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SCANNING ELECTRON MICROSCOPIC ANALYSIS OF INTRAOCULAR OSSIFICATION IN ADVANCED RETINAL DISEASE

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Abstract

Chicken eyes from congenic blind (rd/rd) animals showing early, intermediate, and final stages of ossification, similar to the phthisis bulbi condition in man, were examined using scanning and transmission electron microscopy as well as light microscopy and X-ray microanalysis. Early stages of ossification were devoid of mineralized calcium apatite while intermediate and end stages of the disorder contained large amounts of calcium and phosphorus. This process resulted in metaplastic bone formation. An intact Bruch's membrane appeared to separate the choroid from the degenerated pigment epithelium and the developing bone suggesting that its possible origin was metaplasia of the retinal pigment epithelium and the degenerated sensory retina. The end-stage ossification resulted in "phthisic bone" formation which completely filled the vitreous cavity in a manner very similar to the human condition of phthisis bulbi.

Key Words: Ossification, retina, phthisis, calcification, metaplasia, intraocular, microanalysis, chicken, SEM, bone.

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Introduction

Very advanced degenerative lesions of the eye, endophthalmitis or panophthalmitis sometimes result in generalized ocular atrophy followed by phthisis bulbi in man. Phthisis bulbi is characterized by extreme disorganization of all ocular tissues, gradual replacement of the globe by scar tissue and eventual formation of cancellous bone (Hogan and Zimmerman,1962; Yanoff and Fine,1975; Apple and Rabb,1985; Spencer,1985). The process occurs as the final stage of the particular disease which precedes it. Phthisis is irreversible, non-arrestive, and frequently results in severe pain and/or disfigurement, necessitating enucleation.

We recently discovered that a similar phenomenon occurs in a strain of chickens possessing a hereditary retinal degeneration (Kelley et al,1986; Ulshafer et al,1984; Ulshafer and Allen,1985). In humans the condition is variable in its rate of progression, taking from months to years to develop. In this animal model the phthisic process begins around 18 months of age and takes consistently another 10-15 months to fully develop. The condition usually advances faster in one eye than the other but by about 36 months of age the entire vitreous appears to be filled with a dense translucent or opaque matrix. In the current study we examined eyes from affected animals with the scanning electron microscope (SEM) to describe pathological features of the condition. In order to determine if the solidified matrix was indeed bone, we performed SEM X-ray microanalysis (Kevex) of tissues at various stages in development of phthisis. Some tissues were simultaneously examined by conventional transmission electron microscopy (TEM) and light microscopy (LM) for verification of findings.

Methods

Twelve eyes were removed from affected (rd/rd) chickens at various

stages in which opacification had been previously noted (Kelley et al, 1986). The eyes were sectioned through the vertical meridian to verify the condition and fixed for 72 hours at 4° C by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Two of the eyes were selected for further analysis: one eye demonstrated typical final end stage ossification and the other eye displayed both early and middle stages of phthisis. Small pieces from each eye selected for analysis were prepared for TEM by postfixation in 1% osmium tetroxide for 1 hour at room temperature, dehydrated in acidified 2,2 dimethoxypropane, cleared with propylene oxide and embedded in epon araldite. The two hemi-eyes were dehydrated through a graded series of ethyl alcohol and critical point dried in a Bomar dryer. The dried eyes were then mounted on specimen stubs and coated with 40 nm of silver in a Techniques Hummer X sputter coater prior to SEM, elemental analysis and photography. Following SEM, selected areas of the specimens were removed from the specimen stubs and embedded in epon araldite for TEM and LM. Sections 800 angstroms thick were cut on a LKB Ultratome III using a diamond knife and poststained with uranyl acetate and lead citrate. LM was performed on one micron thick serial sections stained with either toluidine Blue or toluidine Blue plus Alizarin Red S. SEM was performed in a Hitachi 450S SEM equipped with a Kevex X-ray analytical unit interfaced to the scope. TEM was performed using a Zeiss EM9S TEM.

Results

Figure 1 shows the X-ray energy spectra that we recorded from various regions of tissue under study, i.e. the spectrum of a particular region resembled either that of "A" or "B" and are so denoted in the electron micrographs which follow. Regions denoted by "A" were found to be high in phosphorus and calcium while regions labeled "B" were comparatively higher in sulfur and chlorine.

