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Isoelectric Focusing Studies on Immunochemical Change with Age in Chicken Lens Crystallin

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ABSTRACT. Changes of alpha-, beta- and delta-crystallins of chicken lens soluble protein with age from 7-day embryos to 1.5-year-old chickens were examined by isoelectric focusing and by immunochemical studies with anti chicken total lens crystallin serum and anti chicken beta-crystallin serum.

Alpha- and delta-crystallins were isofocused in a narrow pI region (alpha : pI 4.90-5.30 ; delta : pI 4.90-6.60) and this range did not become broader with age. However, the pI range of beta-crystallin gradually became broader during development.

On immunochemical analysis, beta-crystallin gave two types of precipitin line, named beta〔Ls〕and beta〔Lc〕. The beta〔Ls〕from 7-day embryos was split into two pI regions (pI 5.91-6.18 and pI 6.41-6.64), whereas that from 10-day embryos was fused into one line (pI 5.99-6.86) and a new precipitin line, beta〔Lc〕, was detected at pI 6.65-6.86. These two types of precipitin line, beta〔Ls〕and beta〔Lc〕, extended to an alkaline region before hatching, and to all pI regions (pI 4.26-8.19) in chickens of 40-60 days old.

However, materials of over pI 7 of both beta〔Ls〕and beta〔Lc〕had disappeared in chickens of over 1.5 years old.

Key words : immunochemical studies — dependent on age — chick beta-crystallin — isoelectric focusing — pre- and post-hatched chicken lens

Previously, we analyzed chicken soluble crystallin after preparative flat-bed isoelectric focusing by the immunochemical technique.[1] Results showed that chicken alpha-, beta- and delta-crystallins exist in the same pI range (pI 4.60-5.43), but that the pI range of beta-crystallin (pI 4.23-8.86) is wider than that of alpha- and delta-crystallin.

These findings are not consistent with reports of others that each chicken lens crystallin has a different pI value on thin-layer polyacrylamide gel isoelectric focusing[2,3] and that on isoelectric focusing gel, chicken beta-crystallin gives six major beta-crystallin subunits and some minor components.[4]

The ontogeny of chicken lens has been studied by immunohistochemical and biochemical techniques,[5-8] and the subunit composition of chicken lens has been reported.[9,10]

However, we think that it is necessary to re-examine the reported pI value

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of chicken lens crystallin. To obtain more information on the basic structure of chicken lens crystallin, in this work we analyzed chicken lens crystallin by isoelectric focusing and immunochemical analysis. We also examined the changes of chicken beta-crystallin during development and aging.

MATERIALS AND METHODS

Preparation of Water-Soluble Crystallins of Chicken Lens :
Lens tissue was obtained from chick embryos of 7 days (stage 30), 10 days (stage 36) and 15 days (stage 41) and chickens of 1 day (just hatched, stage 46), 40-60 days and 1.5 years old. Stages are numbered according to Hamburger and Hamilton.[11]

Lenses without neighboring connective tissue of the vitreous body were homogenized in 3 volumes of cold phosphate-saline buffer (0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.3) with 0.01% 2-mercaptoethanol (Koch-Light Lab.) to prevent oxidation, and the supernatants were obtained by centrifuging the homogenates at 9,500 g for 20 min. They were then adjusted to 6.8% protein with a refractometer for flat-bed isoelectric focusing in a granulated gel of Sephadex G-75 superfine (Pharmacia Fine Chemicals).

Isoelectric focusing was performed at 300 volts at 10°C for 14-16 hours as described in detail in our previous paper.[1] After isofocusing, the gel was cut into 30 sections and proteins were extracted from each section.

