Formation and distribution of chick lens proteins

Jan Langman and Harry Maisel

The agar diffusion technique of Ouchterlony was used to determine the time sequence in which the three main lens proteins arise during organogenesis and to examine the distribution of these proteins in the lenses of animals throughout the vertebrate series and in those tissues of the eye which possess the capacity to form a new lens upon removal of the original lens. Alpha crystallin is detected in the epithelial cells of the lens placode before the appearance of any other lens antigens. Since it is also present in the pigment layer of the retina and iris of 72-hour-old embryos—that is, those tissues which have a capacity for regeneration of the lens—and is found in identical or partially identical form in the lenses of representative animals throughout the vertebrate series, it may be considered as an "ancient" lens protein which plays a key role in formation of the lens. Beta crystallin appears at the onset of differentiation and growth of the nuclear lens fibers and is characterized by the rapid formation of 4 closely related subfractions. Since it is found in identical or partially identical form only in the lenses of birds and reptiles, but not in those of mammals, amphibians, and fishes, it may be considered a specialized protein which in the course of evolution develops along divergent lines. Its highly differentiated form and rapid formation may make it a target for teratogenic factors, such as German measles virus, which causes cataract only when it acts during growth and differentiation of the nuclear lens fibers—that is, the time of beta crystallin synthesis. Gamma crystallin—the last lens protein to arise during development—takes an intermediate position between alpha and beta crystallin in regard to its species-specific properties.

Analysis of the soluble proteins of the lens by means of agar diffusion and immuno-electrophoretic techniques has demonstrated the existence of at least 5 to 10 such components in the lenses of animals and of human beings.\(^1\)\(^-\)\(^6\)

Little attention, however, has been given to determining precisely at which stage of lens development the first specific protein appears, what the nature of this protein is, and in what order the different lens proteins arise. With a modified form of the precipitin ring technique, ten Cate and van Doorenmaal\(^6\) succeeded in demonstrating the presence of lens antigens in an extract from 30 somite chick embryos, that is, the stage at which the lens has the shape of a vesicle. Langman and co-workers\(^7\)\(^-\)\(^8\) cultured optic vesicles covered with presumptive lens ectoderm from 7 to 20 somite chick embryos in a medium containing lens antibodies. Since degeneration of the presumptive lens cells was found only in explants from embryos with more than 10 somites, it was suggested that the first lens antigens arise at the 11 somite stage, that is, shortly after the induction of the lens has started (9 somites) and shortly before the first morphologic changes characteristic for the lens placode become visible (13 somites). Though these proteins arise. With a modified form of the precipitin ring technique, ten Cate and van Doorenmaal\(^6\) succeeded in demonstrating the presence of lens antigens in an extract from 30 somite chick embryos, that is, the stage at which the lens has the shape of a vesicle. Langman and co-workers\(^7\)\(^-\)\(^8\) cultured optic vesicles covered with presumptive lens ectoderm from 7 to 20 somite chick embryos in a medium containing lens antibodies. Since degeneration of the presumptive lens cells was found only in explants from embryos with more than 10 somites, it was suggested that the first lens antigens arise at the 11 somite stage, that is, shortly after the induction of the lens has started (9 somites) and shortly before the first morphologic changes characteristic for the lens placode become visible (13 somites). Though these
experiments indicate the presence of lens antigens, no information concerning number or nature of proteins involved or the time sequence in which they arise is given.

This work, therefore, was undertaken to determine the number of soluble antigens in the lens of the adult chick, to analyze their nature by comparing them with lens proteins isolated by continuous flow electrophoresis, and to examine the time sequence in which the various components arise during organogenesis. Furthermore, since iris and pigment layer of the retina in the chick embryo have the capacity to form a new lens upon removal of the original lens,8 these tissues were also examined for the presence of lens antigens. Finally, to gain ontogenetic as well as phylogenetic information about lens antigens, the lenses of representative animals throughout the vertebrate series were examined for the presence of proteins characteristic for the chick lens.

Materials and methods

Preparation of tissue extracts. Lens extracts of chick embryos of the following ages were used: (a) 50 hours (19 to 24 somites); (b) 60 hours (28 to 32 somites); (c) 72 hours (35 to 37 somites); (d) 96 hours (43 to 44 somites); and (e) 10 days. In addition, lens extracts were prepared from the following adult animals: chicken (Gallus domesticus), turkey (Meleagris gallopavo), and duck (Anas platyrhynchos) as representatives of birds; turtle (Chrysemys picta), frog (Rana pipiens), and Dory fish (Stizostedion vitreum) as representatives of reptiles and amphibians; rabbit (Oryctolagus cuniculus), fox (Vulpes fulva), pig (Sus scrofa), cat (Felis domestica), and rhesus monkey (Macaca rhesus) as representatives of mammals.