An eye undergoing early and middle stages of opacification is presented in Figure 2. The globe was sectioned through the equator and positioned so that one is viewing towards the ora serrata/ciliary body/lens on the right of the micrograph. The ossification process was more advanced in the upper portion of the eye near the choroid where the bony plate is about 1.0 mm thick. This apparently ossified material appeared to have replaced the neural retina and pigment epithelium in this region of the globe, abutting directly on Bruch's membrane. Its X-ray spectrum was similar to that in Figure 1A. Towards the bottom of the specimen no ossification was present (spectrum 1B) and in the middle a transition area occurred. A cyclitic membrane, characteristic of eyes undergoing atrophy (Hogan and Zimmerman, 1962), had formed behind the lens and occupies the right third of the micrograph. A high magnification view of the surface of the cyclitic membrane (Figure 3) showed that cells associated with fibrillar material and having many filopodial processes were present on or in the membrane. This structure produced an X-ray spectrum represented by Figure 1B.

Figure 4 shows a higher magnification of the transition zone, where the forming bony plate abuts Bruch's membrane and choroid. The plate is composed of fibrillar and opaque material having many small holes representing osteocytes. A small nodule of degenerating retinal pigment epithelium (RPE) is visible on the left of the micrograph between the bony plate and Bruch's membrane. When this specimen was embedded in epoxy resin and sectioned for TEM, the presence of degenerating RPE nodules was verified (Figure 5). When LM sections of this zone were stained with toluidine Blue or Alizarin Red S (Figures 6a and 6b, respectively), one could readily differentiate between regions containing high or low levels of calcium phosphate. Small packets of pigmented cells were also noted in these LM sections. A TEM micrograph similarly demonstrated dense apatite crystals and large bundles of collagen (Figure 7), indicating that only partial ossification had occurred in this transition zone. Extensive bands of fibrovascular material were also observed in the cyclitic membrane, however, no calcium phosphate was observed in this structure following Alizarin Red staining.

Higher magnifications of the bony plate are presented in Figures 8-11. The lacunae present in the bony plate appear to contain blood vessels and osteocytes. The close continuity between bone and choroid suggests that these blood vessels may be of choroidal origin and contribute to the formation of fatty marrow found in later stages of ossification (Figures 12,13). TEM verified the presence of osteocytes or other secretory cells within the dense connective tissue matrix (Figure 10). This figure also shows that the dense crystals are condensing on a collagen based matrix. The presence of erythrocytes in some of the lacunae observed at very high magnifications in the SEM (Figure 11), confirms their being blood vessels.

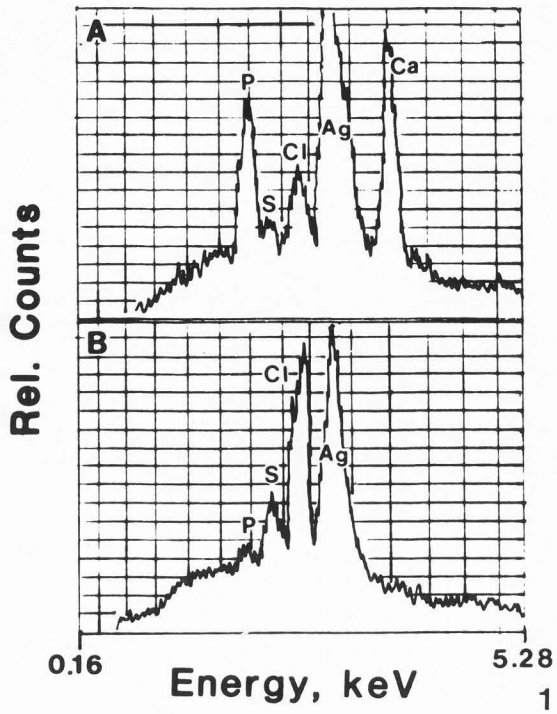


Figure 1. X-ray microanalysis of areas "A" and "B" denoted in several micrographs which follow. The spectrum in A demonstrates areas having high levels of calcium and phosphorus while B's spectrum is comparatively low in these elements but significantly higher in sulfur.