Immunochemical Analysis :
The extracted lens proteins were tested immunochemically against anti chicken total lens crystallin serum (ACLS(T)) and anti chicken lens beta-crystallin serum (ACLS (beta : 5.87-6.33)). Anti chicken total lens crystallin serum was prepared by the method of Zwaan and Ikeda.[6] Anti chicken lens beta-crystallin serum was raised by injection of a mixture of chicken lens beta-crystallin, isofocused at pI 5.87-6.33, with an equal volume of Freund complete adjuvant (Difco Lab., Detroit, Mich.). Specific antiserum for chicken lens beta-crystallin was absorbed with chicken liver and kidney extracts to avoid a non-specific reaction,[12] before use in immunodiffusion tests.

For micro-Ouchterlony tests, a slide (3×12.5 cm) was covered with 1.5% agar (Special Agar Noble, Difco) in Tris-EDTA-boric acid buffer (89 mM-Tris, 2.5 mM-EDTA, 89 mM-boric acid, pH 8.4). Wells (3 mm in diameter) were made in a straight line 4 mm from the groove for antiserum, and the wells were each filled with 7 μl of extracted protein.

RESULTS

Isoelectric Focusing Profiles :
Equal amounts of protein from lenses of chickens of various stages were subjected to flat-bed isoelectric focusing. The profiles of isoelectric focusing are shown in Figs. 1 and 2. One main band and two minor bands were obtained from 7-day embryos (stage 30). The main band was common to all stages and its pI value and relative optical density were similar at all stages from 7-day embryos to 1.5-year-old chickens. Relative to the main band, the two minor

groups of proteins, isofocused at pI 5.91–6.18 and pI 6.41–6.64, increased with age. They seemed to reach a maximum 40–60 days after birth, and those of the higher pI region were not seen in 1.5-year-old chickens. These groups of lens crystallins changed with age, as revealed by immunochemical analysis using anti chicken total lens crystallin serum.

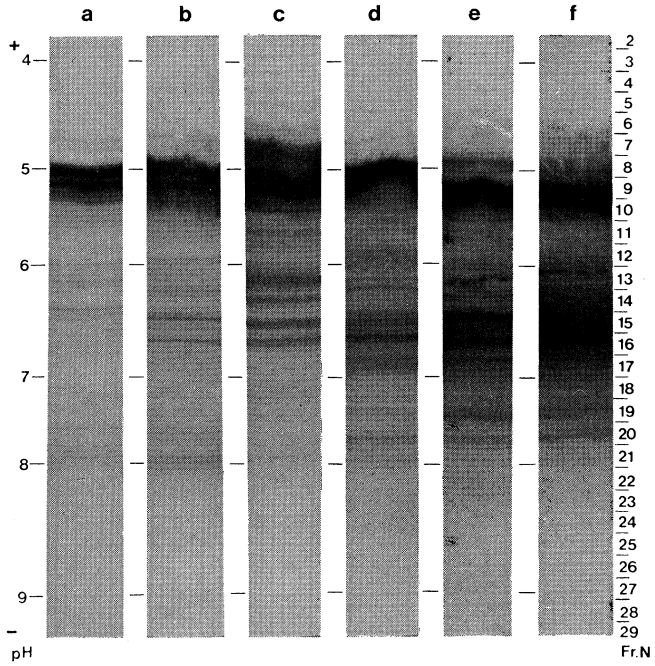

Fig. 1. Changes of isoelectric focusing patterns of chicken lens with age; a) 7-day embryo (stage 30), b) 10-day embryo (stage 36), c) 15-day embryo (stage 41) and after birth, d) 1-day chick (stage 46), e) 40-60-day chicken, f) 1.5-year-old chicken. Stages are numbered according to Hamburger and Hamilton (1951).[11] Samples of 3 ml of lens water-soluble fractions (6.8% protein) were used. Isoelectric focusing (Ampholine, pH 3–10, LKB Product AB) was performed at a constant voltage of 300 v for 14–16 hr at 10°C. α, β and δ : alpha-, beta-, and delta-crystallins.

Immunochemical Analysis :

Lens crystallins extracted from the gel after isoelectic focusing were tested immunochemically against anti chicken total lens crystallin serum as shown in Fig. 3.

