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The Presence of Lens Antigens in the Intra-ocular Tissues of the Chick Eye

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Using polyvalent and monospecific antisera, cross-reacting antigenic determinants between lens crystallins and proteins from other intra-ocular tissues of the chick eye have been detected.

The results of two-dimensional crossed electrophoresis and of line-diffusion electrophoresis with lens antigens and lens antiserum showed immunoprecipitin lines equivalent to α-crystallin, δ-crystallin or FISC (first important soluble crystallin) and electrophoretic "Long Line Material" in extracts of cornea, vitreous body, aqueous humor, iris and retina. Immunoelectrophoresis of these proteins with specific antisera against lens α-crystallin and iris α-crystallin, and FISC demonstrated the presence of these crystallins in all intra-ocular tissues studied.

Isoelectric focusing experiments in thin layer polyacrylamide gels showed a high degree of identity in isoelectric points of the lens crystallins and the proteins present in intra-ocular tissues. The proteins of the FISC-group of the lens, which appeared to contain seven components, were also present in the intra-ocular tissues studied, though in a smaller number and distributed differently.

A protein with serological determinants to a chick serum constituent immunologically similar to albumin, was absent in lens, but present in all other intra-ocular tissues tested, and in extra-ocular tissues like brain, liver, spleen and in hemolyzed blood and in chick serum.

1. Introduction

Anti-lens protein sera show cross reactivity with lens proteins from a number of vertebrates, and this immunologic similarity can be considered as organ specificity. Kodama (1922) was one of the earliest authors who obtained cross reactions of lens with vitreous body and retina, while Burke, Sullivan, Petersen and Weed (1944) also demonstrated that the lens antigens were not strictly organ specific. Between 1954 and 1968 a number of investigators have reported that antisera against lens, also reacted with other tissues. Rao, Kulkarni, Cooper and Radhakrishnan (1955) showed that vitreous body and aqueous humor contained at least two, respectively three proteins immunologically similar to lens antigens. Langman and Prescott (1959) showed with the Boyd's precipitin test a positive reaction of iris, retina and cornea with lens antibodies. Maisel and Langman (1961) observed by agar diffusion technique that iris, retina and cornea contained antigenic substances identical to lens proteins. Cooper, Padukone and Bhatia (1960) applied the same technique and found immunologically identical proteins in bovine aqueous humor, vitreous body and in lens. Maisel (1962) showed that the antigens in the chick iris, cornea and retina, which cross-reacted with the lens antiserum, were proteins with electrophoretic mobilities similar to α-crystallin, δ-crystallin (or FISC) and β-crystallin (or "Long Line Material"). Maisel and Harmison (1963a) isolated an antigen from chick iris which was immunologically and electrophoretically identical to lens α-crystallin, while Maisel and Harmison (1963b) demonstrated by immunoelectrophoresis the presence of

three types of antigens in chick iris, which they considered as identical to α-crystallin, δ-crystallin (or FISC) and β-crystallin (or "Long Line Material"), and as iris-specific. Also an antigen with serum specificity was found in iris. It was shown that for instance chick iris did not contain any tissue specific antigen (Brahma, Bours and van Doorenmaalen, 1971). Little and Langman (1964) showed that in a number of vertebrates, and particularly in human, antiserum against lens proteins demonstrated the presence of antigenic components of the lens in the extracts of different intra-ocular tissues like cornea, vitreous body, aqueous humor, iris and retina.

Several authors detected proteins in ocular tissues which had no serum specificity, but showed (when tested) lens specificity. Perkins and Wood (1963) showed by means of immunodiffusion that cross reactions of guinea pig vitreous body with corresponding pig lens antiserum were not due to serum components. Halbert and Ehrlich (1962) detected six or seven antigens in corneal tissue unrelated to serum proteins. Cooper, Halbert and Manski (1963) performed immunochemical analysis of human and bovine vitreous body, and found that extracts of human vitreous body contained five to six tissue antigens which did not share serological determinants with serum proteins, whereas bovine vitreous body contained nine or more of those antigens. Therefore, the soluble antigens of the vitreous body were not entirely derived from blood plasma. Laurent, Laurent and Howe (1962) also found three out of twelve components of the bovine vitreous body which were not present in blood plasma. Barabanov and Mikhailov (1970) found that chick retina contained six serum antigens and nine proteins considered as tissue antigens. Of these nine tissue antigens two were organ-specific, and seven had inter-organic specificity.

Clayton, Campbell and Truman (1968) studied the cross-reacting antigenic determinants between lens proteins and other tissues of the chick by immunodiffusion, immunoelectrophoresis and by the Osserman technique, and concluded that all tissues tested contained some antigenic determinants similar to those present in lens proteins. Clayton et al. (1968) have summarized in a table the reports of a number of investigators who detected that antisera against lens crystallins also reacted with other eye tissues. There are, as was stated, two possible immunological approaches to the study of this cross reactivity between lens and other eye tissues: one is to employ an antiserum against a pure and specific component of a tissue, which serum may not permit the detection of cross-reactions between lens and other extra-ocular tissues. The second and alternative approach is to recognize as many of the constituents present in the ocular tissues as possible with a polyvalent lens antiserum.

In our studies we applied these two principles. We compared the antigens of the chick lens with the antigens of other intra-ocular tissues by immunoelectrophoresis. The data are presented in five parts: (1) application of the method of Osserman (1960) to compare lens antigens and antigens of other intra-ocular tissues with the concurrent reaction of anti-total chick lens serum; (2) Laurell's two-dimensional crossed electrophoresis of the intra-ocular tissues with anti-total lens serum; (3) the reaction of electrophoretically separated proteins of intra-ocular tissues with anti total chick lens serum and anti-total chick iris serum; (4) the reaction of the proteins of intra-ocular tissues with specific antisera to chick lens α-crystallin and chick iris α-crystallin; and (5) the reaction of intra-ocular tissue proteins with two very specific "tolerant" antisera to chick lens α-crystallin and chick lens FISC (first important soluble crystallin, Rabaey, 1962). Finally we compared the proteins of the intra-ocular tissues of the chick by thin layer isolectric focusing on polyacrylamide gel.

2. Materials and Methods

Preparation of antigens from intra-ocular tissues

Ten chick eyes were removed within 20 min after death and the following tissues were dissected at 4°C: iris, lens, cornea, vitreous body and retina. The aqueous humor was aspirated. All tissues were washed in cold saline and homogenized in a glass homogenizer at 4°C in de-ionized cooled water. Subsequently these extracts were centrifuged during 30 min in a refrigerated ultracentrifuge at 80,000 **g**. Each supernatant was lyophilized and used in the present studies. Wet weights of every tissue in mg, dry weights in percentage and relative amounts of the tissues were determined.

Preparation of antisera by means of subcutaneous injections

Fifteen mg chick lens extract in 0·5 ml "double distilled water" was mixed with Freund's adjuvant (complete) in a ratio of 1:1 and injected subcutaneously into rabbits at 2 week intervals for a period of 6 weeks. Serum was collected 10 days after the last injection.

Preparation of antisera by injections of antigens into the popliteal lymph nodes of the rabbit

This immunization technique was a modification of the methods described by Newbould (1965) and Goudie, Horne and Wilkinson (1966). One mg of the sample was dissolved in 2·5 ml saline; 0·5 ml of this solution was then incorporated in an equal volume of Freund's adjuvant (complete) and 0·1 ml of this emulsion was injected into the popliteal lymph node on either side, and the remaining volume (0·8 ml) was then injected intraperitoneally. A drawing of the position of the knee lymph node in the muscular tissue was given by Greene (1959). After 3 weeks 0·5 ml of the sample solution mixed with an equal volume of adjuvant was injected on the back of the rabbit at four different sites. This was repeated after 3 weeks. Ten days after the last injection the serum was taken from the marginal ear vein and tested against the antigen by immunoelectrophoresis. When the antibody titer was found to be low, the last injection could be repeated after 3 weeks. We found that the first three injections were sufficient to produce an antiserum with a high titer. Following this method three antisera were prepared, i.e. anti-total iris, anti-lens α-crystallin, and anti-iris α-crystallin.

Preparation of specific antisera against closely related proteins using immunological tolerance (Dixon and Maurer, 1955)

Anti FISC "tolerant" serum was available from the previous work of van Doorenmaalen, Brahma and Hoenders (1968), and anti α-crystallin "tolerant" serum was from experiments by Brahma and van Doorenmaalen (1971).

Immunoelectrophoresis

Immunoelectrophoresis was carried out according to Scheidegger (1955) using purified DIFCO bacto agar and Tris(hydroxymethyl)aminomethane—Ethylenediaminetetra acetic acid—boric acid buffer of pH 8·9 according to Aronsson and Grönwall (1957).