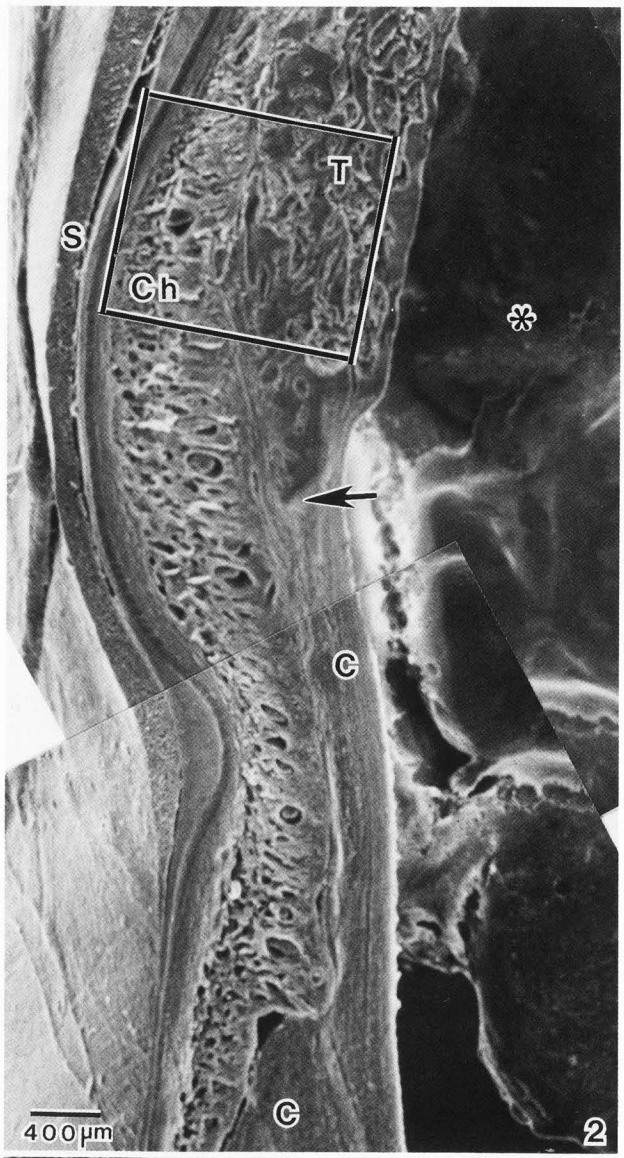


Figure 2. SEM of a chicken eye undergoing ossification. The eye is cut through the equator and is viewed from the vitreous cavity towards the anterior globe. The sclera (S) is the outermost tunic. An ossified trabecular plate (T) is extending from the choroid (Ch) and represents an intermediate stage of ossification. A cyclitic membrane (*) has formed which obstructs our view of the lens and anterior globe. Early stages of phthisis characterized by extensive collagen (C) is shown extending from the choroid in the bottom of the micrograph. When specifically analyzed, the intermediate and early stages of ossification gave X-ray spectra represented by spectra A and B respectively. A transition area (arrow) exists between the early non-ossified and intermediate ossified regions. The boxed area is shown in magnified detail in Figure 8.

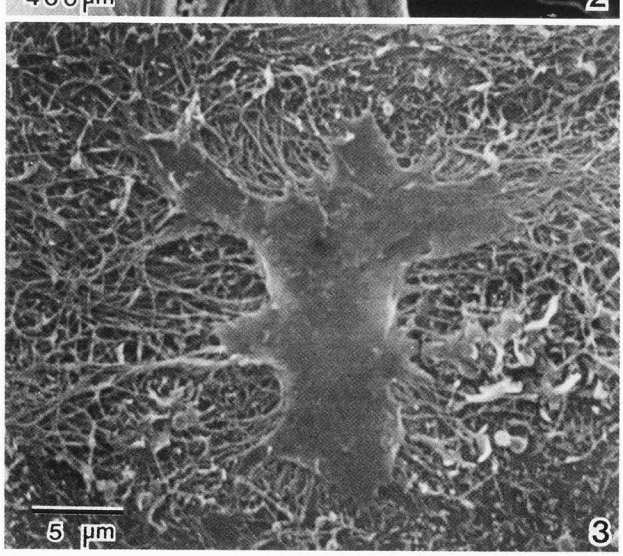


Figure 3. SEM surface view of cyclitic membrane (* in Figure 2). A large cell having many fibrous filopodia is shown. X-ray analysis gives a spectrum represented by spectrum B.

