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Abnormal accumulation of sulphated materials in lens tissue of mice with the aphakia mutation

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SUMMARY

Sulphated materials were tested for in the eyes of late gestational and postnatal normal mice and mice with the aphakia mutation using Spicer's high iron diamine staining method. Qualitative identification of these materials was attempted with bovine testicular hyaluronidase and nitrous acid digestion methods. The grossly abnormal morphology of the aphakia lens made it necessary to confirm identification of lens-derived tissue by testing for lens crystallins using standard immunohistological methods. As seen in normal mouse lens maturation, accumulated inter- and intracellular sulphated materials were observed in aphakia lens tissue from just before birth through juvenile maturation. Large cyst-like structures consisting of lens-derived tissue were commonly seen in the eyes of young postnatal mutant mice. Sulphated materials formed basal lamina-like structures on many of these lens-derived units, but a well-defined lens capsule never formed. Abnormal fibrillar structures rich in sulphated materials were seen in the intraocular cavity in many older mutant specimens, most of which were largely resistant to both digestion methods. These results indicate that the potential to elaborate sulphated materials qualitatively similar to those seen in normal mouse lens maturation is present in the aphakia mutant, although the mode of accumulation is grossly disturbed.

INTRODUCTION

The aphakia mutation in mice, first described by Varnum & Stevens (1968), involves abnormal differentiation and maturation of the ocular lens (Zwaan & Kirkland, 1975; Zwaan, 1975). Early morphogenesis of the lens rudiment in this mutant strain is relatively normal; however, morphological abnormalities are seen in this tissue shortly after lens vesicle formation (Zwaan & Kirkland, 1975; Zwaan & Webster, 1984).

Compositional changes in the extracellular matrix between the optic vesicle and presumptive lens are temporally correlated with morphogenetic changes in these tissues in normal mice (Webster, Silver & Gonsalves, 1983). Histochemical

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abnormalities involving this extracellular matrix have been observed during and correlated with the development of congenital eye defects in the anophthalmia (Webster, Silver & Gonsalves, 1984) and aphakia (Zwaan & Webster, 1984) mutants in mice. In the aphakia mutant, intercellular sulphated glycosaminoglycans are observed in the lens rudiment from the lens cup through the lens vesicle stages. The appearance of these abnormal intercellular materials is temporally correlated with the malorientation of many mitotic figures (Zwaan & Kirkland, 1975) and abnormally large intercellular spaces seen at the ultrastructural level (Konyukhov, Kolesova & Nonchev, 1983) in these mutant lens rudiments.

The abnormal lens rudiments of the aphakia mice have basal lamellae that are ultrastructurally (Zwaan, 1983; Konyukhov *et al.* 1983) and histochemically (Zwaan & Webster, 1984) similar to those seen as precursors to the capsule during early lens morphogenesis in normal mice (Webster *et al.* 1983; Zwaan & Webster, 1984). However, a thick and well-defined lens capsule does not form in these mice.

During normal mouse lens maturation, discrete zones of sulphated materials in the lens capsule become increasingly apparent as the capsule becomes increasingly thicker (Webster, Searls, Hilfer & Zwaan, submitted). While it is readily apparent that the collagenous component in older aphakia lens 'capsules' is largely deficient or lacking, the situation of the sulphated components is not known. The results reported here are to describe the temporal appearance and localization of sulphated materials with properties similar to those seen in normal mouse lens maturation in older prenatal and postnatal aphakia mice.

MATERIALS AND METHODS

Mutant strain aphakia mice were routinely mated (Zwaan, 1975) in the animal rooms at the Massachusetts Eye and Ear Infirmary and normal eyed Icr:Ha(ICR) timed-pregnant females were obtained from the Fox Chase Institute for Cancer Research (Philadelphia, PA). Pregnant females were sacrificed by exposure to chloroform to obtain 16 and 19 days gestation specimens; postnatal mice of 7, 15, 30 and 90 days were similarly sacrificed. Tissues were prepared for sectioning and histochemical analysis as described (Webster *et al.* 1986). Also, normal mice at each age were autoradiographically tested for $Na_2{}^{35}SO_4$ incorporation into the lens capsule as described (Webster *et al.* 1986). Sulphated materials were tested for histochemically using Spicer's high iron diamine staining method (Spicer, 1965).

Selected sections were challenged with total mouse lens crystallin antisera (Zwaan, 1983) and the reactions were visualized by the indirect immunofluorescent method as described by Ikeda & Zwaan (1967) or by the indirect immunoperoxidase method as described by Wordinger, Miller & Nicodemus (1983). The morphology of postnatal aphakia lens tissue is usually extremely disturbed; immunohistological testing for lens crystallins was done to ensure that tissues or cells being evaluated were of lens origin.

Bright- and dark-field photomicrographs were filmed on Kodak 2415 technical film and immunofluorescent photomicrographs were filmed on Kodak Tri-X film using a Leitz Orthoplan microscope equipped with a high-pressure mercury arc lamp and Ploemopak incident-light fluorescence. Photomicrographs were printed on Ilford resin-coated paper.

RESULTS*

16 days gestation

Normal lenses at this stage demonstrated a regular spherical geometry (Fig. 1A), and autoradiographic evidence indicated that sulphate was being incorporated into the lens capsule area (Fig. 1B). Histochemically, the capsule was strongly high iron diamine positive (HID+) (Fig. 2).

Aphakia lenses observed at this stage were morphologically abnormal in that they were usually contorted and lacked a regular spherical geometry. Patches of cells were positive for lens crystallins in most of the abnormal lens rudiments, appearing as a mosaic of fluorescence (Fig. 3). Two of the specimens contained lenses in which all of the cells were positive for crystallins. Patches of very weakly staining HID+ materials were occasionally seen on the lens rudiment basal lamina, most often near the pupillary margin of the optic cup/lens interface (Fig. 4). Intercellular HID+ materials were rarely seen, and stained very weakly when present. Intracellular HID+ materials were not obvious at this stage.

19 days gestation

All mutant specimens examined contained single lens-derived structures. Crystallins were seen in some, but not all, of the specimens. Most lens rudiments contained some cells that were positive for crystallins and others that were not (Fig. 5A). Fluorescence patterns were not identical in cells of the same specimen. Occasionally, a uniformly fluorescent region was bordered by a fibrillar fluorescent region (Fig. 5B).

Normal lens capsules at this stage were strongly HID+ (Fig. 6A); although the staining appeared to be uniform throughout the regions of the lens capsule, there were differences in staining intensity and capsular thickness between anterior, equatorial and posterior regions. In the mutant specimens, patches of strongly staining HID+ materials were observed on the outer parts of most lens rudiments at areas where the presumptive capsule would normally be. Intercellular deposits of HID+ materials were also seen in lens tissue of most specimens (Fig. 6B,C). These materials in both normal and mutant specimens were largely resistant to both nitrous acid and hyaluronidase digestion methods.