Studies with anti chicken total lens crystallin serum showed that the main components of alpha- and delta-crystallins had narrow pI ranges from 7-day embryos to 1.5-year-old chickens (alpha: 4.90–5.30; delta: 4.90–6.06). On the contrary, the precipitin lines of beta-crystallin changed with age. The two groups of precipitin lines at pI 5.91–6.18 (fr. 13) and pI 6.41–6.64 (fr. 15) obtained from 7-day embryos extended to a higher pI range till 15 days of embryonic life and to a lower pI range in 1-day chicks. Moreover, the precipitin lines that extended to a higher pI range until 40–60 day after birth had disappeared in 1.5-year-old chickens. These findings are in partial agreement with

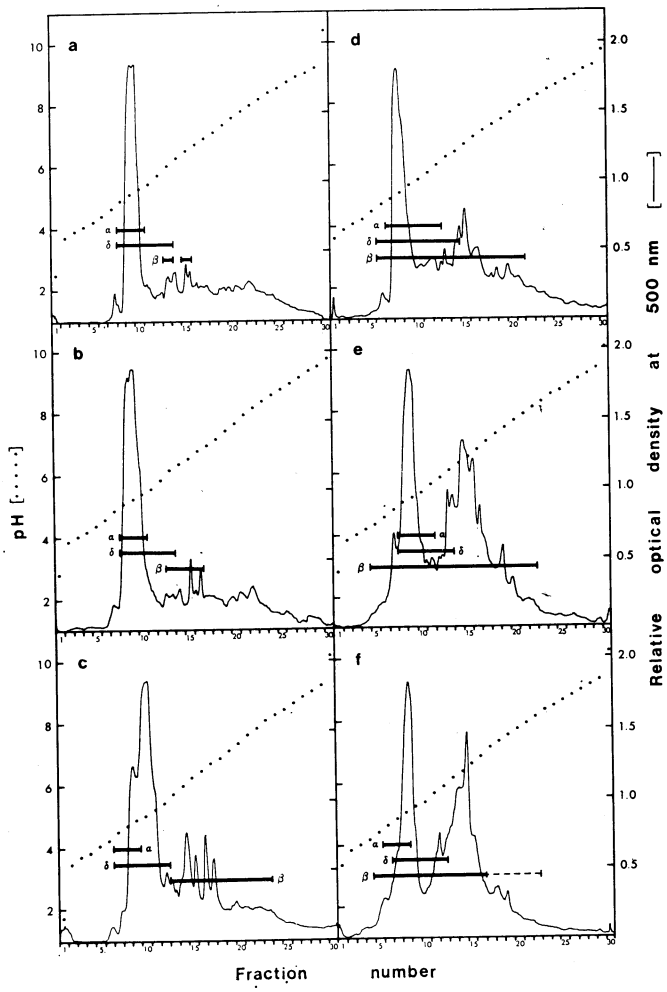

Fig. 2. Densitometric scanning profiles of isoelectric focusing patterns of chicken lenses, a) 7-day embryo (stage 30), b) 10-day embryo (stage 36), c) 15-day embryo (stage 41) and after birth, d) 1-day chick (stage 46), e) 40-60-day chicken, f) 1.5-year-old chicken. The photographic recording method described previously (Mishima and Ikeda, 1981)[1] was used. α, β and δ: alpha-, beta-, and delta-crystallins. The solid lines, pI ranges based on the results of the immunochemical tests shown in Fig. 3.

those of others.[6,10,13,14] But other workers did not describe the beta-crystallin in the lowest pI range and/or that in the highest pI range found in young chickens that disappeared in old adult chickens. To determine how and when lens crystallin was synthesized and what its composition was, we examined the change of chick beta-crystallin with age using pure anti chicken lens beta-crystallin serum.