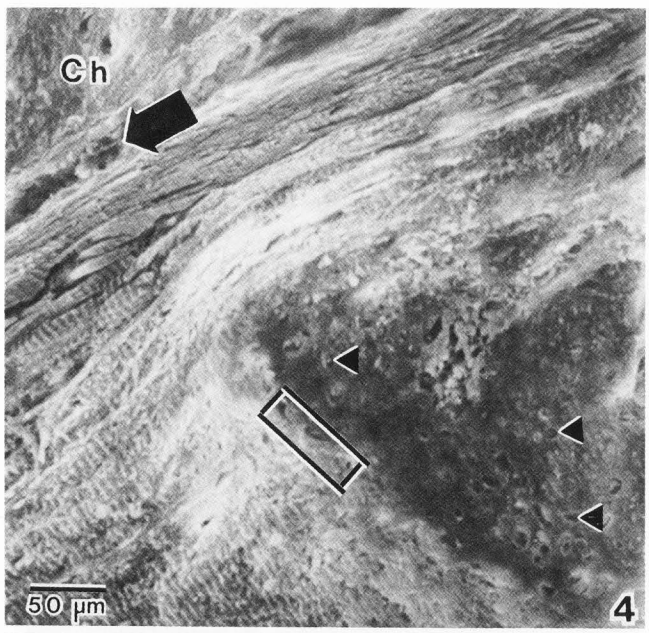
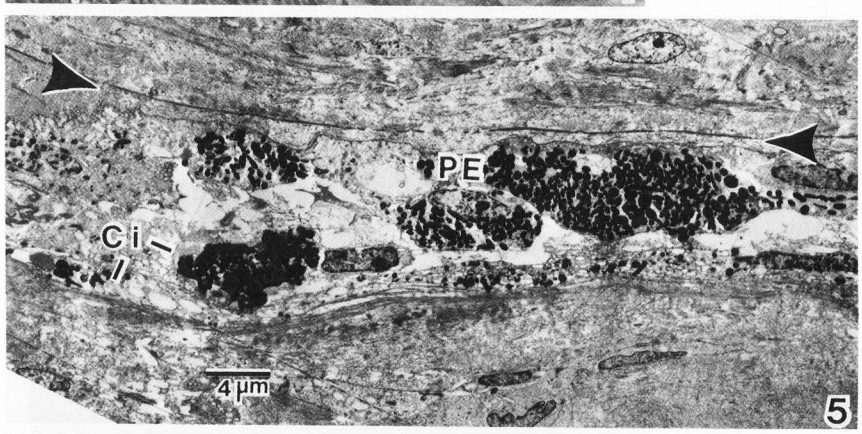
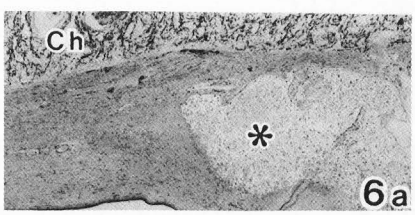


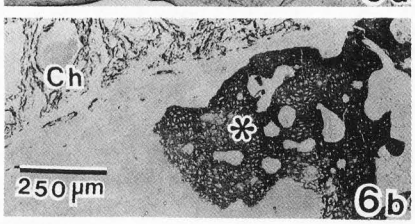
Figure 4. SEM of transition zone (Arrow in Figure 2). The trabeculum, in various phases of ossification, occupies the majority of the view. A pocket of intact RPE (arrow) separates the choroid (Ch) from the trabeculum. The bony plate of the trabeculum contains many small holes (arrowheads), representing osteocytes. Boxed area is shown in Figure 7.

Figure 5. TEM of RPE pocket (arrow Figure 4). An intact layer of degenerating RPE cells (PE), containing melanin granules and resting on Bruch's membrane (arrowheads), is present. Other inclusion-laden cells (Ci) are also seen in the vestigial subretinal space.