7 days postnatal

Most mutant specimens seen at this stage possessed single lens-derived units, while a few demonstrated what appeared to be fragmented or fragmenting lens rudiments. Large cyst-like structures were seen in lens tissue of some of the specimens at this stage. These structures contained morphologically different cells. Typically, the outer cells were morphologically squamous or low cuboidal

* Highlights of sulphate accumulation in normal lenses will be presented where appropriate to permit direct comparison with abnormalities seen in the aphakia mutant. A more detailed analysis of sulphate incorporation and accumulation in normal maturing mouse lenses can be found elsewhere (Webster *et al.* 1986).

epithelial and non-vacuolated, while the inner cells were mesenchyme-like and vacuolated (Fig. 7). The inner cells were sparsely distributed in larger cysts. Some, but not all, of the cells in these structures were positive for lens crystallins (Fig. 8).

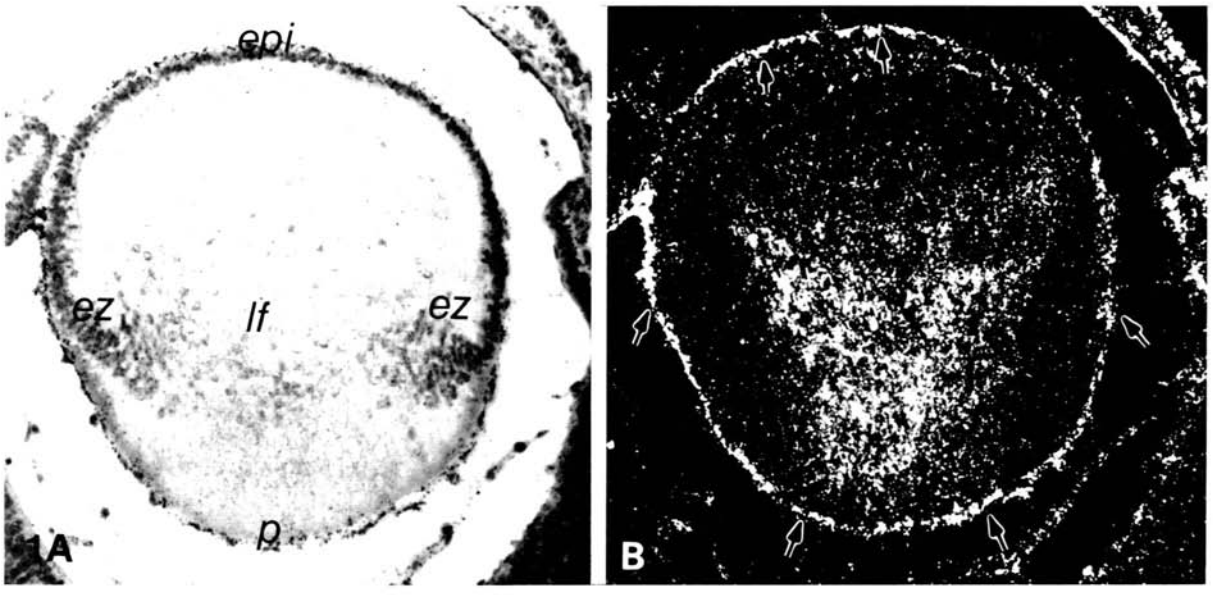

Fig. 1. 16 days gestation normal lens tested autoradiographically for sulphate incorporation under bright- (A) and dark- (B) field illumination. The lens has a regular spherical geometry, and newly incorporated sulphate can be seen in the lens capsule area (B, arrows). *epi*, lens epithelial cells; *ez*, equatorial zone; *lf*, lens fibres; *p*, posterior lens capsule. Both ×100.

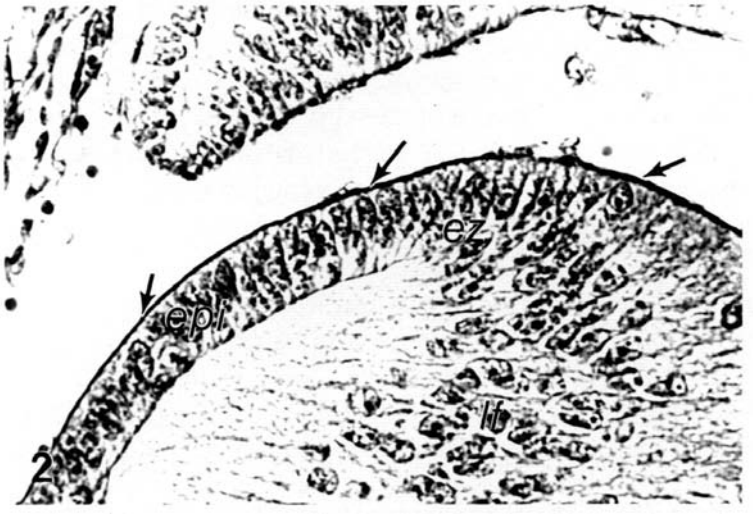

Fig. 2. 16 days gestation normal lens tested histochemically for sulphate using the HID method. The capsule (arrows) is strongly staining throughout. *epi*, lens epithelial cells; *ez*, equatorial zone; *lf*, lens fibres. ×800.

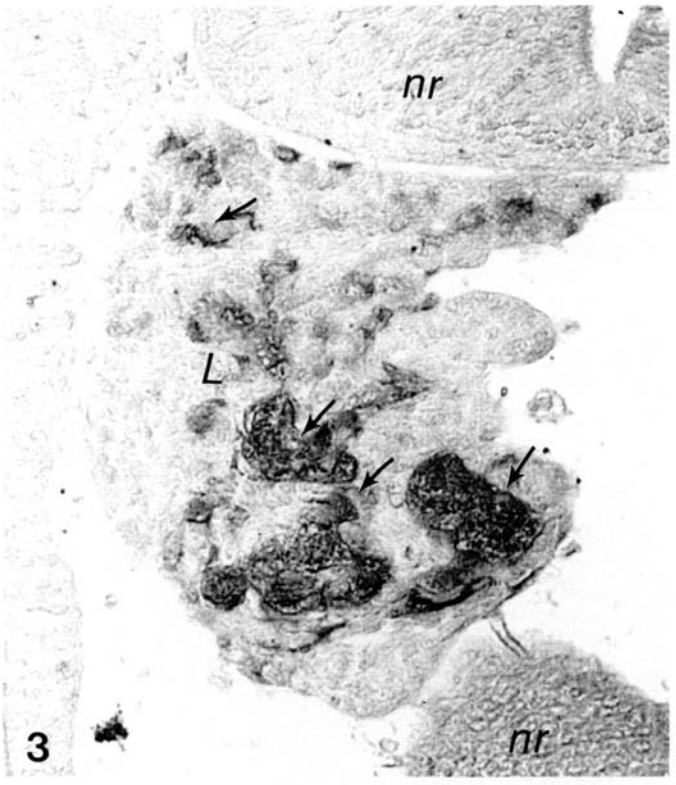

Fig. 3. Section from a 16 days gestation aphakia lens tested for lens crystallins by the immunoperoxidase method. Patches of cells demonstrate lens crystallins (arrows) within this abnormal rudiment. *nr*, neural retina; *L*, lens. ×600.