Anti chicken lens beta-crystallin was absorbed with chicken liver and kidney before use to avoid non-specific reactions. This absorbed antiserum is

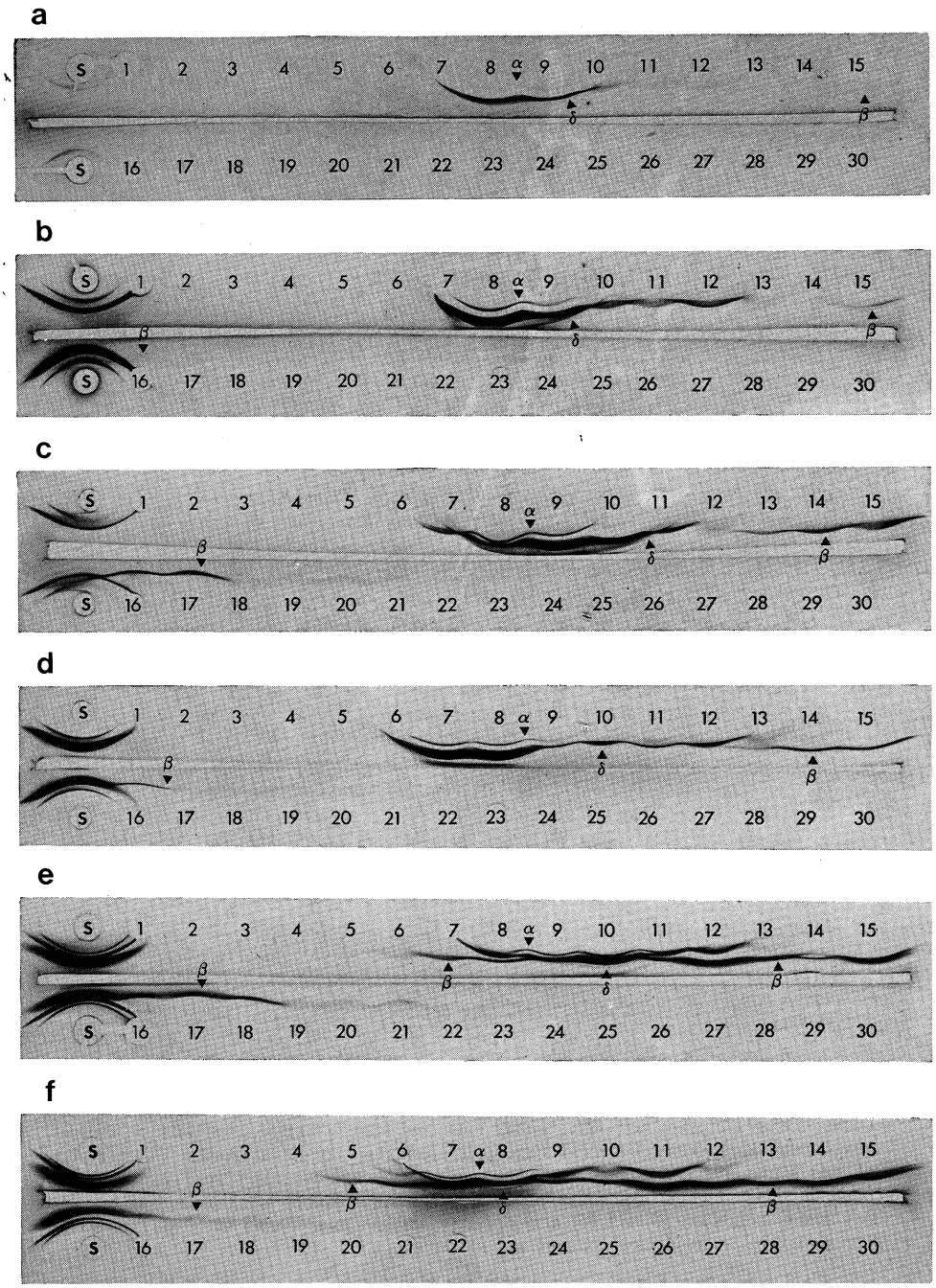

Fig. 3. Immunodiffusion tests against anti chicken lens total crystallin serum on lens crystallins after isoelectric focusing from chickens at different stages of development. In micro-Ouchterlony plates (3×12.5 cm), wells of 3 mm in diameter were made in a straight line 4mm from the groove for antiserum, and each well (section 1-30) was filled with 7 μl of extract. The samples are of lens water soluble protein (s) at various stages. Extracts of 30 sections were used (1-30, from the anodal side to the cathodal side): a) 7-, b) 10- and c) 15- day embryos (stages 30, 36 and 41) and d) 1-day chicks (stage 46), e) 40-60-day chickens and f) 1.5-year-old-chickens (from top to bottom.