Figure 6. LM micrographs of serial sections of Figure 4. (a) Toluidine Blue stain;(b) Toluidine Blue plus Alizarin Red S stain. The ossified region (*) does not stain with Toluidine Blue but does stain with Alizarin Red S indicating the presence of calcium.







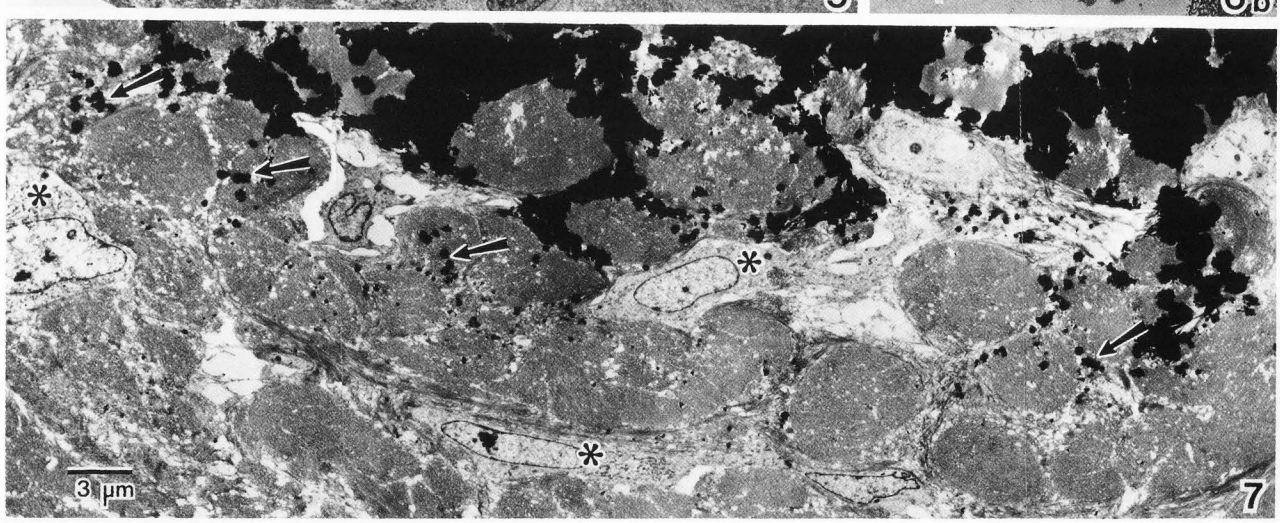
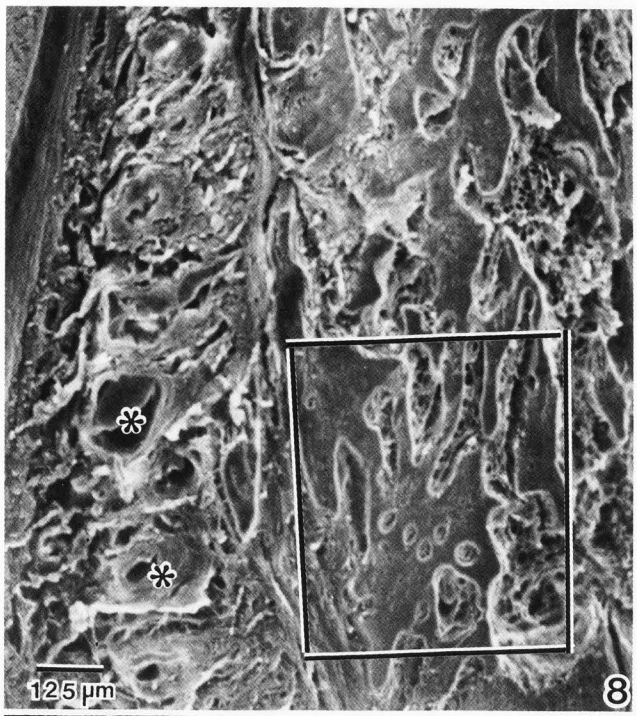
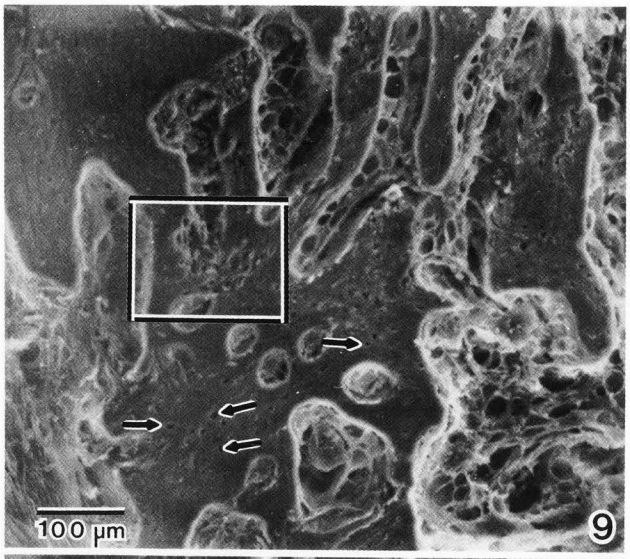
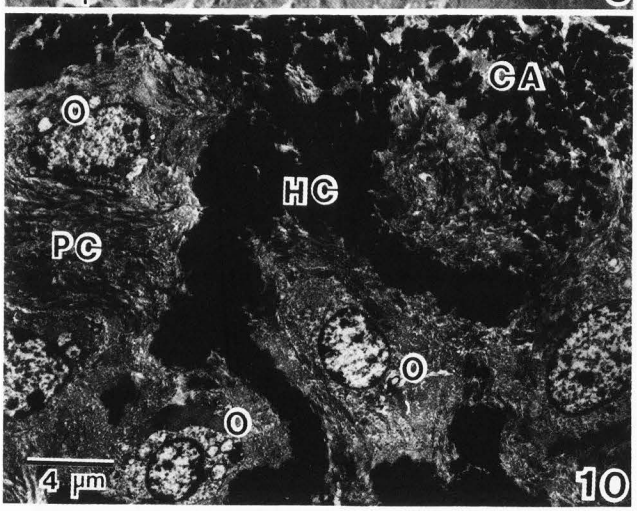


