and aggregation which affect proteins. Although the glycogen in the lens may contribute to the refractive index, no study has evaluated the relative contributions of glycogen and protein except for the estimate that the pigeon lens nucleus contains 1.7 times as much glycogen as δ-crystallin (14). Also, no detailed study has been made of the glycogen spatial distribution in the pigeon lens other than the initial finding (1, 5, 14) that glycogen is highest in the nucleus, almost as high in the intermediate zone, somewhat lower in the cortex and completely absent from the annular pad. The precise distribution and other morphological characteristics are of interest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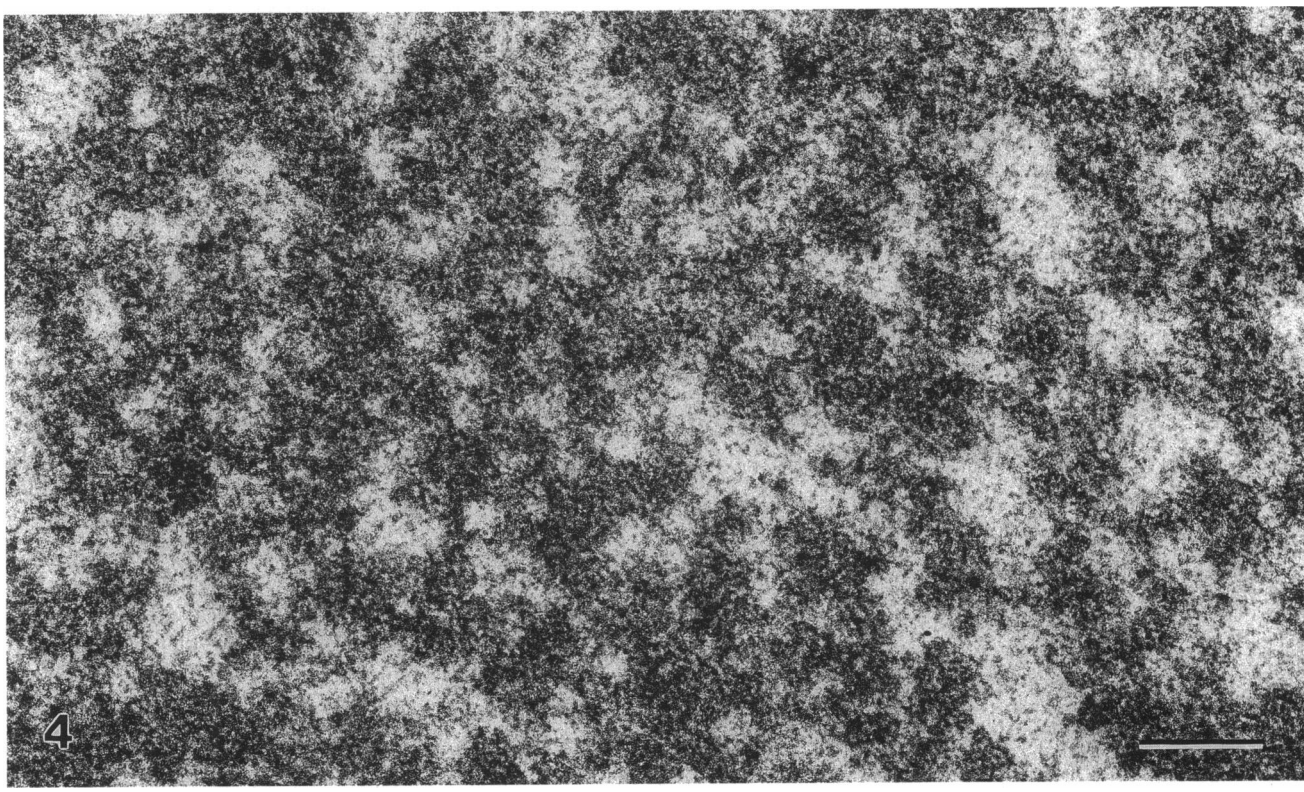
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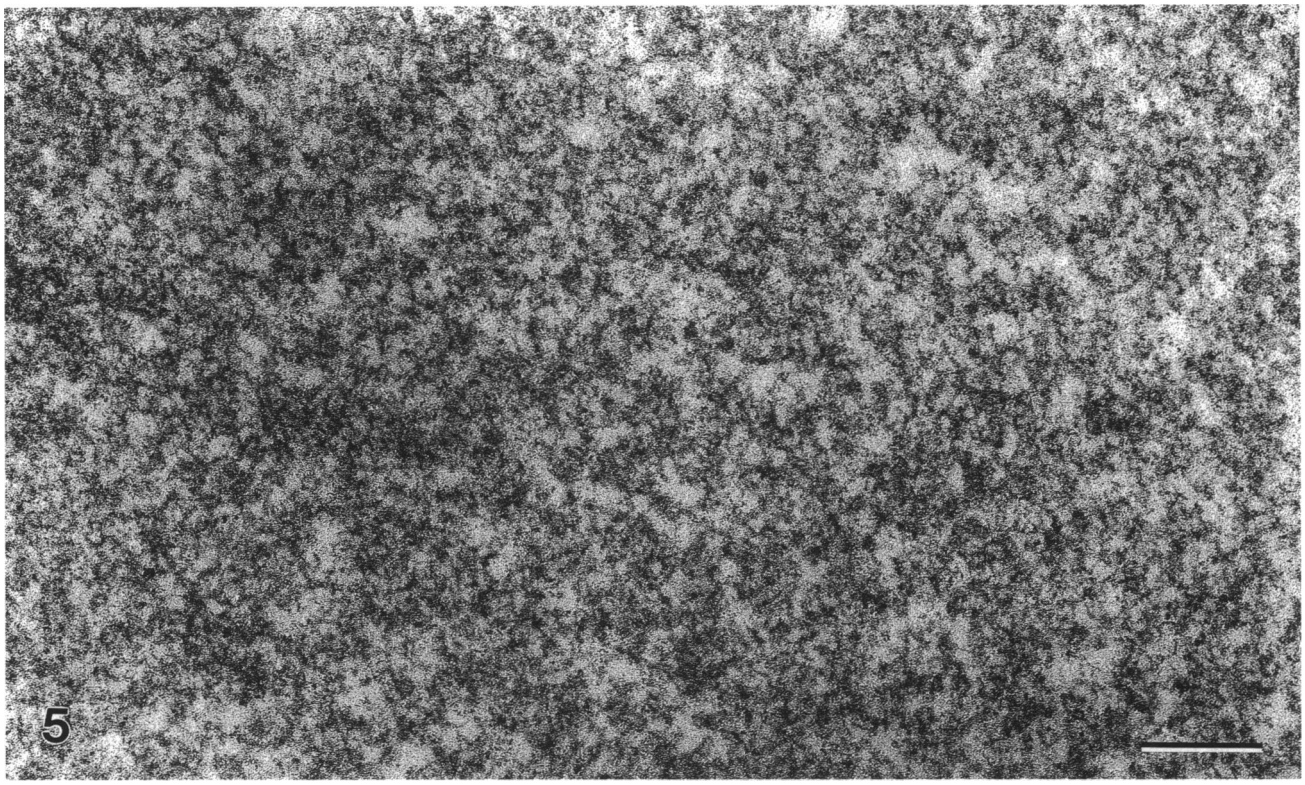