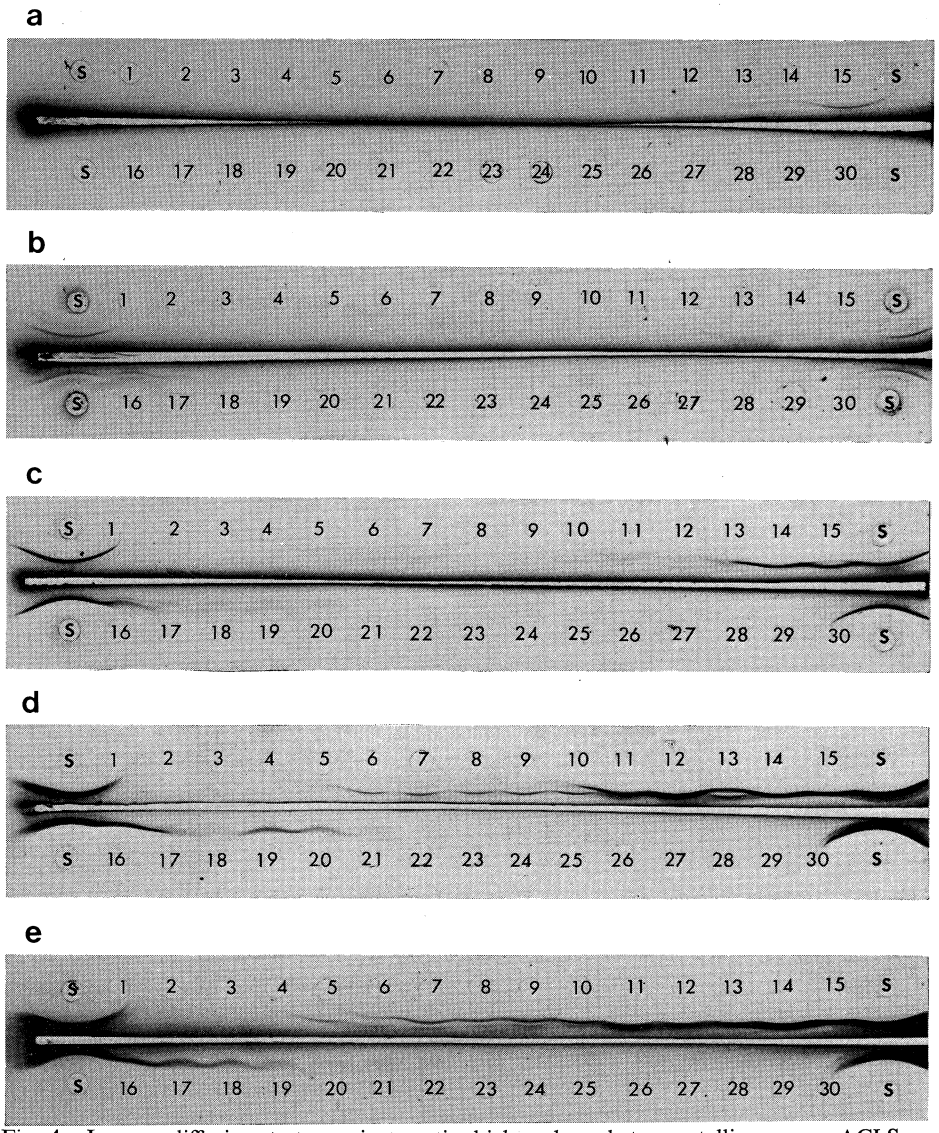

Fig. 4. Immunodiffusion tests against anti chicken lens beta-crystallin serum ACLS (beta : 5.87–6.33), absorbed with chicken liver and kidney extracts, on lens crystallins after isoelectric focusing from chickens at different stages of development. The samples of lens water soluble protein (s) were obtained at various stages and are extracts from 30 sections (1-30, from the anodal side to the cathodal side); a) 7- and b) 10-day embryos (stages 30 and 36) and c) 1-day chicks (stage 46), d) 40-60-day chickens and e) 1.5-year-old chickens (from top to bottom).

named ACLS (beta : 5.87–6.33). In immunochemical tests, two types of precipitin line, named beta [Ls] and beta [Lc] were observed. Beta [Ls] is a precipitin line showing spur formation, and beta [Lc] is a continuous line without spur formation over all the pI range. The beta [Ls] of 7-day embryos was split into bands at pI 5.91–6.18 and pI 6.41–6.64. This confirmed the densitometric scanning profiles (Fig. 2). In 10-day embryos, two precipitin lines

were observed, beta [Ls] at pI 5.99–6.86, like that of 7-day embryos but without a split, and a new precipitin line, beta [Lc], detected at pI 6.65–6.86. These two precipitin lines were observed at stages from 10-day embryos to 1.5-year-old chickens, as summarized in Fig. 5. In 15-day embryos, both precipitin lines extended to the alkaline region, and at birth they also extended to the acidic region. During maturation of chickens, these two types of line extended, showing the widest range from an acidic to alkaline region, pI 4.44–7.99 and pI 4.25–8.19, in 40–60 day old chickens. But material in the high alkaline pI region had disappeared in 1.5-year-old chickens; namely, beta [Ls] and beta [Lc] of over pI 7 had disappeared.