Normal lens capsules were thicker in all regions than at earlier stages, and were strongly HID+ (Fig. 9A). As seen at earlier stages, there were differences in staining intensity in the different regions of the lens capsule (see Webster *et al.* 1986, for more details). The outside of the lens tissue in most mutant specimens contained strongly staining patches of HID+ materials, forming what appeared to be a partial basal lamina (Fig. 9B). Intracellular granular deposits of HID+ materials were also seen in most of these cells. Through observations of serial sections, it was found that cells demonstrating these intracellular HID+ materials also contained lens crystallins. These materials in both strains were largely resistant to both digestion methods.

15 days postnatal

While most mutant specimens at this stage demonstrated single lens-derived rudiments, there was evidence of fragmentation in a few cases. Lens crystallins were seen in some, but not all, cells of each specimen. Morphologically, some cells appeared elongated and, together with adjacent cells, formed roughly spherical three-dimensional structures that were variable in size and resemble hollow cysts (Fig. 10). HID+ materials were seen in the cell-free interiors of some of these

cysts. Most of the lens-derived cysts contained a basal lamina-like region that stained strongly for HID+ materials (Fig. 11), and slight fibrillar strands of HID+ materials were frequently seen in the adjacent intraocular cavity. Intracellular granular HID+ materials were also present in many of these cells, both in those demonstrating crystallins and in those that did not (as seen through serial sections). Inter- and intracellular HID+ materials in both strains were largely resistant to both digestion methods.

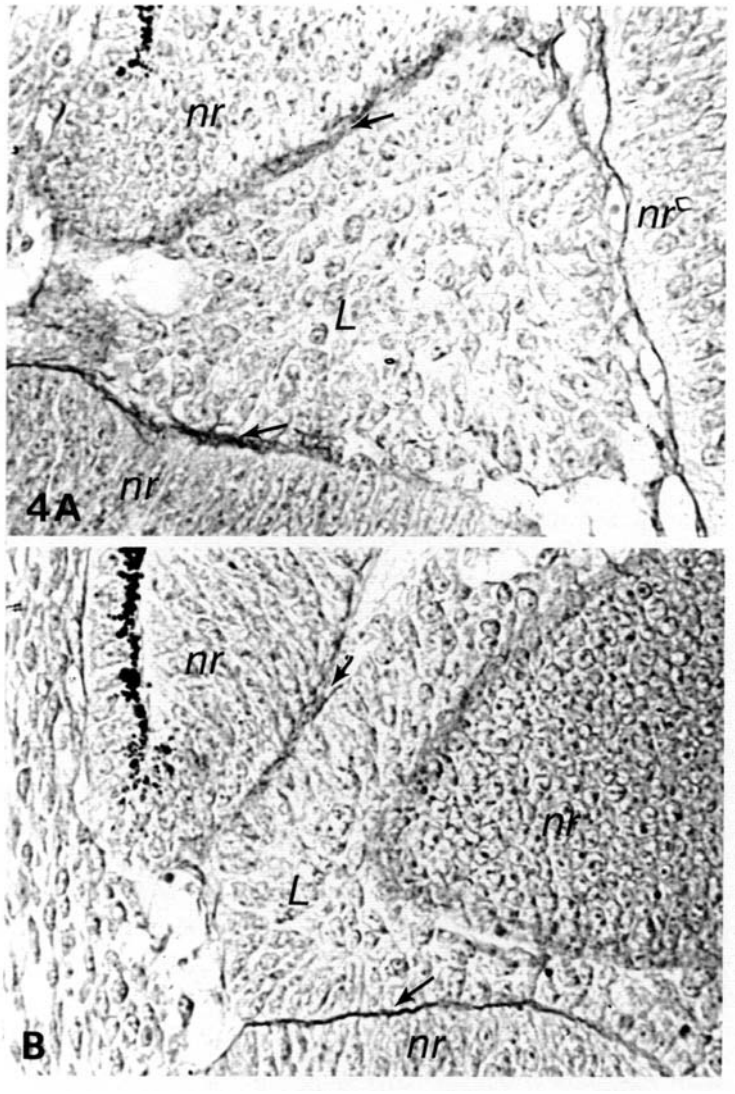

Fig. 4. Sections from 16 days gestation aphakia specimens stained with HID and nuclear fast red. Aphakia lens rudiments at this stage demonstrate wide variations in lens morphology. Neither section demonstrates intercellular sulphate in the lens rudiment. Moderately strong staining is seen between the tips of the optic cup at the site of the presumptive ciliary body and the adjacent lens rudiment (arrows). *nr*, neural retina; *L*, lens. Both ×600.

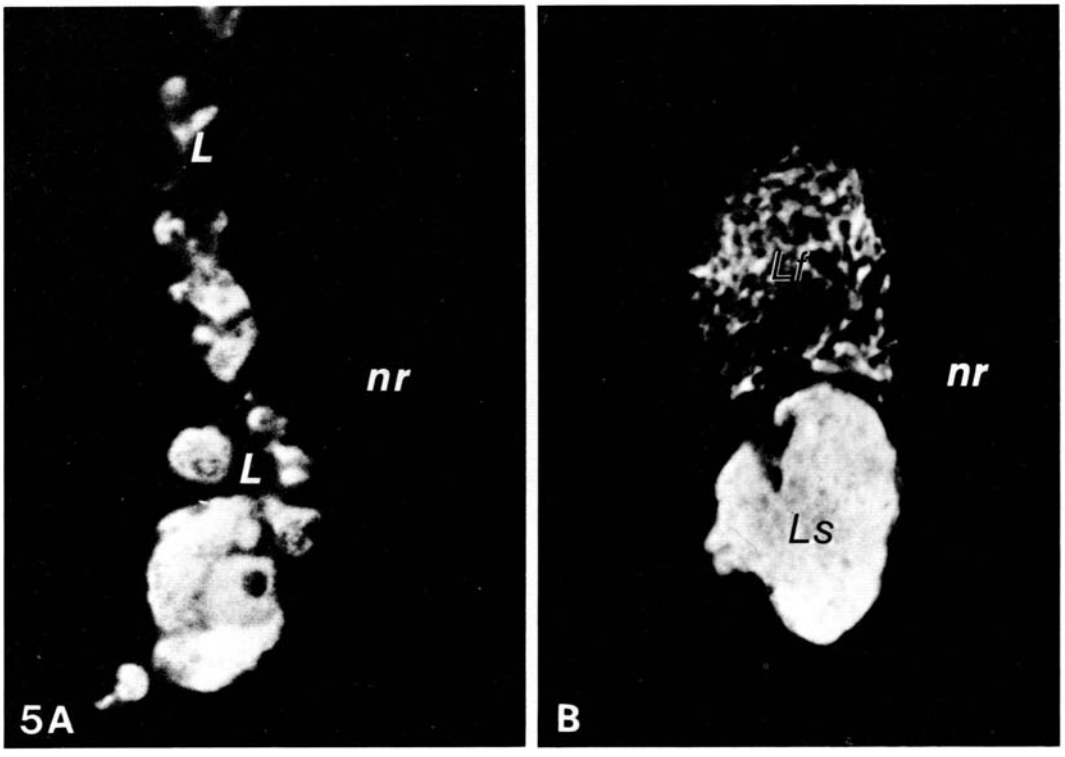

Fig. 5. (A) Section from a 19 days gestation aphakia specimen tested by the immunofluorescent method for lens crystallins. This lens rudiment (*L*), crushed between layers of the neural retina (*nr*), demonstrates a mosaic of fluorescence. (B) Section from a 19 days gestation specimen demonstrating two (morphologically) distinct types of fluorescence for lens crystallins. A solidly fluorescent region (*Ls*) is bordered by a fibrillar fluorescent region (*Lf*). The reason for this difference is not known. Both ×550.