All extracts of the isolated intra-ocular tissues like lens, cornea, vitreous body, aqueous humor, iris and retina, and brain as an extra-ocular tissue were tested with various antisera. These antisera were: anti-total chick lens, anti-total chick iris, anti-lens α-crystallin, anti-iris α-crystallin, "tolerant" anti-lens α-crystallin, and "tolerant" anti-lens FISC.

Line-diffusion immunoelectrophoresis according to the Osserman-method (1960)

This procedure was carried out in the same way as described for normal immunoelectrophoresis. Proteins isolated from intra-ocular tissues were tested against anti-total chick lens serum. After electrophoresis for 1·5 hr with a constant current of 25 mA and a voltage

of 250 V the additional antigen trough was filled with 0·5% lens extract. Immunodiffusion was allowed to continue for 16 hr at room temperature. The upper trough containing the antigen was not included in the picture.

Laurell's two-dimensional antigen/antibody crossed electrophoresis

Extracts of all ocular tissues mentioned before were tested by this technique of Laurell (1965), and Clarke and Freeman (1968), against anti-total chick lens serum in 1% Agarose A 37 (Industrie Biologique Française, Product No. FF 1769). A buffer solution of pH 8·9 according to Aronsson and Grönwall (1957) was used. The first dimension of this electrophoresis was at a constant voltage of 300 V for 45 min. The second dimension was at a constant voltage of 150 V for 22 hr, also carried out at 4°C. Plates were then washed in saline, dried and stained in Amido Black.

Density gradient isoelectric focusing

α-Crystallins of iris and lens were isolated by isoelectric precipitation in an electrofocusing column of 110 ml contents. The electrofocusing equipment consisted of a column (LKB 8100–10), a power supply (LKB 3371) and an UV-monitor "Uvicord-II" (LKB 8300), as described by Haglund, (1967). The electrofocusing column was filled with a sucrose-gradient containing samples of 60 mg chick lens α-crystallin fraction from Sephadex G-200 chromatography (Bours, Hoenders and van Doorenmaalen, 1970) or 100 mg chick iris extract respectively. The concentration of "Ampholine" carrier ampholytes pH 3 to 10 (No. 8141, LKB-Produkter, A.B., Bromma, Sweden) was 1%, the voltage applied was 300 V during 2 days, with a gradual drop in amperage from 6 mA to 0·4 mA. After this time the α-crystallin was precipitated in the column due to low solubility at the isolectric point. The column was drained through an ultraviolet absorptiometer and 1 ml fractions were collected. The pH of all fractions was measured with a Radiometer combined electrode (Type GK 2321C), and the pH-gradient curve was plotted on the transmission diagram read on the recorder. This way the isoelectric point of the α-crystallin present was determined. The α-crystallin of chick lens or of chick iris was taken from the fractions where precipitation was observed. The pooled α-crystallin fractions were dialyzed and re-focused in the same way to eliminate contamination of other lens crystallins which were still present in these samples, though in small quantities. After re-precipitation by isoelectric focusing all contaminating crystallins were removed. The fractions were pooled likewise and each α-crystallin preparation purified in this way was dialyzed and lyophilized. Purity was checked by immunoelectrophoresis (Bours, Hoenders and van Doorenmaalen, 1970).

Isoelectric focusing in thin layer polyacrylamide gels

The intra-ocular tissues and brain were isofocused on a polyacrylamide gel plate, according to the method of Awdeh, Williamson and Askonas (1968), with some modifications (Bours and van Doorenmaalen, 1970) and described in detail (Bours, 1971). Polyacrylamide gels were defined by the notation of Hjertén (1962) in which $T = 5{\cdot}5\%$ and $C = 2{\cdot}6\%$ respectively denoting the total concentration of the gel and the cross-linking concentration. These gels were prepared with a final concentration of 2% "Ampholine" carrier ampholytes covering a pH range from 3 to 10 and consisting of ampholytes of pH ranges 3–5: 5–7 : 7–10 in a ratio of 2:1:1. Lyophilized samples of dialyzed extracts of intra-ocular tissues and brain were applied on the gel in the following quantities: 0·5 mg lens, 1·2 mg cornea, 1·0 mg vitreous body, 1·5 mg aqueous humor, 1·0 mg iris, 1·0 mg retina and 1·0 mg brain. Each of these samples was dissolved in 50 μl distilled water and they were applied on sample papers of 1 cm² Whatman no. 3 mm, which were placed on the gel 15 mm from the anodic side. The current initially applied was 4 mA during 24 hr at an increasing voltage starting at 45 to 250 V. The experiment was carried out in a humid chamber (Bours, 1971) at 4°C. After the run the pH-gradient established was determined

with a flat membrane glass electrode (Radiometer type G 242C), at points 20 mm apart along the gel, and at the same time marking positions with a 0·5 mm diameter punch. The gel slab was washed extensively for several days, starting with a 10% and ending with a 3% solution of trichloroacetic acid, to remove the "Ampholine" carrier ampholytes. Staining was done in a 0·05% solution of Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in a mixture of methanol—acetic acid—water in a ratio of 45:9:46 during 2 hr, followed by washing in the same solvent until the background appeared to be colourless. The gel was then photographed.

3. Results

Determination of tissue wet and dry weights

From 52 chick eyes we obtained after freeze-drying extracts of the following intra-ocular tissues: 1·0 g lens extract (wet weight 96 mg, dry weight 20·5%), 30·6 mg iris extract (wet weight 22 mg, dry weight 2·7%), 340 mg vitreous body extract (wet weight 351 mg, dry weight 1·9%), 168 mg retina extract (wet weight 122 mg, dry weight 2·7%), and 28·6 mg cornea extract (wet weight 43 mg, dry weight 1·3%). For aqueous humour this amount was 144 mg extract (wet weight 188 mg, dry weight 1·5%). From brain 102 mg extract was obtained, (wet weight 3·76 g, dry weight 2·7%). Parenthesis denotes data from single tissue.

The soluble proteins from extracts of these intra-ocular tissues of the chick were studied (Plates 1–9): lens (a), cornea (b), vitreous body (c), aqueous humor (d), iris (e), and retina (f). For comparison brain (g) was taken as an extra-ocular tissue. In Plate 8 the immunoelectrophoresis of lens extract against anti total lens serum was used as a reference, indicated as (a)*.

Immunoelectrophoretic test against anti-total lens serum

The immunoelectrophoretic patterns of the extracts a–g tested against anti-total lens serum was shown in Plate 1. The precipitin lines formed represent the lens crystallins: α-crystallin, FISC, and electrophoretic "Long Line". In all intra-ocular tissues (a–f) the α-line and the electrophoretic "Long Line" were clearly visible, in aqueous humour (d) the FISC-line was half the original size of this precipitin line detected in lens. Brain (g) showed no reaction with anti-total lens serum.

Line-diffusion immunoelectrophoresis according to Osserman, against anti-total lens serum

The method of Osserman (1960) was used to compare the electrophoretically separated primary antigens from intra-ocular tissues and a known protein constituent like the crystallins from total chick lens, with the concurrent reaction of anti-total lens serum, which was shown in Plate 2. Between the two longitudinal troughs filled with 0·5% chick lens extract and anti-total lens serum three straight precipitin lines were formed, namely one for precipitating α-crystallin, one for FISC and one for "Long Line Material", in an order of decreasing molecular weights (Zwaan, 1968b). These lines fused with the corresponding antigen present in the intra-ocular tissue tested. The ocular tissues demonstrate the presence of α-crystallin, FISC and Long Line. The FISC-line in retina (f), however, was not visible due to low concentration of this protein; this will be discussed later [compare Plate 3(f)]. In aqueous humor (d) the FISC-line was half the original size of this line detected in lens. In brain (g) lens antigens could not be detected: all three precipitin lines were straight. Only on the