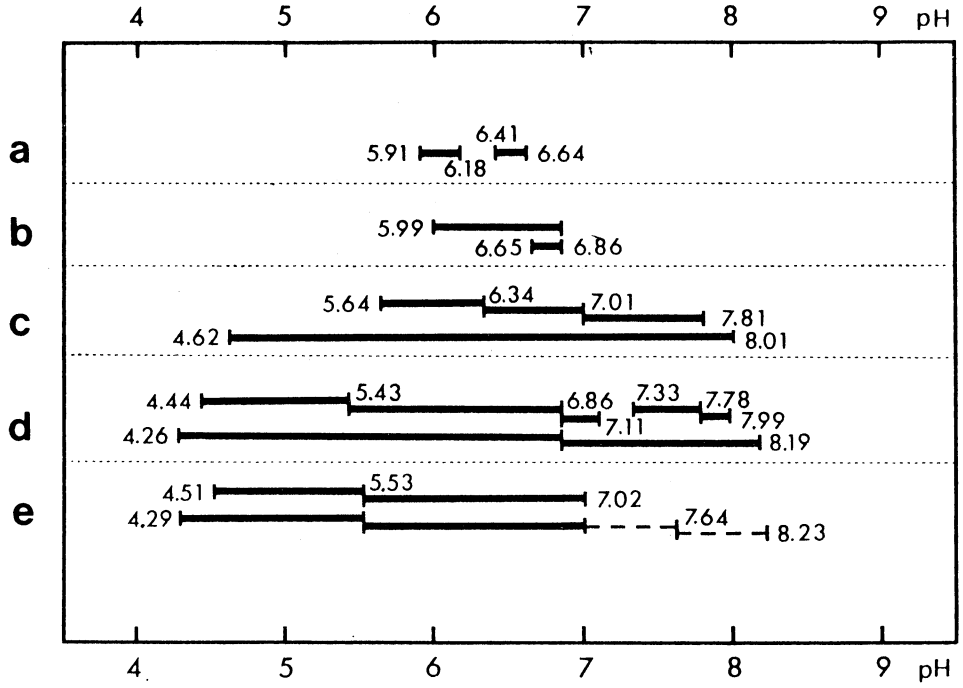

Fig. 5. Summary of changes in pI ranges of beta-crystallins with age. The solid lines indicate pI ranges deduced from the immunochemical tests shown in Fig. 4. The pI ranges are indicated as actual values at the ends of the lines, and the solid lines are bordered by two or three sections at the point of immunochemical spur formation. a) 7–, b) 10-day embryos (stages 30 and 36) and c) 1-day chicks (stage 46), d) 40–60-day chickens and e) 1.5-year-old chickens (from top to bottom).

It is interesting that the two types of precipitin line were seen with or without spur formation, respectively, suggesting that there are two types of process in synthesis of chicken lens beta-crystallin.

DISCUSSION

In this work, we used a preparative flat-bed isoelectric focusing technique, because with this technique it is easy to recover isofocused protein from the

gels after electrofocusing. We obtained enough material from the gels to analyse the structure, amino acid sequence and molecular weight of lens crystallins. Extracting from 30 fractions, the proteins were analysed immunochemically and the changes in pI ranges of beta-crystallins with age were analysed in comparison with those of other crystallins.