30 days postnatal

Lens-derived units of most mutant specimens at this age were fragmented, although a few appeared to be single structures. Patches of cells within these units were positive for lens crystallins, and many of these crystallin-positive cells also demonstrated intracellular HID+ materials. Each lens-derived unit also demonstrated a basal lamina-like structure that was strongly staining for HID+ materials. Fibrillar strands of HID+ materials were frequently seen in the presumptive vitreous cavity adjacent to these lens-derived units. Again, these materials were largely resistant to both digestion methods.

Aphakia control slides tested by the immunoperoxidase method demonstrated a moderate level of background staining in lens-derived tissue. Test slides treated with crystallin antisera demonstrated unmistakeably stronger reactions, probably due to a combination of the inherent background staining plus the specific reaction for the crystallins. Background staining of this type was not seen with the immunofluorescent method, and attempts to reduce the background in sections

tested by the immunoperoxidase method using conventional methods (e.g. pretreatment of sections with preimmune serum or 1 % serum albumin) were futile. The background seen with the immunoperoxidase method may be due to some type of endogenous peroxidase activity present in aphakia lens-derived tissue at this time.

90 days postnatal (adult)

Normal lens capsules were significantly thicker than at earlier stages and discrete laminar zones were histochemically evident in the capsule. The type of zonation seen differed from anterior and equatorial regions from that seen in the posterior region (Fig. 12).

All mutant specimens observed at this stage demonstrated fragmented lens rudiments. Scattered cells were positive for lens crystallins (Fig. 13), and most of these cells also demonstrated intracellular deposits of HID+ materials. Moderate background staining using the immunoperoxidase method, similar to that seen at 30 days postnatal, was also evident at this stage; this background staining was not seen with the immunofluorescent method. Fibrillar strands of HID+ materials were seen in all specimens, some of which virtually spanned the diameter of the intraocular cavity (Fig. 14). HID+ materials formed basal lamina-like structures around some of these lens-derived masses. All of these HID+ materials in both strains were found to be largely resistant to both digestion methods.

DISCUSSION

Technical considerations

Cetylpyridinium chloride (CPC) was purposely not added to the fixative used in this study. CPC binds to polyanions, particularly sulphate and carboxylate groups (Scott, Quintarelli & Dellovo, 1964). CP-compounds have been successfully used to precipitate sulphated glycoconjugates for biochemical analysis after extraction and solubilization of these materials from tissue (Toole & Gross, 1971; Kosher & Searls, 1973). They have also been successfully used to preserve apical cell surface anionic glycoconjugates for scanning electron microscopic observation in chick embryos (Hilfer & Yang, 1980; Yang & Hilfer, 1982). In each case, the rationale behind the use of CP-compounds was to precipitate soluble anionic glycoconjugates. In theory, this reagent would seem ideally suited for maximal preservation of anionic glycoconjugates for tissue sections; in principle, it probably does. In practice, where visualization of a coloured product is the criteria for a positive reaction, CPC is terrible to use. The reason for this is as follows: CPC binds to sulphate and carboxylate groups and effectively blocks these functional groups from reacting with the staining reagents (Scott *et al.* 1964; Quintarelli & Dellovo, 1965). Even though these materials may be more effectively preserved in sections, they cannot be seen. Previous testing using CPC in a fixative for extracellular matrix histochemical analysis in mouse embryos (Webster, A. Silver & Gonsalves,

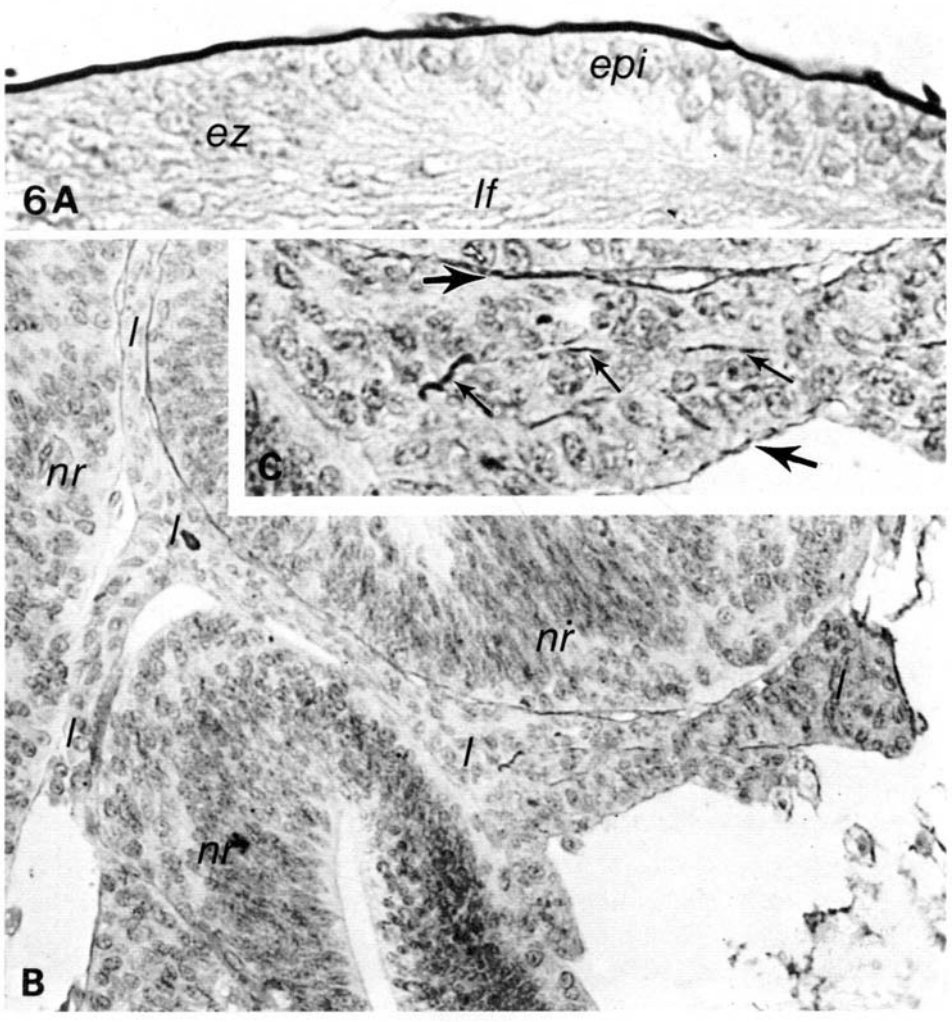

Fig. 6. Sections from 19 days gestation specimens stained with HID and nuclear fast red. (A) The normal lens demonstrates a thick and uniformly staining lens capsule over both epithelial cells (*epi*) and lens fibres (*lf*) over the equatorial zone (*ez*). (B) This aphakia lens is extremely contorted between layers of the neural retina (*nr*); (C), an enlargement of (B), demonstrates both intercellular sulphated materials (small arrows) and sulphated materials at the region of the presumptive lens capsule (large arrows). (A) ×1000. (B) ×375. (C) ×725.