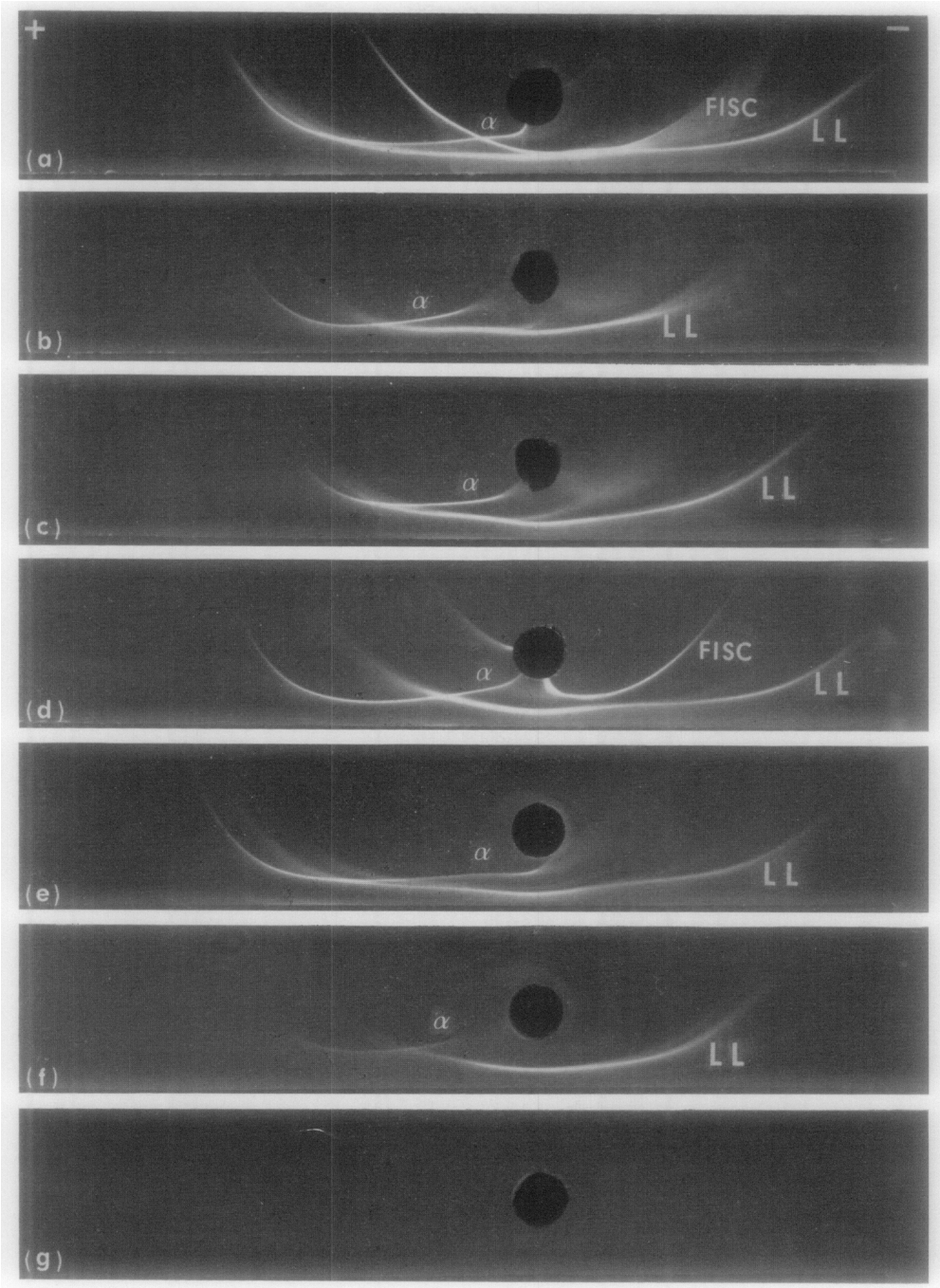

PLATE I. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues using anti-total lens serum. In all intra-ocular tissues the α-line and the "Long Line" were present. FISC in aqueous humor was half the original size of this line in lens. Brain showed no reaction. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin; FISC = first important soluble crystallin; LL = electrophoretic "Long Line Material".

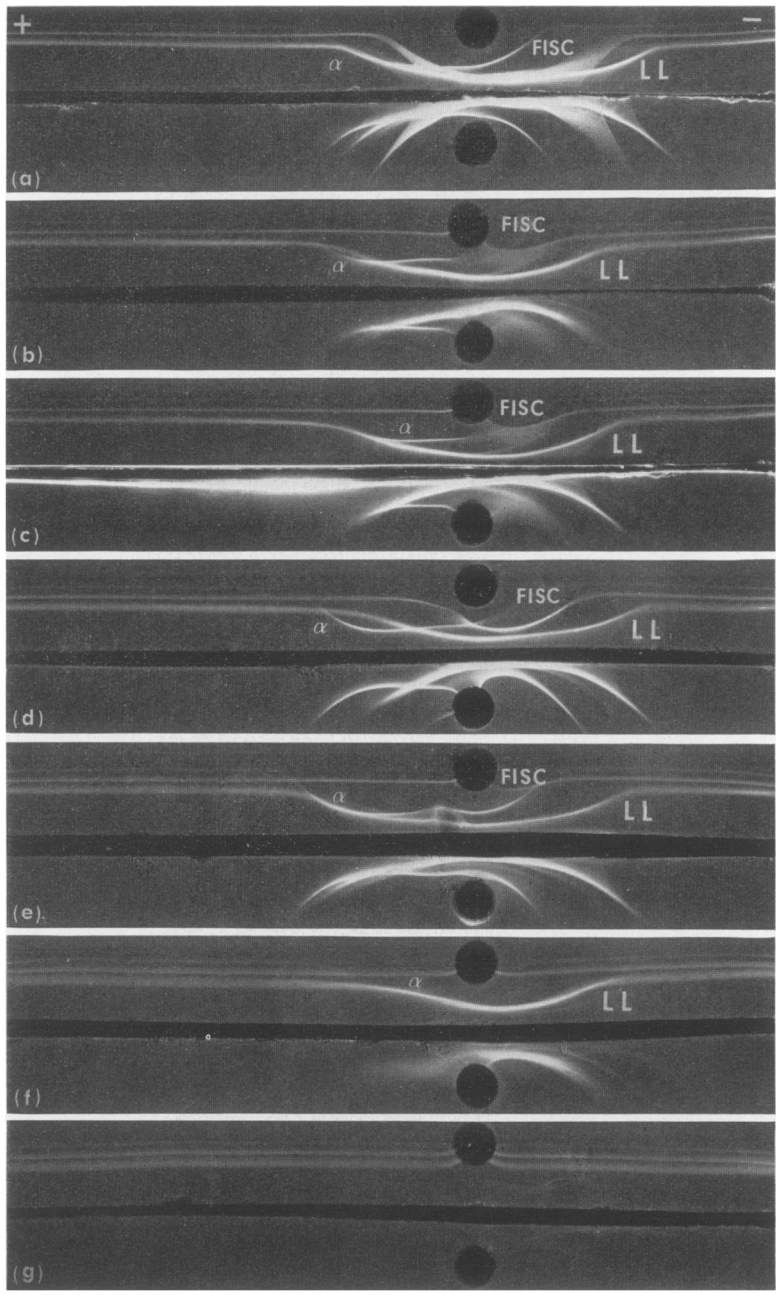

PLATE 2. Osserman tests of extracts of chick intra-ocular tissues compared with chick lens extract, diffused from the upper trough which is not reproduced, using in the lower trough anti-total lens serum. In all intra-ocular tissues the α-line, the FISC-line and the "Long Line" were present, except in retina where the FISC-line was not visible. In brain, lens antigens were not detected. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin; FISC = first important soluble crystallin; LL = electrophoretic "Long Line Material".