Figure 7. TEM of boxed area in Figure 4 showing large bundles of collagen surrounding small, electronlucent cells (*), probably osteocytes. This collagen matrix is overlain with electron dense calcium apatite crystals in the top of the micrograph. Incipient crystal formation (arrows) is present in the middle of the photograph.







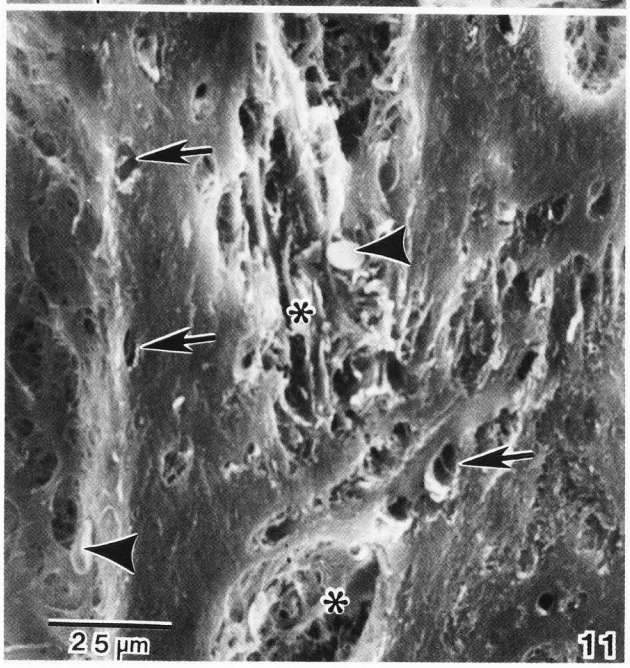


Figure 8. SEM of boxed area in Figure 2. The choroid is degenerating although a few capillaries (*) are still recognizable. In the right of the micrograph is an area undergoing ossification. Lacuna of varying size are present and the matrix surrounding the lacuna are high in calcium and phosphorus (spectrum A). The boxed area is shown in Figure 9.

Figure 9. SEM of the boxed area in Figure 8. The enlarged lacuna indicate the development of fatty marrow. Numerous individual osteocytes (arrows) are scattered throughout the ossified matrix. The entire view is represented by spectrum A. The boxed area is shown in figure 11.