unpublished results) confirmed the blocking effect earlier described (Scott *et al.* 1964; Quintarelli & Dellovo, 1965).

The high iron diamine method is reportedly specific for sulphated materials (Spicer, 1965). Autoradiographic results of localized $^{35}SO_4$ incorporation into normal mouse eye lens agrees well with observed HID+ staining patterns in this tissue (Webster *et al.* submitted); this parallel between autoradiographic labelling patterns and HID+ staining patterns has also been seen in cartilage, cornea

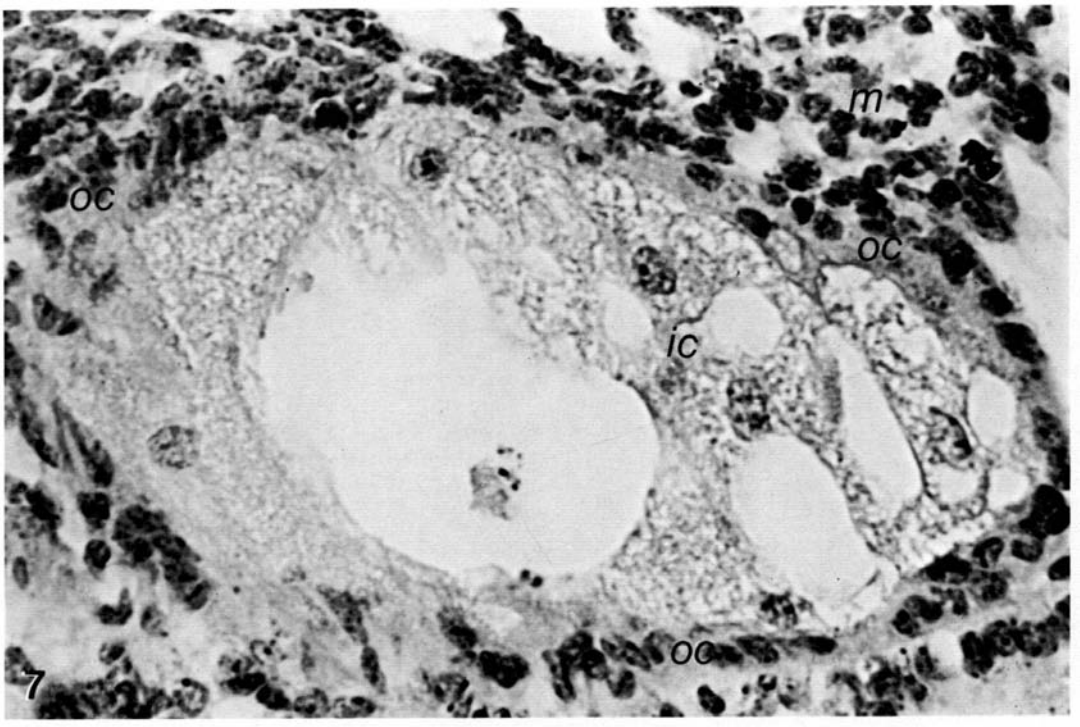

Fig. 7. Section from a 7 days postnatal aphakia eye stained with nuclear fast red, only. Cyst-like structures consisting of morphologically distinct cell types are clearly seen. The outer cells (*oc*) appear to form a squamous-like epithelium around the inner cells (*ic*); the inner cells are mesenchyme-like, appear vacuolated and may be necrotic. True mesenchyme cells (*m*) are seen adjacent to the cyst. ×800.

(Webster, Searls & Hilfer, unpublished results) and in other tissues (see Spicer, 1965, for references). Based on these parallel observations and the nature of the reagents used (Spicer, 1965), it seems reasonable to refer to the HID+ materials seen in aphakia lens-derived tissue as sulphated materials.

Consideration of results

Normal maturational changes in the aphakia lens rudiments, both biochemical and morphological, are markedly altered. During the early stages of lens morphogenesis, a relatively normal invagination of lens ectoderm into a lens vesicle is followed by a lack of lens fibre cell morphogenesis (Zwaan, 1975; Zwaan & Kirkland, 1975; Zwaan & Webster, 1984) and lens crystallin appearance (Zwaan, 1975; Malinina & Konyukhov, 1981). Subsequently, α-crystallin appears in some, but not all, cells in these morphologically abnormal lens rudiments at least 3 days later than in normal mice (Zwaan, 1980; Malinina & Konyukhov, 1981). β and γ-crystallins, markers of lens fibre cell differentiation, are observed much later than normal in some cells of lens origin from just before birth through early postnatal development (Zwaan, Webster & Cooper, in preparation). The implications from these data is that while morphogenesis of this tissue does not have to

be followed by differentiation (since a morphologically normal optic cup and lens vesicle form in the aphakia mutant), the differentiation of this tissue, likewise, does not have to be preceded by normal morphological development (since the fibre cells do not undergo normal morphogenesis but cells expressing their differentiative markers do appear later). The separation of morphogenesis and differentiation during development of an organ anlage has also been observed elsewhere. *In vitro* work with developing pancreas (Spooner, Cohen & Faubion, 1977) has demonstrated that α-amylase, a digestive enzyme indicative of differentiation in this tissue, is synthesized by cultured cells that have not undergone normal morphogenesis. Results from aphakia lens-derived tissue, which is essentially in an *in vivo* culture system, clearly indicate that morphogenesis and differentiation can be separated into discrete yet normally temporally coinciding developmental phenomena during ocular morphogenesis. The extent to which this separation of morphogenesis and differentiation can be applied to other tissues remains to be shown.

Sulphated materials, resistant to both testicular hyaluronidase and nitrous acid digestion, were observed histochemically and autoradiographically in normal mouse lenses from mid-foetal stages through maturation.