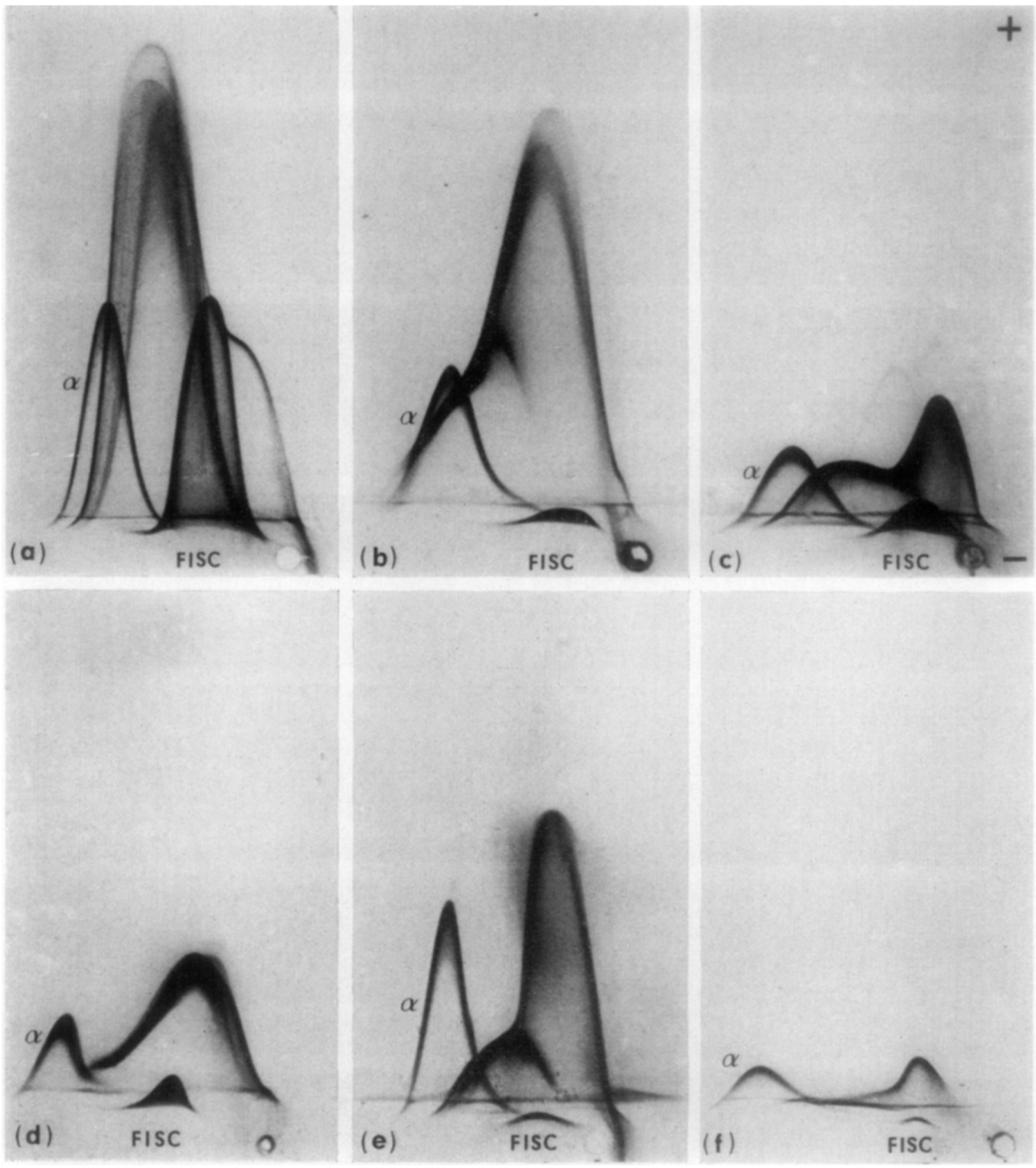

PLATE 3. Two dimensional antigen/antibody crossed electrophoresis of chick intra-ocular tissues against anti-total lens serum. In all intra-ocular tissues, the 3 main constituents of lens were detected. Mobilities of α-crystallin and FISC were identified by the application of monospecific antisera to each of these crystallins, as published earlier in references 8 and 7. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina. α = α-crystallin; FISC = first important soluble crystallin.

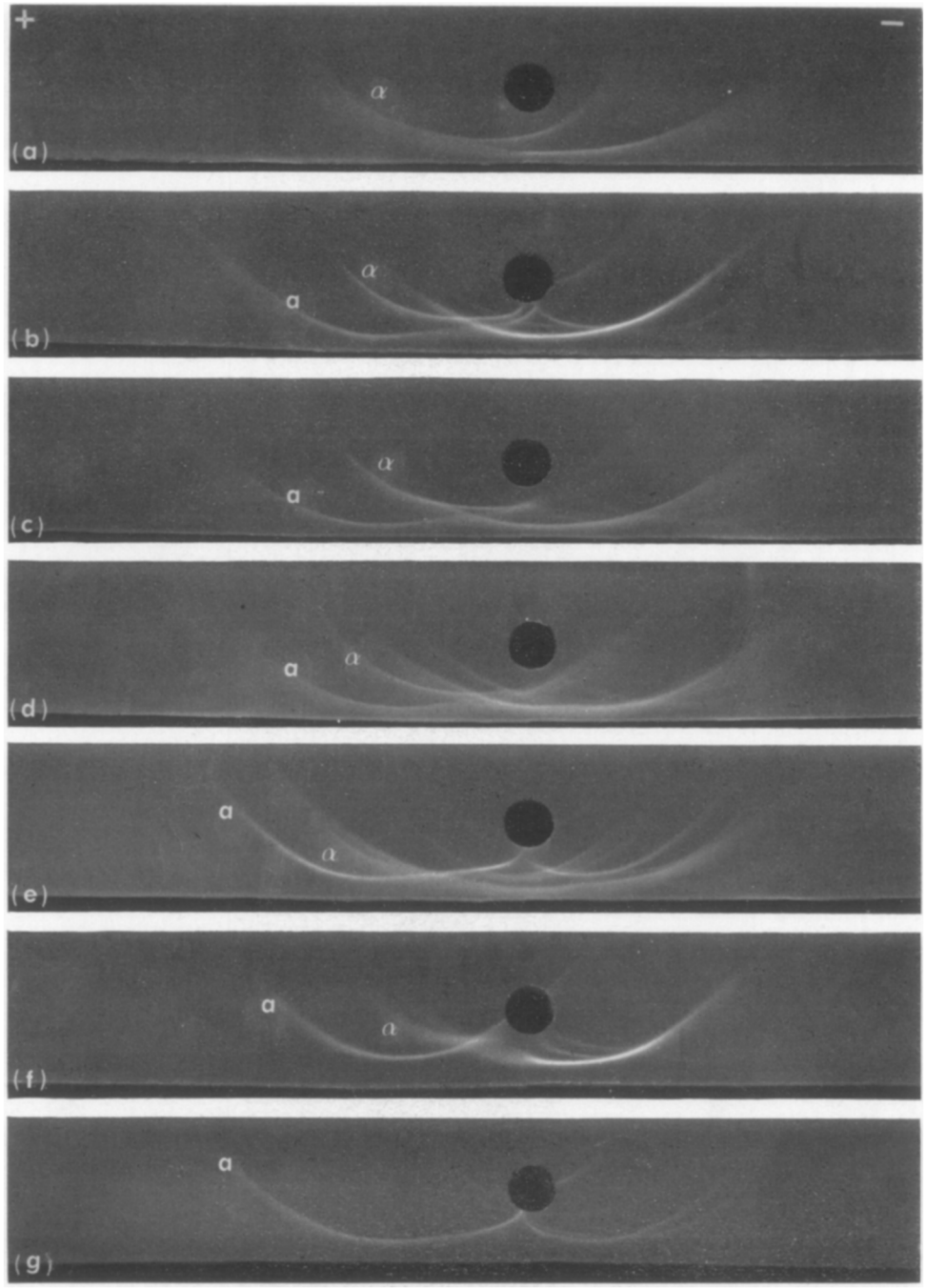

PLATE 4. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues, using anti-total iris serum. Besides other lines, lens only showed 1 α-line. All other intra-ocular tissues showed 2 anodic lines: the α-line and the *a*-line. Brain only showed the *a*-line, besides 2 cathodic lines. (a) = lens; (b) cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin; *a* = a protein immunologically similar to serum albumin.