The lens tissue of the embryos and adult animals was carefully dissected and cleaned of adherent iris epithelium, and subsequently weighed and homogenized to a concentration of 100 mg. wet weight per milliliter in sterile 0.9 per cent saline solution. Furthermore, the iris and pigment layer of the retina were dissected from the eyes of adult chicks, carefully washed and cleaned from adjacent tissues, and cut into small fragments. The tissue fragments were then homogenized in 0.9 per cent saline solution to a concentration of 250 mg. wet weight per milliliter. After centrifugation at 3,000 r.p.m., the collected supernatant was used for agar diffusion tests.

Adult chick lens extract (15 mg. protein per milliliter) was run in the Spinco Model C.P. continuous flow electrophoresis apparatus for 36 hours to isolate the three main lens proteins—alpha, beta, and gamma crystallin. The analysis was made at 4° C. in Veronal buffer at pH 8.6 and ionic strength 0.02. A 60 Ma. current was used and the sample flow rate was adjusted filterate 2.4 ml. per hour. The lower curtain was equilibrated for 3 hours before the sample was applied. Fractions were collected, dialyzed, and concentrated to a protein value of 38 to 82 mg. per 100 ml., and tested for homogeneity with the Spinco Model E ultracentrifuge (protein concentration 0.38 to 0.82 mg. per 100 ml., pH 8.6, speed 60,000 r.p.m., temperature 20° C.).

Preparation of lens antibodies. A 10 per cent lens extract prepared from adult chickens was suspended in Freund's adjuvant in a ratio of 3 to 2, and 3 ml. of this suspension was injected subcutaneously in five different sites into a number of rabbits. This procedure was repeated 3 to 7 times at 3 week intervals. The serum of the rabbits collected 2 weeks after the last injection will be referred to as lens antiserum.

Agar diffusion technique. The agar diffusion method of Ouchterlony10 was used for the tests. Agar plates were prepared as previously described11 with 2 per cent dialyzed filtered agar (Difco B140; pH 7.2) to which 0.01 per cent Merthiolate had been added. Peripheral wells of different shapes were made at a distance of 5 to 20 mm. from the central well. The tests were carried out at 4° C. When critical concentrations of the diffusing antigen and antibody meet, a precipitin band is formed in the agar. Neither antigen nor antibody can diffuse beyond the precipitin zone which acts as a virtual barrier to this particular antigen-antibody system, but other antigens and antibodies go through.12,13 When a mixture of several antigens and antibodies is used, as in our experiments, a precipitin spectrum may be seen, each precipitin band corresponding to an antigen-antibody system. Coalescence or fusion of bands observed when various extracts are tested against the same antiserum in one agar plate indicates identity of antigenic components, while total intersection of the lines indicates nonidentity of the antigens. Partial coalescence of the precipitin bands indicates that the antigenic components have some reactive groupings in common, but they differ in others.

Results

Analysis of adult chick lens extract. Figs. 1 and 2 show the precipitin bands formed when 10 per cent lens extract of adult chickens was tested with lens antiserum. It
is evident that the adult chick lens contains substantial amounts of at least 7 to 10 water-soluble antigenic components, grouped into three main fractions (Fractions I, II, and III). Whereas Fraction I contains 1 antigen, Fraction II consists of at least 4 closely related subfractions, and Fraction III of 2 subfractions.

When alpha crystallin isolated by continuous flow electrophoresis and total lens extract were tested simultaneously in one agar plate, it was found that the precipitin line formed by alpha crystallin coalesced with the line corresponding to Fraction I of total lens extract (Fig. 3). It was thus concluded that Fraction I is identical to alpha crystallin. Likewise, it was found that Fractions II and III are identical to beta and gamma crystallin, respectively (Fig. 4).

Analysis of embryonic lens extract

**Fifty-hour embryos (19 to 24 somites).**

During the 19 to 24 somite stage the lens placode begins to invaginate and acidophilic fibers appear in the cytoplasm of the cells (Fig. 5). When an extract of the invaginating lens placode was tested with lens antiserum, one vague precipitin band appeared. Fig. 6 shows that this “placode band” fused completely with the “alpha crystallin band” indicating antigenic identity. It was thus concluded that at the 50 hour stage alpha crystallin is present in the lens placode.

**Sixty- and seventy-two-hour embryos.**

When lens extracts of 60 hour (28 to 32 somites) and 72 hour embryos (35 to 37 somites) were tested with lens antiserum, 3 to 4 precipitin bands became visible which indicated the presence of substantial amounts of 3 to 4 lens antigens. The appearance of the additional antigens coincided with the formation and growth of the acidophilic fibers in the cytoplasm, the appearance of basophilic granules at the base of the cells, and the formation of the first nuclear lens fibers (Fig. 7). When the nature of the additional bands was examined, it was found that these bands coalesced with the band formed by beta crystallin (Fig. 8). Hence, while the first-appearing lens antigen is formed at the placode stage and is identical to alpha crystallin, the additional bands associated with the formation of nuclear lens fibers are identical to beta crystallin.