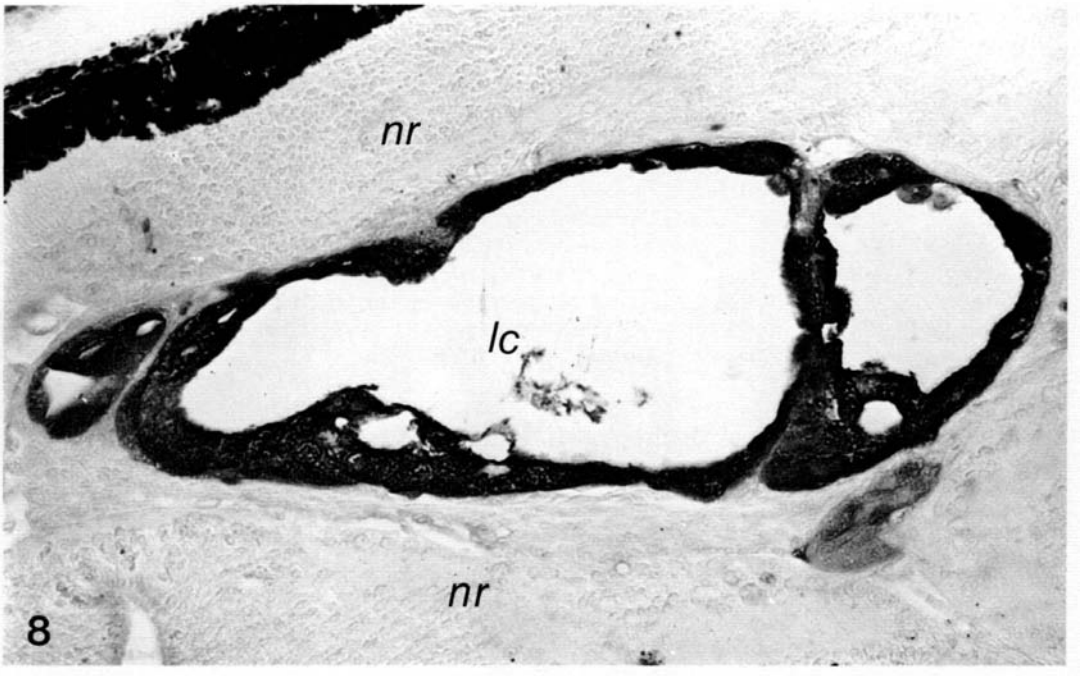

Fig. 8. Section from a 7 days postnatal aphakia eye tested for lens crystallins by the immunoperoxidase method. Numerous compartments, referred to in this paper as cysts, are seen to comprise lens-derived tissue. Virtually all of the cells comprising these cysts, both inner and outer, demonstrate crystallins. *nr*, neural retina, *lc*, lens cyst. ×175.

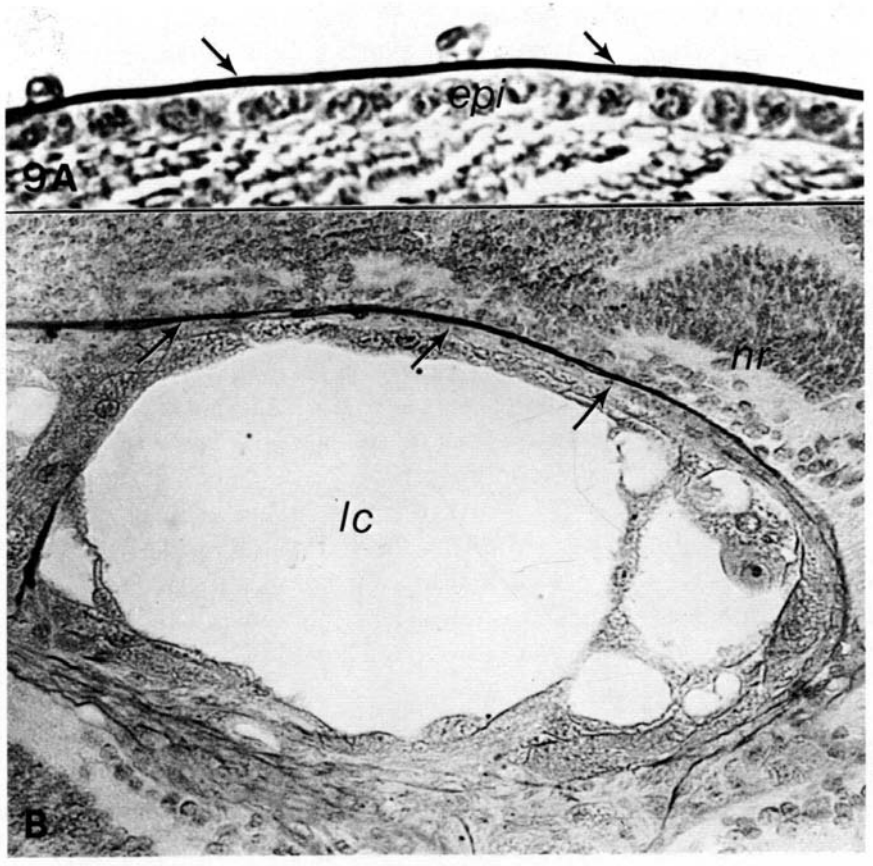

Fig. 9. (A) Normal-eyed 7 days postnatal specimen stained with HID and nuclear fast red. The anterior lens capsule over the lens epithelial cells (*epi*) is rather thick and strongly staining. (B) A serial section from the same mutant specimen as seen in Fig. 8 stained with HID and nuclear fast red. Strongly staining sulphated materials appear to form a basal lamina at the top part of these cysts (arrows); this is not seen in the bottom portion. *nr*, neural retina; *lc*, lens cyst. (A) ×800. (B) ×250.

Sulphated materials, with properties similar to those seen in normal mouse lenses, were found to accumulate at slightly later developmental stages in the aphakia mutants than in normal lenses. In aphakia lens-derived tissue, these sulphated materials were observed inter- and intracellularly and formed basal lamina-like structures on many lens-derived units at the site of the presumptive lens capsule. This mode of accumulation was also seen in normal mouse lens maturation (Webster *et al.* 1986). Based on the appearance of these sulphated materials with properties that differ from those seen with glycosaminoglycans (Webster, Silver & Gonsalves, 1983; Kosher & Searls, 1973; Toole & Gross, 1971) with respect to enzyme susceptibilities, it may well be that these materials may represent sulphated glycoproteins that are further differentiative markers of lens. Heathcote & Orkin (1984) recently described eight different sulphated glycoproteins that are secreted by rabbit lens epithelial cells into culture medium; these