Figure 10. TEM of a calcifying area. Several osteoblasts (O) are identified in the collagen matrices of pre-calcified (PC), calcifying (CA), and heavily calcified (HC) tissue. In the PC region, a framework of collagen fibrils has been secreted between two cells. In the CA region, hydroxy apatite crystals have been deposited among the fibrils while in the HC region dense crystals appear to have completely impregnated the collagenous matrix.

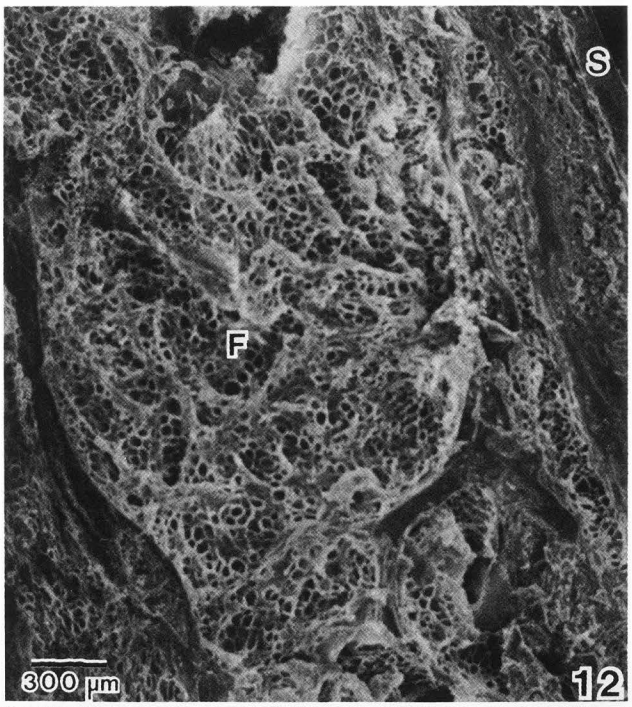
Figure 11. SEM of boxed area in Figure 9. Red blood cells (arrowheads) are present in the blood vessels within the ossified matrix. Osteocytes (arrows) and developing fatty marrow (*) are evident.

In advanced stages of ossification, the bony matrix has completely replaced the choroid and vitreous cavity (Figure 12), resembling cancellous woven bone. High magnification shows that the bone contains a fatty marrow laden with adipocytes, blood vessels and small, bony trabeculae (Figure 13).

Discussion

This report represents the first SEM study of end-stage, advanced ocular disease having a genetic origin. The abnormalities found in the animal model are not unlike that noted in advanced disease such as endophthalmitis in humans, in that phthisis bulbi with ossification is present. In our model, ossification commences only after complete atrophy of the retina and pigment epithelium (Ulshafer et al, 1984; Ulshafer and Allen, 1985). It appears that the entire organ is gradually replaced by a bony matrix composed of fatty marrow and cancellous trabecular bone. The mechanism involved in the initiation of this phthisic condition is questionable. Massive inflammation of the degenerating eye may allow infiltration by osteoblasts possibly from the choroidal vasculature. The presence of a degenerating pigment epithelium between the bony plate and choroid and pigmented cells in the opacifying vitreous in early and intermediate stages of phthisis suggests that metaplasia or transdifferentiation of the RPE cells may have occurred. Stages of bone deposition in the eye include the formation of a collagenous matrix by osteoblasts and the condensation of calcium apatite crystals onto the collagen fibrils eventually covering them to form mineralized bone. In this phthisic condition, the deposition of calcium apatite is not uniform throughout the eye but begins in the vitreous body or atrophied retina adjacent to the choroid. Trabeculae of osseous material then span the vitreous, occupying more and more of that structure until the entire vitreous is replaced by bone.