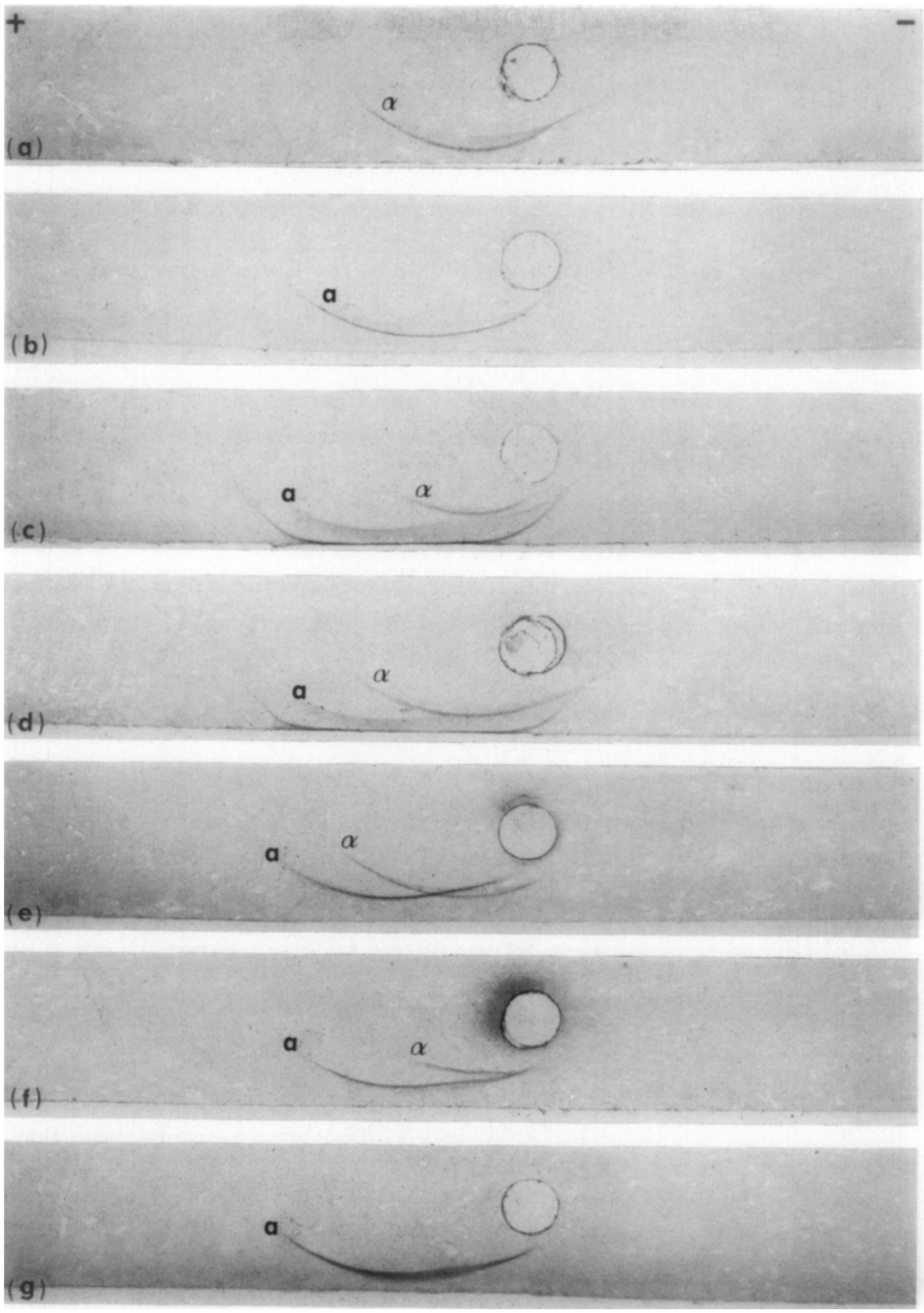

PLATE 5. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues, using anti-iris α-crystallin serum. Lens only showed 1 α-line. All other intra-ocular tissues showed 2 anodic lines: the α-line and the *a*-line. Brain only showed the *a*-line. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin; *a* = a protein immunologically si serummilar to albumin.

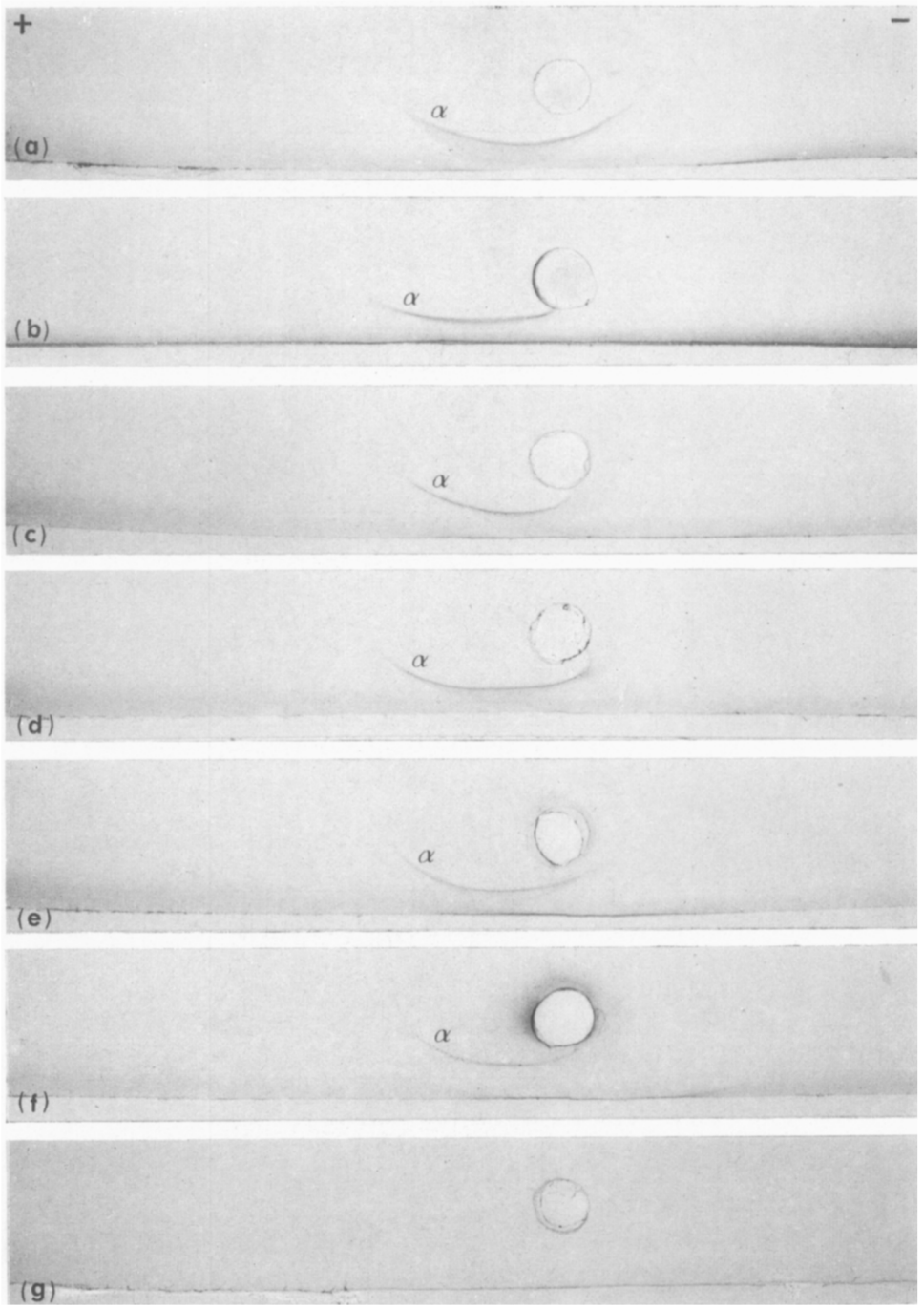

PLATE 6. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues, using lens anti α-crystallin serum. A single precipitin band located at the anodic side was formed with extracts of the intra-ocular tissues. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin.