The development of lens crystallin in chicks has been well established, and reviewed by Clayton.[15] Zwaan and Ikeda[5,6] reported that delta-crystallin was synthesized in the lens from stage 14, and matured in 5.5-day embryos, whereas beta-crystallin appeared at stage 16 and alpha-crystallin at stage 19. Clayton et al.[12] reported immunofluorescence, immunelectrophoretic and physicochemical analyses of two classes of chicken beta-crystallin, cathodal and anodal groups, and suggested that these two groups were synthesized at somewhat different times. But there are few reports on the ontogeny of chick beta-crystallins.

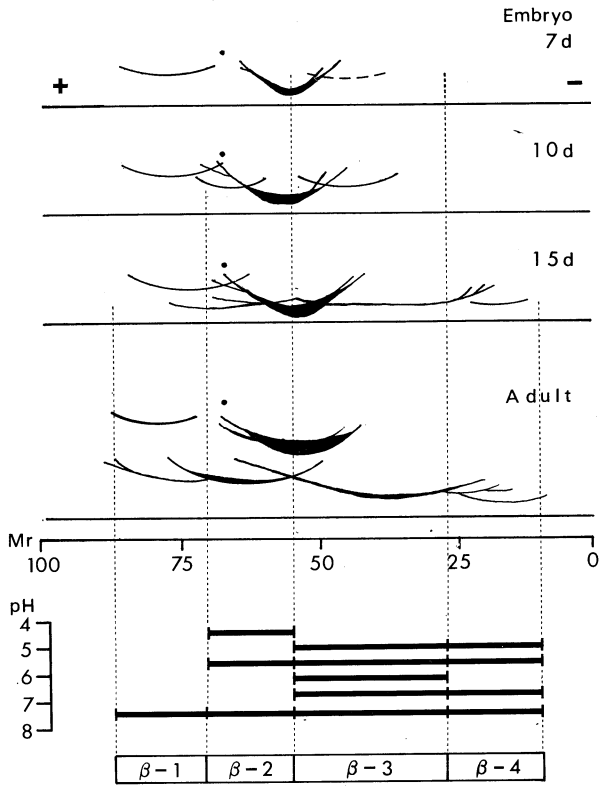

Fig. 6. Combination of our results and those of Zwaan (1963).[16] Our results are based on isoelectric focusing (Mishima and Ikeda, 1981).[1] Changes in immunoelectric mobilities with age are the results of Zwaan (1963).[16]

There have been several recent studies on lens crystallins by isoelectric focusing. Bours[3] found by polyacrylamide thin-layer focusing that beta-crystallin has a pI of 5.58-7.58. Using the isoelectric focusing gel method, Thomson et al.[4] confirmed results on purified beta-crystallin obtained by gel chromatography.