**Ten-day embryos.** At this stage the 3
main proteins of the lens—alpha, beta, and gamma crystallin—are present and each fraction is represented in the agar plate by one or more precipitin lines.

It was thus concluded that the various antigenic components of the lens arise in consecutive order—that is, first, alpha crystallin, second, beta crystallin, and, finally, gamma crystallin. When the sedimentation coefficient of each of the crystal-
Table I. Sedimentation constant of lens proteins

<table>
<thead>
<tr>
<th>Protein (mg./100 ml.)</th>
<th>pH</th>
<th>S0</th>
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</thead>
<tbody>
<tr>
<td>Total chick lens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>0.3</td>
<td>8.6</td>
</tr>
<tr>
<td>Fraction B</td>
<td>0.6</td>
<td>8.6</td>
</tr>
<tr>
<td>Fraction C</td>
<td>0.82</td>
<td>8.6</td>
</tr>
<tr>
<td>Isolated fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-cristallin</td>
<td>0.51</td>
<td>8.6</td>
</tr>
<tr>
<td>β-cristallin</td>
<td>0.38</td>
<td>8.6</td>
</tr>
<tr>
<td>γ-cristallin</td>
<td>0.82</td>
<td>8.6</td>
</tr>
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When 25 per cent extracts of the iris and pigment layer of the retina were tested with lens antiserum, three distinct precipitin bands were observed. From Fig. 9 it is evident that the precipitin lines formed by iris extract show complete coalescence with those formed by the pigment retina indicating that both tissues contain substantial amounts of three identical lens antigens. Further analysis showed that these bands coalesced completely with those formed by isolated alpha, beta, and gamma crystallin (Figs. 10 and 11). It is thus evident that the cells of the iris and pigment retina of adult chickens contain antigens identical to the 3 main proteins of the lens.

Chick lens antigens in the lenses of various animals throughout the vertebrate series

**Mammals.** When lens extracts of man, monkey, fox, cat, and hog were tested with chick lens antiserum, one precipitin band was formed by each of the extracts. By placing the various extracts in a series of wells surrounding the antibody well, the precipitin bands were found to fuse with each other and to form a precipitin ring.
(Fig. 12). Further analysis showed that the antigen responsible for the formation of this band was partially identical to the alpha crystallin found in the lens of the chick, but did not have any immunologic relationship with the beta or gamma crystallin (Fig. 13). It was thus concluded that alpha crystallin is the only lens protein with reactive antigenic groupings common to the mammalian and chick lens.
Birds. When the precipitin lines formed by the lens extracts of turkey and duck were compared with those of the chick, it was found that the bands formed by turkey lens fused completely with those of the chick. The lines formed by the duck lens, however, coalesced with the alpha and gamma crystallin bands of the chick lens but showed a reaction of partial identity in regard to the beta crystallin fraction. This indicates that the lens of the turkey does not differ from that of the chick in any of the lens proteins, whereas the lens of the duck shows a distinct antigenic difference with regard to beta crystallin.

Reptiles. When 10 per cent turtle lens extract was tested against chick lens antiserum, three precipitin bands were formed. While two of these bands showed coalescence with the alpha and gamma crystallin bands of the chick, the third band showed a reaction of partial identity with the beta crystallin band (Fig. 14).

Amphibians. When frog lens extract was tested with chick lens antiserum, two precipitin lines showing partial identity with the alpha and gamma crystallin bands of the chick were found (Fig. 15). The frog lens apparently does not contain an antigenic component comparable with beta crystallin as found in the chick lens.

Fishes. When fish lens extract was tested with chick lens antiserum one distinct precipitin line which showed a reaction of partial identity with the "alpha crystallin band" of the chick was observed (Fig. 16). Thus, the fish lens does not contain any antigenic components identical to beta and gamma crystallin as found in the chick lens.

These experiments show that alpha crystallin—the first formed protein in the lens of the chick—is present in the lenses of animals throughout the vertebrate series in identical or partially identical form which indicates its basic importance for the lens. With regard to beta crystallin, however, a considerable difference may be noticed. While the duck shows only minor differences from the chick in regard to the beta crystallin fraction, the turtle—phylogenetically more distant from the chick than the duck—shows considerably more difference. Mammals, amphibians, and fishes do not possess any beta components antigenically identical to that in the chick. Beta crystallin, therefore, appears to be a lens protein with rather strong species-specific characteristics. Gamma crystallin seems to take an intermediate position with regard to its species-specific properties.