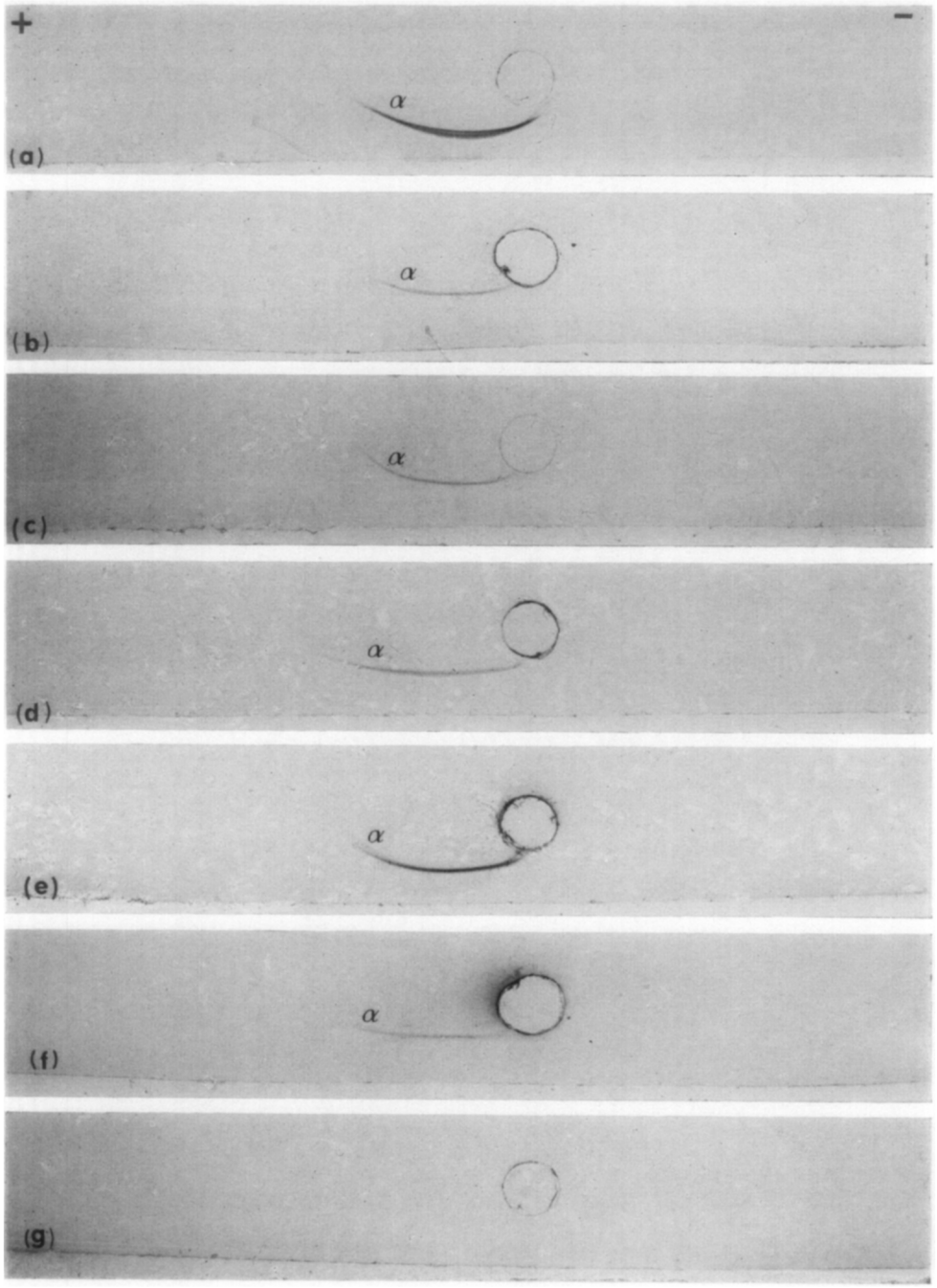

PLATE 7. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues, using tolerant antiserum against chick lens α-crystallin. A single precipitin band located at the anodic side was formed with extracts of the intra-ocular tissues. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin.

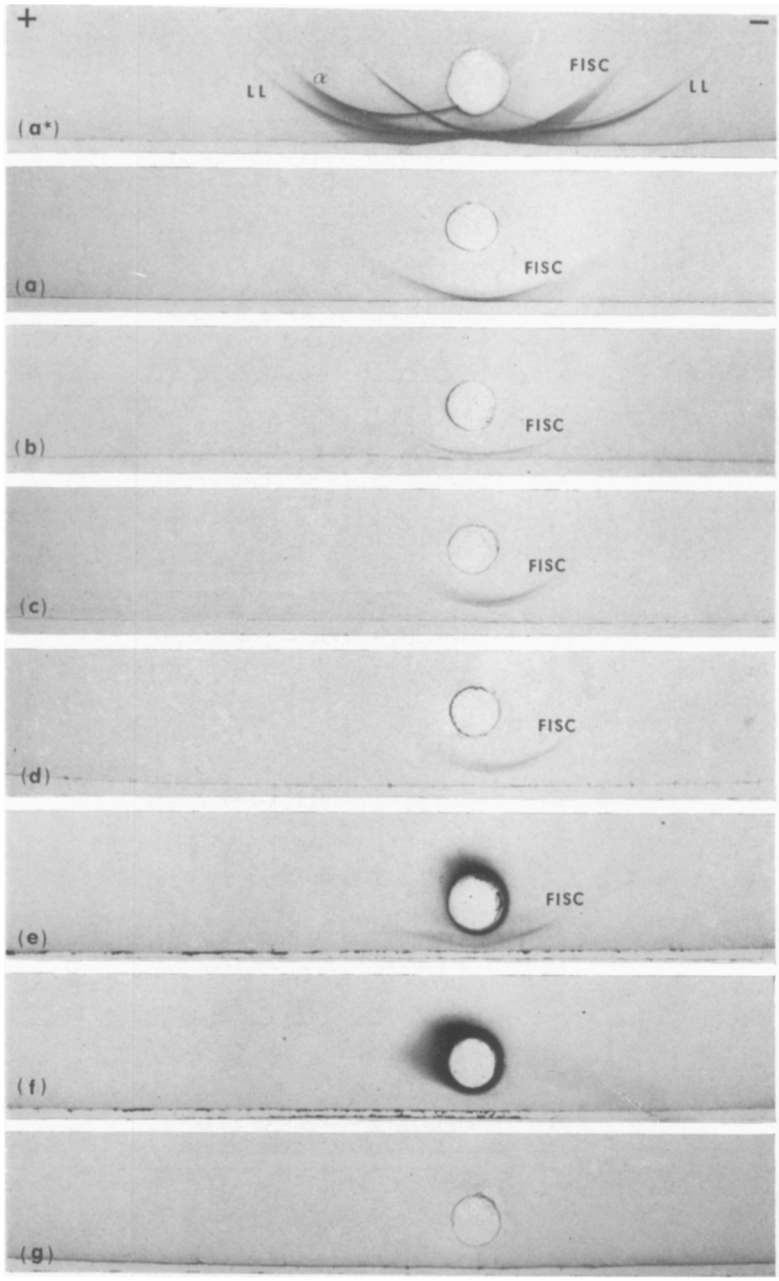

PLATE 8. Immunoelectrophoretic patterns of extracts of chick intra-ocular tissues, using tolerant antiserum against chick lens FISC. A single precipitin band located around the antigen well was formed with extracts of the intra-ocular tissues, except in retina. (a*) = chick lens extract against anti-total lens serum, as reference. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain, as extra-ocular tissue. α = α-crystallin; FISC = first important soluble crystallin; LL = electrophoretic "Long Line Material".