On the contrary, our previous immunochemical studies on water-soluble crystallin from the lens of chickens of 40-60 days old suggested that the three types of chick lens crystallin coexisted in a certain pI range and that beta-crystallin gives four groups of precipitin lines localized in a wide pI range of 4.23-8.86, with spur formations. Therefore, we suggested that many workers had overlooked some beta-crystallin localized in an acidic pI range. Moreover, we observed that four immunoheterogenous groups of beta-crystallin can be separated according to their pI values. Our results[1] and those of Zwaan[16] are modified and combined in the scheme shown in Fig. 6.

In the present study we examined the relation between the four immunoheterogenous groups of beta-crystallin and their pI values in embryos, chicks at hatching, and adult and old chickens, by flat-bed isoelectric focusing and immunochemical analysis. The results are shown as densitometric scanning profiles to facilitate understanding of relative changes with age. Beta-crystallin showed increase in density at over pI 6 in chickens until 40-60 days after birth and decrease in density at over pI 7 in 1.5-year-old chickens, possibly due to changes in synthesis and degradation of the protein. The strongest evidence for this change was that in immunodiffusion tests, a new precipitin line at over pI 6 was seen with material from chickens of 40-60 days old, and a weak one at over pI 7 with material from 1.5-year-old chickens.

No marked changes in the relative amounts of alpha- and delta-crystallins were seen during development from 7-day embryos, and immunochemical analyses showed no age-dependent change in the pI values of alpha- and delta-crystallins after the stage of 7-day embryos. This finding that the syntheses of alpha- and delta-crystallin were complete in 7-day embryos confirmed the results of immunofluorescence analysis by Zwaan and Ikeda.[5,6] In contrast, before hatching beta-crystallin had a pI of 5.58-8.03, but after hatching it extended from pI 4.26 to pI 8.19. The pI of beta-crystallin, beta[Ls] of 7-day embryos showed two narrow split regions, pI 5.91-6.18 and 6.41-6.64, which fused in 10-day embryos to pI 5.99-6.86. At about the same time, some group of beta-crystallins showed spur formation, and a new precipitin line, beta [Lc], appeared at pI 6.65-5.86. After hatching, the two types of beta-crystallin were localized at pI 4.44-7.99 and pI 4.26-8.19 but material of over pI 7 had disappeared in 1.5-year-old chickens. Our results suggest that beta-crystallin consists of two types of protein : one type, beta[Lc], has common antigenicity all over the pI range, while the other, beta[Ls], has some common antigenicity but also shows spur formation.

Clayton et al.[17] suggested that beta-crystallin was synthesized as two groups of subunits, anode and cathode groups, consisting of subunits with common antigenicity to adjacent subunits. This discrepancy between our findings and those of others raises the problem of the relation between the growth and maturation of chickens and the isoelectric point of their beta-crystallin. It is possible to consider that (1) beta-crystallin of all pI values is not synthesized simultaneously ; (2) beta-crystallin of neutral to high pI range is synthesized progressively before hatching and to that of a low pI range after hatching ; (3) at all pIs, some beta-crystallin group has the same antigenicity, and (4) another beta-crystallin group has some antigenicities in common, as suggested by Clayton et al.[17]

Moreover, we observed that the beta-crystallin with a high pI had disappeared in 1.5-year-old chickens. This disappearance could be due to degradation of beta-crystallin, because it is reported that the high-molecular weight protein (HM-protein) of chicken lens consists entirely of beta-crystallin (Gan et al.[18]), and we demonstrated that the HM-protein has an acidic pI of 4.15-4.70 (unpublished data). This pI range corresponds to the most acidic pI region of beta-crystallin, in which the beta-crystallin will ultimately be degraded. As it was difficult to obtain further information on the structure of beta-crystallins by SDS-dissociation, we are now studying specific subunits of beta-crystallin by monoclonal antibody analysis.

Acknowledgment

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