Discussion

Examining the self-differentiation capacity of the chick lens, McKeehan found that a lens placode of a 21 somite embryo is capable of independent lens formation when transplanted into the celomic cavity of another embryo. At this stage of development the lens placode cells are believed to possess the basic chemical inventory required for lens differentiation. From our experiments it appears that alpha crystallin is the first lens antigen detectable during organogenesis, that is, at the lens placode stage. It would appear, therefore, that alpha crystallin is a protein "essential" for lens formation. Once this protein is formed the lens placode is able to proceed with its development independently of the surrounding tissues.

Additional evidence for the importance of alpha crystallin in lens development was found in experiments in lens regeneration. When van Deth removed the lens primordium of a 53 hour chick embryo and explanted the remaining eye cup in a tissue culture medium, a new lens was formed by the pigment layer of the retina and by the rim (iris) of the eye cup. Although this regenerating capacity of the lens was thought to be restricted to the embryo, recent unpublished experiments in which the lenses of newly hatched chickens were removed showed that even in the newborn chick lens fibers can be formed by the cells of the iris 2 to 7 weeks after removal of the original lens. In view of the latter observations it is not surprising that the cells of the iris and pigment layer of the retina...
were found to contain substances antigenically identical to alpha, beta, and gamma crystallin. Although it was first thought that the presence of these antigens was due to contamination with lens material, this possibility was excluded when extracts of the iris and retinal pigment of eyes from which the lenses had previously been removed yielded the same number of precipitin bands as extracts from eyes with lenses. Thus, antigenic components identical to the three main proteins of the lens are incorporated in the cells of the iris and pigment layer of the retina of newly hatched and adult chickens. While under normal conditions these antigens are fully integrated in the molecular population of the cells, it is probable that under specific conditions, such as removal of the lens, this intracellular pattern can change, resulting in loss of pigment and formation of lens fibers.

Since it is not clear from the above experiments whether alpha crystallin is the only lens protein involved in the transformation of iris cells into lens fibers, the appearance of lens antigens in the course of development of the iris and pigment of the retina was examined. It was found that the first lens antigen appears in the cells of the eye cup at the 60 hour stage and that this component is identical to alpha crystallin. Since at this stage of development, and even slightly earlier, the prospective pigment of the retina and iris cells already possess the capacity to form a lens, it seems likely that alpha crystallin is a protein essential for formation and regeneration of the lens.

Whereas, until recently, lens proteins have been considered as identical throughout the vertebrate series, application of the agar diffusion technique shows that this is not the case. From our experiments it is evident that the number of lens antigens common to the chick and other animals tested depends on the phylogenetic relationship of the particular animal to the chick. While chick and turkey show complete identity in regard to the lens antigens, the duck differs in its precipitin spectrum from that of the chick in respect to the beta crystallin fraction. While this difference is only minor, the turtle, which is more distant from the chick in the phylogenetic scale, appears to have considerably greater differences in regard to this antigen. Mammals, amphibians, and fishes do not contain any antigenic component identical to beta crystallin of the chick lens. It was thus concluded that beta crystallin has considerable species-specific properties, while alpha crystallin appears to be present in the lenses of animals throughout the vertebrate series with only minute species differences. Thus, alpha crystallin is not only the first lens antigen formed during organogenesis but is also the most widely distributed since it is present in the lenses of all animals investigated and in those tissues of the eye capable of regeneration of the lens. It may, therefore, be considered as an "ancient" basic lens protein with almost no species-specific properties.

Though originally the beta crystallin fraction was considered to be one homogeneous protein, recent observations favor the existence of a number of closely related components within the beta crystallin fraction. When François and Kaminski tested bovine lens extract by means of immuno-electrophoresis, two beta fractions were demonstrated. Firfarova, using free electrophoresis, reported the presence of 2 to 3 beta fractions, while Resnik and co-workers who analyzed bovine lens extract by means of free-boundary electrophoresis, suggested the presence of 6 closely related components. Our experiments, likewise, indicate the existence of 4 to 5 components in the beta crystallin fraction. It is further evident from our work that these components arise one after the other in a short time interval during which invagination and vesicle formation of the lens placode occurs. Since this is the time during which most likely synthesis and differentiation of the various beta components takes place,
it may very well be one of the most sensitive stages in lens development. As such, the time of formation of the various beta crystallin subfractions might be the period during which environmental factors that interfere with protein synthesis may affect the development of the primary lens fibers. Indeed, German measles virus seems to cause destruction of the nuclear lens fibers only if acting during formation of these fibers—that is, during the time when synthesis and formation of the various beta components occur. Therefore, considering the great number of subfractions in the beta crystallin group which appear in the short time interval during which chemical differentiation of the lens fibers occurs, and considering the species-specific properties of this protein, beta crystallin seems to be a well-differentiated, complicated, and highly specialized substance which plays an essential role in the formation of lens fiber.

REFERENCES