FIGURE 5 TEM of nuclear fiber cells of chicken lens showing the lack of glycogen granules. Bar = 0.2 μm.

as evidence bearing on the function of glycogen as a structural component, a metabolic store or a combination of the two.

Based on electron microscopic examination, two types of glycogen particles are distinguishable morphologically: (*a*) the beta particles usually appear as single granules of 15–40 nm in diameter and (*b*) the alpha particles, or rosettes, whose morphology is comprised of numerous beta particles with a rosette diameter of 60–200 nm (17). Previous studies have shown that alpha particles tend to predominate in cells storing large quantities of polysaccharide (i.e., liver) whereas the beta particles are characteristic of musculature and spermatozoa (9, 17). Our examinations and measurements of the glycogen particles in the dove lens nucleus have led us to believe it to have the beta structure (Fig. 3).

FIGURE 3 Thin-section electron micrograph of ring-neck dove lens showing the distribution and structure of glycogen particles in the nuclear region. Glycogen particles, ~35 nm in diameter, were dispersed randomly within the cytoplasmic matrix and were classified as the beta structure. Bar = 0.2 μm.

FIGURE 4 TEM of fiber cells in the nuclear region of the dove lens after a 2 h enzyme digestion with amylo-1,6-glucosidase. Glycogen particles were completely digested, thereby leaving artificial pockets or gaps in the cytoplasmic matrix. Bar = 0.2 μm.

Our finding that the glycogen particles are of the beta type suggests that the metabolic function is predominant. However, the situation is unique in that the larger alpha particle would be of a size which could scatter light and its existence in the transparent lenses could not be tolerated. Furthermore, it occurs at a much higher concentration in the nucleus where it is less accessible to the metabolically active young fiber cells and the epithelial cells of the annular pad. That is, if glycogen were to act as a reserve carbohydrate in the lens, then it would be stored in the cortical region of the lens where, once broken down into glucose monomers, it could diffuse more easily to the metabolically active young fiber cells or epithelial cells in the superficial regions of the lens. Such a structural function, if true, does not follow the established glycogen classification, previously defined by its morphology (17), but rather its tissue source (18, 19).

Other factors supporting the idea that glycogen has a structural function in the lens are the lack of glycogen phosphorylase activity as well as the location of glycogen

along the visual axis of the nucleus where, because of its relatively high concentration, it contributes significantly to lens refractivity. The asymmetrical shape of the deposit, i.e., longest along the visual axis, would have a greater effect on lens refractivity than a spherical deposit. However, this elongated shape could result from the effect of the annular pad in restricting glycogen deposition from the equatorial cortex; the annular pad is devoid of glycogen at all ages including the embryonic stages. Because the pad is nowhere near the visual axis, the presence of glycogen there would serve no refractive function but it would certainly be available for metabolism, if that were its purpose. It is also apparent that the metabolism of large amounts of glycogen is not necessary in all bird lenses because the lenses of ground-running birds like the chicken have no glycogen. The idea that lens glycogen is primarily a structural material is in conflict with that of Hockwin who emphasized the metabolic role (5). However, it should be pointed out that Hockwin studied glycogen metabolism only in the bovine lens which has the lowest glycogen content of several species observed. It is conceivable that in the bird lens the structural function is dominant while it is negligible in the bovine lens.

The mass of evidence leads to the conclusion that glycogen in the lens nucleus of flying birds is a structural material which serves to maintain the refractive index in a lens which lacks the γ-crystallin of most vertebrates.

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