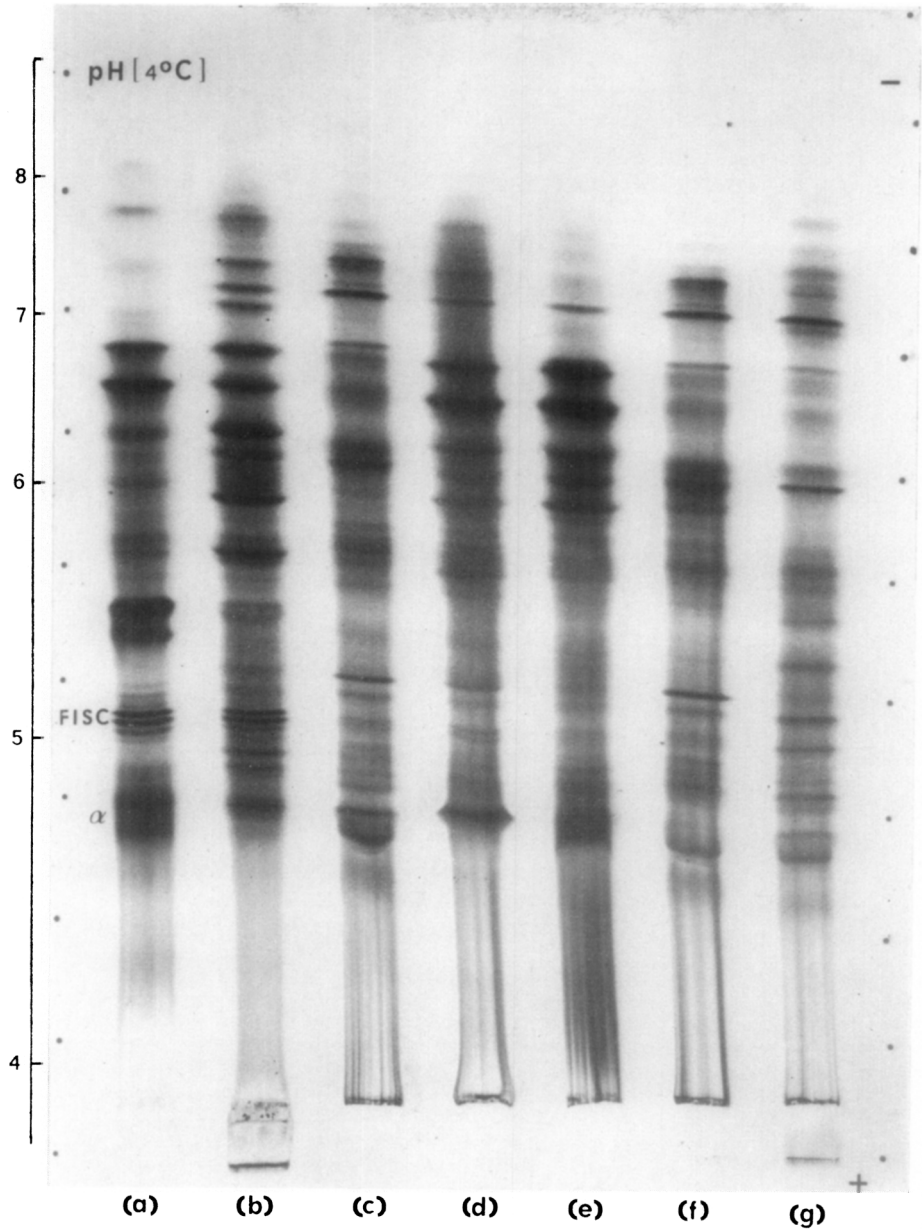

PLATE 9. Thin layer isoelectric focusing of proteins extracted from different intra-ocular tissues of the chick, in a 5% polyacrylamide gel plate (T = 5·5% and C = 2·6%) using 2% "Ampholine" carrier ampholytes, pH 3–5, 5–7, 7–10. The current initially applied was 4 mA, with an increasing voltage from 45 to 250 V during 24 hr (see text). Each intra-ocular tissue was dissected within 20 min after death of the animals, to avoid possible leakage of antigens from the lens. The samples amounted to 1 to 2 mg of dialyzed and lyophilized extracts. The pH was measured with a flat-membrane glass electrode on the spaces between adjacent marks (see text). The pH-gradient curve compiled from these data was a straight line. The scale on this Plate shows the pH-values along the gel. The number of focused bands in the FISC region appeared to be less in the intra-ocular tissues. Also in other regions protein components with the same isoelectric points were detected. (a) = lens; (b) = cornea; (c) = vitreous body; (d) = aqueous humor; (e) = iris; (f) = retina; (g) = brain. α = α-crystallin; FISC = first important soluble crystallin.

cathodic side some precipitin lines were observed, which did not fuse with the longitudinally applied antigen.

Laurell's two-dimensional antigen/antibody crossed electrophoresis against anti-total lens serum

In all intra-ocular tissue extracts (a–f) tested, the three main constituents of chick lens (α-crystallin, FISC and Long Line Material) were detected as shown in Plate 3. Even the FISC-line which appeared to be present in a very low concentration in retina (f) as mentioned before, was demonstrated with this technique. The mobility of α-crystallin and FISC in agarose, as indicated in Plate 3, was identified by the application of "tolerant" antisera specific to α-crystallin, and FISC, exactly as published earlier by Brahma and van Doorenmaalen (1971) and Brahma, Bours and van Doorenmaalen (1971). The line with lowest mobility nearest the antigen well is "Long Line Material".

Through antigen/antibody crossed electrophoresis of brain extract (g) versus anti-total lens serum, only three very faint lines in the FISC- and cathodal region were observed, but proved to be too faint to be photographed. These immunoprecipitin reactions could be attributed to the presence of antibodies against enzymes, glycoproteins, lipoproteins or other antigenic macromolecules (Clayton, Campbell and Truman, 1968).

Immunoelectrophoretic test against anti-total iris serum

The antiserum against chick iris extract was prepared as described under Methods subsection 2. The immunoelectrophoretic patterns of the tissue extracts (a–g) tested against anti-total iris serum was shown in Plate 4. The iris/anti-iris system (e) served as a reference. The lens tissue only showed one α-crystallin precipitin line. All other intra-ocular tissues showed two anodic precipitin lines: the α-crystallin (indicated as α) and a protein with a higher anodic mobility (indicated as *a*). Brain showed only the *a*-precipitin line, besides two cathodic lines.

Isolation of lens α-crystallin and iris α-crystallin by means of isoelectric focusing

α-Crystallin of chick lens and chick iris was isolated by density gradient isolectric focusing as described under Methods subsection 7. The isoelectric point determined in this way for both lens- and iris- α-crystallin equalled 4·85, as communicated by Bours, Hoenders and van Doorenmaalen (1970). The α-crystallins of chick lens and chick iris, served as an antigen for injection with the "lymph-node technique", described under Methods subsection 2.

Immunoelectrophoretic test against anti-iris α-crystallin serum

The antiserum against chick iris α-crystallin was prepared as described under Methods subsection 2. The immunoelectrophoretic patterns of the tissues tested (a g) against anti-iris α-crystallin serum was shown in Plate 5. Lens (a) only had one α-crystallin, while all other intra-ocular tissues showed one α-crystallin precipitin line and one *a*-precipitin line with higher anodic mobility, as also was found with anti-total iris serum. Brain (g) showed only the *a*-precipitin line.

Immunoelectrophoretic test against anti-lens α-crystallin serum

The antiserum against chick lens α-crystallin was prepared as described under

Methods subsection 2. The immunoelectrophoretic patterns of the tissue extracts (a–g) tested against anti-lens α-crystallin serum was shown in Plate 6. All intra-ocular tissue extracts demonstrate one precipitin line: α-crystallin. Brain extract (g) proved to be negative with this antiserum.

Immunoelectrophoretic test against "tolerant" anti-lens α-crystallin serum

Using immunological unresponsiveness the antiserum against chick lens α-crystallin was prepared by Brahma and van Doorenmaalen (1971). With this method antibodies against one particular lens antigen could be obtained. The immunoelectrophoretic patterns of the tissue extracts (a–g) tested against this antiserum was shown in Plate 7. In all extracts of intra-ocular tissues one α-crystallin precipitin line could be found with this antiserum. Brain (g) showed no reaction.

TABLE I

The presence of a protein with high anodic mobility in extra-ocular tissues of the chick

	Antiserum applied, directed to the following antigens from:			
Extra-ocular tissue	Iris	Iris α-crystallin	Lens	Lens α-crystallin
Brain	+	+	—	—
Hemolized blood	+	+	—	—
Serum	+	+	—	—
Liver	+	+	—	—
Spleen	+	+	—	—
Iris (reference)	+	+	—	—

+ indicates the presence of a protein, immunologically similar to serum albumin.
— indicates the absence of a protein, immunologically similar to serum albumin.

Immunoelectrophoretic test against "tolerant" anti FISC serum

Using immunological unresponsiveness the antiserum against chick lens FISC was prepared by van Doorenmaalen et al. (1968). The immunoelectrophoretic patterns of the extracts of the tissues (a–g) tested against this antiserum was shown in Plate 8. All intra-ocular tissues (a–f) showed one immunoprecipitin line in the FISC region. The lens (a), cornea (b) and iris (e) showed a precipitin curve situated symmetrically around the antigen well, while with vitreous body (c) and aqueous humor (d) a part of the FISC line was present at the cathodic side only, due to incompleteness (Clayton, 1970) of the FISC-components (Plate 9), like the immunoelectrophoretic patterns shown in Plate 2(b, c, d), and in Plate 1(d). With retina (f) the amount of antigen was beyond observation. Brain showed no reaction.

Immunoelectrophoretic test of chick serum against "tolerant" lens anti-iris serum, and "tolerant" iris anti-lens serum

Tolerant lens anti-iris serum and tolerant iris anti-lens serum were prepared by Brahma, as described in Brahma et al. (1971). These sera were tested by immunoelectrophoresis against chick total serum and against chick serum albumin. Tolerant

iris anti-lens serum was negative to both chick serum and chick serum albumin, and tolerant lens anti-iris serum was negative to chick serum albumin, and showed lines with β- and γ-mobilities with chick total serum.

Immunoelectrophoretic test of extra-ocular tissues against several antisera

Extra-ocular tissues were tested against anti-total iris serum, anti-iris α-crystallin serum, anti-total lens serum and anti-lens α-crystallin serum. The same anodic a-precipitin line which was demonstrated in iris [cf. Plate 4(e), Plate 5(e)] and in brain [cf. Plate 4(g), Plate 5(g)] was present in all tissues tested. In Table I, where iris was mentioned as a reference, results were summarized.

Isoelectric focusing in thin layer polyacrylamide gels

The isoelectric focusing experiments which were made with samples of dialyzed extracts of intra-ocular tissues (a–f) and brain (g) are shown in Plate 9. All tissues showed lines in the α-crystallin region between pH 4·70 and pH 4·85. Lens FISC had seven components with isoelectric points between 5·10 and 5·35, while any other intra-ocular tissue examined showed in the FISC region a lower number of focused bands, described as an incomplete FISC with a lower number of components. Protein bands representing the electrophoretic "Long Line" were situated at isoelectric points higher than 5·50, and showed a great deal of conformity.

4. Conclusion

The results of all experiments described are summarized in Table II. Antigens immunologically similar to lens crystallins are demonstrated in cornea, vitreous body, aqueous humor, iris and retina. A protein with serological determinants similar to serum albumin, is found to be absent in lens, but present in all intra-ocular tissues studied and in extra-ocular tissues like brain, liver, spleen and in serum.

5. Discussion

The results of the experiments described above show that chick lens antigens are not strictly localized to the lens, but can also be found in all intra-ocular tissues of the chick studied here, though in very low concentrations. All antigenic constituents present in lens are not as organ specific as they were considered to be, since these are also found in the extra-lenticular tissues of the chick eye [Plates 1–3].