Metaplasia has been defined as 'the transformation of an adult or fully differentiated tissue of one kind into a differentiated tissue of another kind in response to abnormal circumstances' (Haines and Mohuiddin, 1968). Examination of metaplastic bone development underlying articular cartilage shows that the metaplastic tissue is derived from cartilage by progressive mineralization of the matrix and inclusion of cells without multiplication or hypertrophy. The development of metaplastic bone has received considerable review and



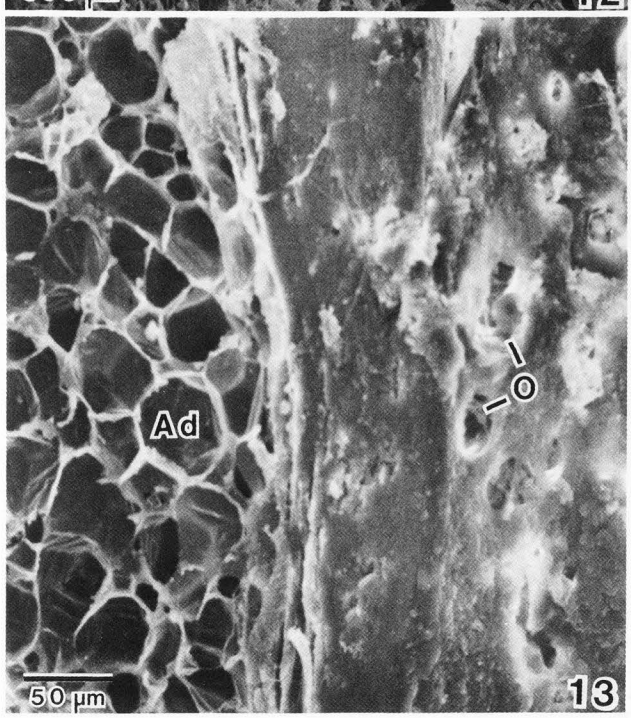


Figure 12. SEM of a piece of chicken eye with advanced ossification. The entire choroid and retina have been replaced by bony material. A fatty marrow (F) containing small trabeculae is present within the bony matrix. Sclera (S).

Figure 13. SEM of fatty marrow (left) and trabeculum (right). Many thin-walled adipocytes (Ad) constitute the marrow. The trabeculum contains many osteocytes (O).

discussion without definitive conclusions however,certain criteria are required for metaplastic bone: It has been described as resembling unglazed porcelain and being denser and firmer than ordinary bone. It stains like other bone; has similar chemical properties and is sharply demarcated from the unaltered soft tissues (Haines and Mohuiddin,1968). One may describe metaplastic bone with reference to its location or tissue of origin eg. articular bone, tendon bone, ligament bone etc. Such descriptions would parallel the customary approach when reference is made to periosteal, endosteal and endochondral bone, thereby incorporating the tissues that form these kinds.

There is sufficient evidence presented to refer to the bone formation in this animal model as a metaplastic type of bone. While definitive origins of the ossification process are not aptly demonstrated, evidence does associate the osseous metaplasia with the pigment epithelium. There was no evidence of a cartilage model preceding the bone in this study. Conformation to the above conventions in descriptive nomenclature of the bone would suggest "epithelial bone" however this conclusion may be premature. The osseous formation described here is similar to phthisis bulbi in humans and the type of bone formed in this process might more aptly be termed "phthisic bone" thereby referring to the condition rather than the tissue which precedes it.

Acknowledgments

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Discussion with Reviewers

G.K. Klintworth: The X-ray spectra shown in Figure 1 appear to have been taken from part of the eye that includes the sclera. Since the sclera of chickens normally contains bone, what steps were taken to exclude the contribution of endogenous scleral ossification from the intraocular ossification of phthisis bulbi?

Authors: The X-ray spectrum shown in Figure 1B was not specifically taken from a region containing sclera but depicts a generalized spectrum representative of all the spectra taken from areas of the specimen in which no calcium was detected. Likewise, the spectrum showing high calcium and phosphorus is also a generalized spectrum representative of all the spectra taken from areas of the specimen which did show calcium and phosphorus.

P.Versura: What information has SEM added (with respect to other morphological procedures) in evaluating the progression of mineralization process?

Authors: The differences between the mineralized "bony" areas of the specimen and the non-mineralized collagen laden areas as well as the choroid are clearly differentiated by the use of SEM. It is also possible to identify the transition area, that area of non-mineralized collagen interfacing with mineralized collagen, using the SEM. The X-ray analysis is not necessary for the determination of mineralized regions but serves to confirm the absence or presence of mineral.