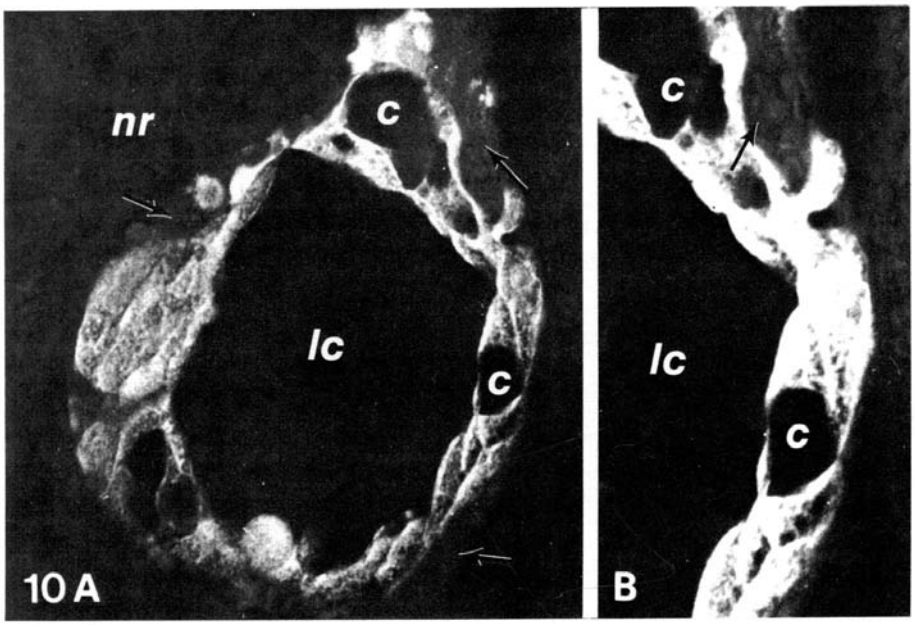

Fig. 10. 15 days postnatal aphakia lens cyst tested immunofluorescently for lens crystallins. As seen in (A), a large cyst is surrounded by elongated cells (more easily seen in the enlargement in (B)) and several smaller cysts (*c*) are seen between cells of the cyst wall. *nr*, neural retina. (A) ×300. (B) ×850.

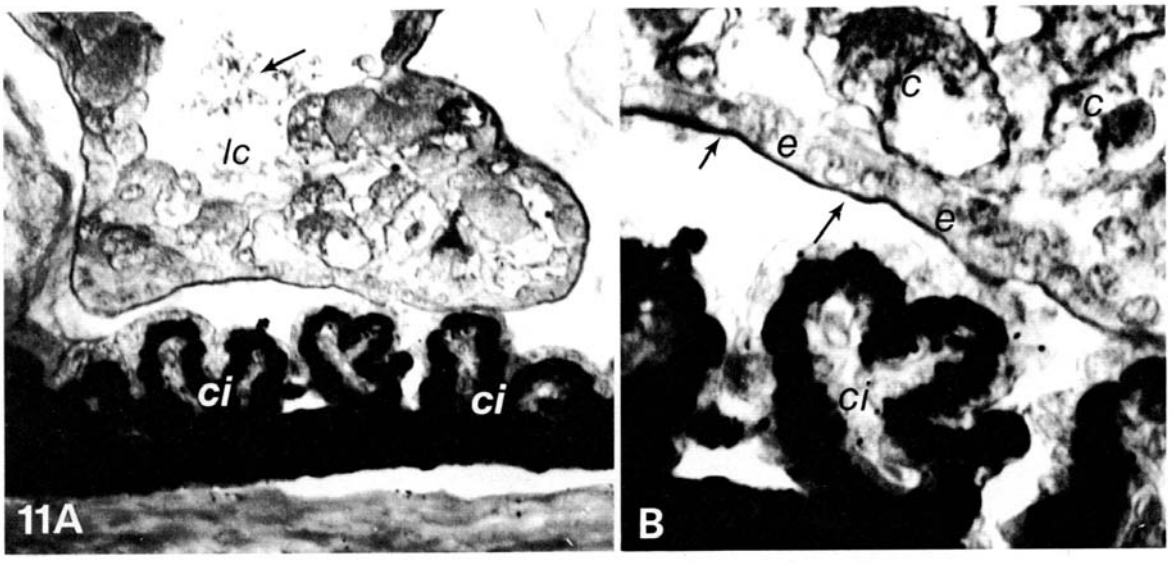

Fig. 11. Section from a 15 days postnatal mutant specimen stained with HID and nuclear fast red. This large lens cyst (*lc*), close to the apparently normal ciliary process (*ci*), stains strongly for sulphate in many regions. Granular sulphated materials are seen within the large cyst (A, arrow). (B) An enlargement of (A), demonstrates a basal lamina-like structure (arrows) as do smaller cysts (*c*). The outer cells (*e*) appear to be cuboidal epithelial in morphology. (A) ×225. (B) ×650.

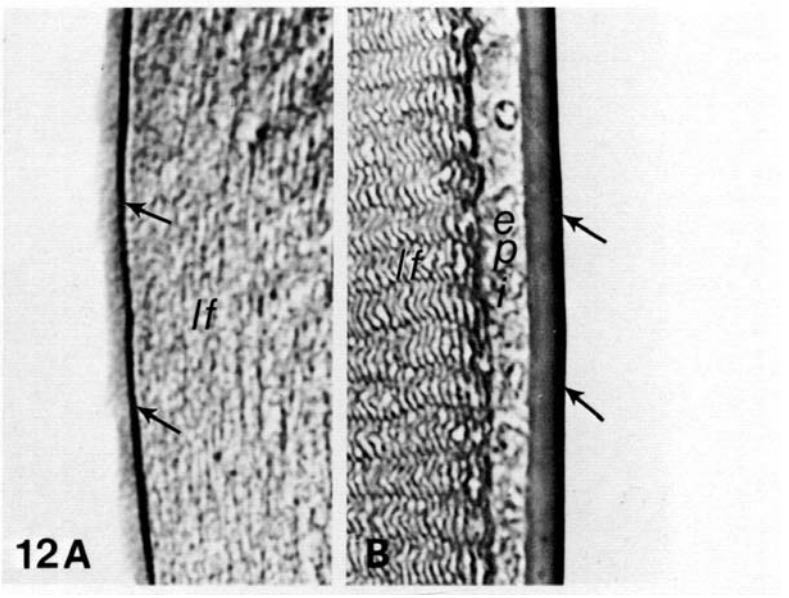

Fig. 12. Adult normal lens stained with HID and nuclear fast red. The posterior lens capsule (A) is thinner than the anterior lens capsule (B). The anterior capsule typically demonstrated a strongly staining outer zone (arrows), a thick and weakly staining middle zone and occasionally a thin, moderately staining zone at the capsular–epithelial cell (*epi*) interface. The posterior capsule typically demonstrated a bilaminar zonation, with a strongly staining region at the capsular-fibre cell (*lf*) interface. Both ×700.