Our results [Plate 1] are in accordance with the reports of Maisel (1962, 1963), who concluded that extracts of iris, retina, cornea and aqueous humor contained antigens with electrophoretic mobilities similar to the lens proteins of chick, and that the extra-lenticular antigens were also immunologically identical to the corresponding lens antigens. Little and Langman (1964) found that each of the intra-ocular tissues of the human eye tested contained one or more antigenic components identical to those of the human lens. Clayton et al. (1968) re-examined the organ specificity of lens antigens of the chick, and found that all intra-ocular tissues tested contained some antigenic determinants similar to those found in lens proteins.

So far these authors did not use antisera specific to one of the lens crystallins. We tested antisera specific to lens α-crystallin, and found that all intra-ocular tissues contained antigenic determinants similar to lens α-crystallin [Plate 6]. We also tested the intra-ocular tissues with an antiserum specific to iris α-crystallin [Plate 5] and found that the lens tissue only responded to α-crystallin [Plate 5(a)]. All other

TABLE II

The presence of lens antigens in the intra-ocular tissues of the chick eye, and the presence of a protein immunologically similar to serum albumin in these tissues and in brain, detected by monospecific and polyvalent antisera

Crystallins demonstrated by antisera	Intra-ocular tissues						Extra-ocular tissue:	Antiserum applied	Monospecific	Polyvalent	Referred to
	Lens	Cornea	Vitreous body	Aqueous humor	Iris	Retina	Brain				
α-crystallin	+	+	+	+	+	+	−	anti lens α-crystallin serum	m		Plate 6
								anti lens α-cryst. (tolerant) serum	m		Plate 7
FISC (7 components or less)	+	+	+	+	+	+	−	anti FISC (tolerant) serum	m		Plate 8
Long Line Material	+	+	+	+	+	+	−	*anti* total lens serum		p	Plates 1, 2, 3
								anti total iris serum		p	Plate 4
"ALBUMIN" (a protein immunologically similar to serum albumin)	−	+	+	+	+	+	+	anti total iris serum		p	Plate 4
								anti iris α-crystallin serum	m		Plate 5

\+ indicates the presence of antigen(s).
− indicates the absence of antigen(s).
m, monospecific antiserum.
p, polyvalent antiserum.

ocular tissues contained, besides α-crystallin, a protein with a higher anodic mobility, immunologically similar to serum albumin [Plate 5].

Using immunological unresponsiveness two very specific tolerant antisera directed to chick lens FISC and α-crystallin antigens respectively were prepared to confirm the presence of these antigens in the different intra-ocular tissues [Plates 7, 8]. By means of the isoelectric focusing technique in polyacrylamide gel [Plate 9] a great deal of similarity especially in the α-crystallin and FISC region is emphasized for the intra-ocular tissues studied.

With chick *cornea* we detected by immunoelectrophoresis against anti-total lens serum the presence of α-crystallin and the electrophoretic "Long Line Material" [Plate 1(b)]. By line-diffusion electrophoresis according to Osserman, which is a very sensitive method, the FISC component is localized in cornea [Plate 2(b)]. The presence of this crystallin in cornea is confirmed by two-dimensional antigen/antibody crossed electrophoresis [Plate 3(b)], and with tolerant antiserum to FISC [Plate 8(b)]. By immunoelectrophoresis Maisel (1962) could not detect the FISC component in cornea. This could possibly be due to low concentration of this antigen in cornea. Although François, Rabaey and Evens (1958) detected albumin, β-globulin and γ-globulin as serum proteins in human *aqueous humor*, only one report by Little and Langman (1964) was available on the detection of lens antigens in human *vitreous body* and aqueous humor. As in accordance with our results with chick, the patterns of vitreous body and aqueous humor are very similar [Plate 2(c), (d)]. We observed the same phenomenon that FISC from vitreous body and aqueous humor was incomplete [Plate 2(c), (d), Plate 8(c), (d)]. Clayton (1970) also found that in early embryonic stages FISC was antigenically incomplete.

Maisel and Harmison (1963a) isolated from *iris* a protein with antigenic specificities of α-crystallin. We confirmed this result. FISC, a "β-mobility component" (Clayton et al., 1968) was found by these authors to be absent in iris, using Osserman tests with iris extract as diffusional antigen in the longitudinal trough. Our electrophoresis experiments of iris against anti-total lens serum [Plate 1(e)] did not show either the presence of FISC or a part of it in iris. However, our Osserman test of iris extract against anti total lens extract in the longitudinal trough [Plate 2(e)], with anti total lens serum does show the presence of FISC in iris. Using other techniques like two-dimensional crossed electrophoresis, and by isoelectric focusing, the same presence was confirmed. Also with tolerant antiserum specific to FISC we detected FISC in iris [Plate 8(e)]. Tolerance experiments with chick lens and iris (Brahma et al., 1971) showed also that serological determinants directed to lens and present in lens were also present in iris, and vice versa. It is assumed that all proteins present in iris, which are not shared by serum proteins, have lens determinants. On the other hand we detected by immunoelectrophoresis of chick *serum* against tolerant lens anti-iris serum, that chick *serum determinants* were present in iris. The reverse experiment testing chick serum as antigen against tolerant iris anti-lens serum proved to be negative, because this antiserum did not contain any serological determinant specific to chick serum, due to the acquired tolerance against chick serum proteins present in iris.

In *retina* the concentration of FISC was very low, and could not be detected either with the Osserman test [Plate 2(f)] nor by immunoelectrophoresis with anti-lens serum [Plate 1(f)] and specific anti-FISC serum [Plate 8(f)]. By two-dimensional crossed electrophoresis, however, FISC or a part of it could be detected, though in a very low quantity [Plate 3(f)].

Brain as an extra-ocular tissue was studied to detect cross-reactivity of lens antibodies with brain antigens. We hardly found any antigens shared by brain and lens proteins [Plate 1(g), Plate 6(g), Plate 7(g)]. By the Osserman test of brain extract against anti lens serum no lens antigens fusing with brain constituents could be detected [Plate 2(g)]. Only some cathodic lines were found, which did not fuse with the lens antigens put in the longitudinal trough [Plate 2(g)]. Clayton (1970) found reaction of identity of chick brain with lens components of the same cathodic mobilities that we observed in Plate 2(g). With Laurell's crossed electrophoresis only a very faint a-specific reaction of brain extract with anti-lens serum was observed. Possibly Davies, de Saram, Perkins and Wood (1965) observed in non-ocular tissues of the guinea pig the same antigens with components identical to lens antigens having β- and γ-mobilities, which were the same antigens having γ-mobilities with anti total iris serum [Plate 4(g)].

Using anti-total iris serum and anti iris α-crystallin serum brain and iris shared a constituent, immunologically identical to serum albumin [Plate 4(g), Plate 5(g)]. The same holds for other extra-ocular tissues supplied by the blood stream. Maisel and Harmison (1963b) found the same anodic component in chick serum by electrophoretic test of these antigens with anti-iris serum. These authors detected the same component also in 8-day iris extract tested with the same antiserum. Conclusive results concerning further studies of this albumin-like constituent found in intra-ocular tissues will be communicated in another article (Bours, in preparation).

A possibility of *leakage* of the antigens from the lens to the other intra-ocular tissues, as to iris was suggested by Zwaan (1963, 1968a). This, however, could be excluded by the fact that time between the collection of chicken heads and the dissection of iris was reduced to less than 20 min. Moreover, had there been a diffusion, we could have expected all the components of lens FISC into the iris and other intra-ocular tissues [Plate 9].

In isoelectric focusing *microheterogeneity* is frequently observed, and described by various authors [Susor, Kochman and Rutter, 1969; Hayes and Wellner, 1969; Drysdale, 1970; Righetti and Drysdale, 1971]. In the present isoelectric focusing experiments we found microheterogeneity in chick lens crystallins. For example, FISC (δ-crystallin), which appeared homogeneous on electrophoresis (Bours and van Doorenmaalen 1970) and showed heterogeneity on prolonged electrophoresis as described by Rabaey (1962) and Zwaan (1968a, b), could be resolved by isoelectric focusing into seven components [Plate 9(a)]. These seven components showed a reaction of identity (Bours and van Doorenmaalen 1970). Hayes and Wellner (1969) presented evidence that this microheterogeneity could be attributed to differences in the primary structure of the various protein components observed.

It may be emphasized that by means of *immunofluorescence* experiments the presence of lens antigens in intra-ocular tissues cannot be detected due to either a low concentration or masking of these antigens in the tissues concerned.

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