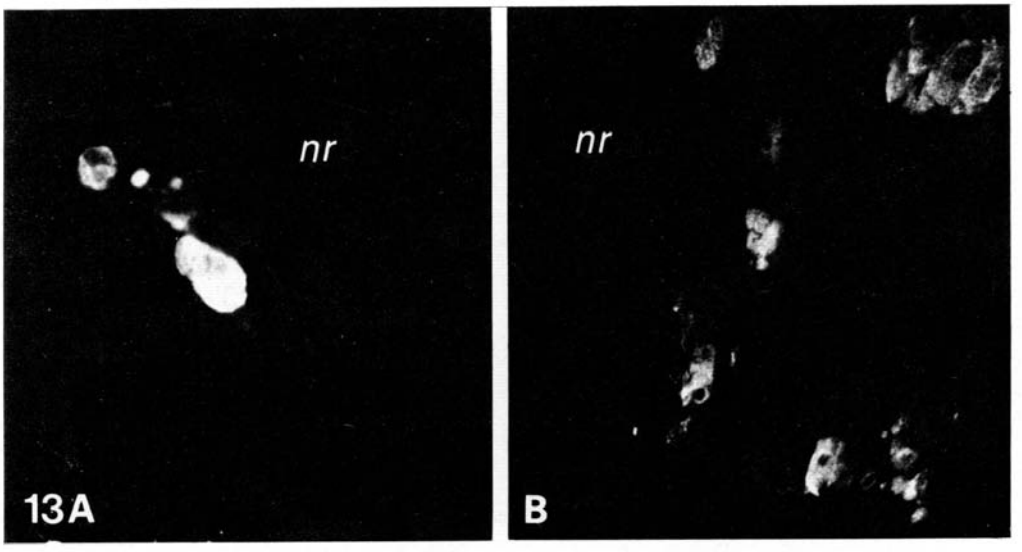

Fig. 13. Sections from different adult aphakia eyes tested immunofluorescently for lens crystallins. Patches of cells in each eye demonstrate lens crystallins. *nr*, neural retina. Both ×275.

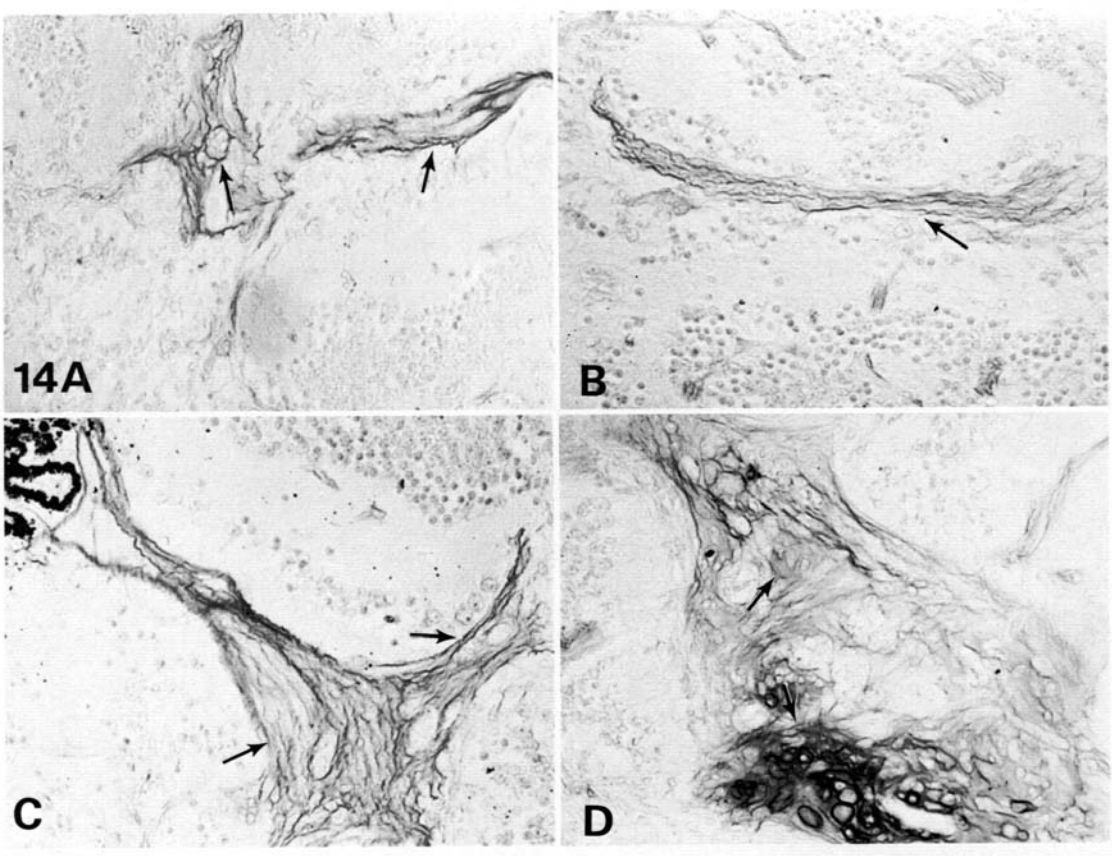

Fig. 14. Sections from different adult aphakia eyes stained with HID and nuclear fast red. Note the distinct, strongly staining fibrillar structures (arrows) within the intraocular cavity in each specimen. All ×125.

components are only partially characterized and information regarding their structure and properties is scant and regarding their function is non-existent.

Cystic structures composed of lens-derived tissue were seen in aphakia eyes shortly after birth. Cystic structures in the lens fibre cell region were often seen in young postnatal normal mouse lenses during early maturational stages (Webster *et al.* submitted). The formation of these structures is commonly attributed to fluid accumulation both in ocular structures (Von Sallman, 1951; Friedenwald & Rytel, 1955; Kinoshita, 1965; Kuwabara, Kinoshita & Cogan, 1969) and other tissues (for examples, see Damsky, Richa, Solter, Knudsen & Buck, 1983; Martin & Evans, 1975). The presence of these cysts in aphakia lens tissue provides further evidence that some type of fluid pumping, perhaps related to the rapid increase in lens size shortly after birth, may be present in the lens at this period of maturation.

Early differentiation of the normal mouse lens is characterized by the appearance of three families of lens crystallin proteins at specific developmental stages (Zwaan & Silver, 1983; Webster & Zwaan, 1984). The appearance of all types of crystallins is delayed, yet present, in the aphakia mutant (Zwaan *et al.* in

preparation). Maturation of the normal mouse lens is characterized by the appearance of sulphated materials (Webster *et al.* submitted) that are qualitatively different from those seen in early lens morphogenesis in mice (Webster *et al.* 1983). The appearance of sulphated materials with properties similar to those seen in normal mouse lens maturation is delayed, yet present in the aphakia mutant. Cysts that may play a role in the rapid growth of the lens during late prenatal and early postnatal stages are seen in the lens fibre region of normal mice (Cohen, 1958; Webster *et al.* submitted). Cystic structures are seen in aphakia lens-derived tissue at slightly later stages than are observed in normal mouse lens maturation. These cystic structures contain cells that are elongated, have inter- and intracellular sulphated materials associated with them, demonstrate hydropic characteristics and produce β and γ-crystallins (Zwaan *et al.* in preparation); these are all characteristics demonstrated by maturing and differentiated lens fibre cells. It is, therefore, possible to conclude that virtually all known characteristics associated with normal mouse lens maturation and differentiation are present in aphakia lens-derived tissue. These delays in differentiative expression may be useful in investigating the role(s) of factor(s) involved in triggering normal mouse lens differentiation and maturation.

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