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DEVELOPMENTAL CHANGES IN PROTEINS OF PURIFIED MEMBRANES OF CHICKEN LENSES AND EVIDENCE FOR CONTAMINATION BY CYTOPLASMIC δ-CRYSTALLIN

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Summary

Urea-washed membranes from embryonic chick lenses (15 days old) and from the cortical and nuclear regions of adult chicken lenses (1 year) have been prepared by repeated centrifugation through discontinuous density gradients. The protein components of the isolated membranes have been examined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and urea. Proteins with molecular weights of 75 000, 56 000, 54 000, 48 000, 34 000, 32 000, 25 000, and 22 000 were present in all the membrane preparations, although their proportions changed during development. One additional protein, molecular weight 70 000, was seen only in the embryonic lens membranes. The greatest developmental change was the increase in 25 000 molecular weight protein from 12% in the embryonic lens to about 45% in the adult lens. Since it has been suggested that this protein is associated with gap junctions, its increase during development may reflect a corresponding increase in the number of gap junctions in the lens.

The 50 000 molecular weight protein of embryonic lens membranes and membranes of adult nuclear lens fibers consisted at least partly of δ-crystallin, since δ-crystallin peptides could be identified in tryptic pepetide maps of the isolated protein after in vitro radioiodination. Peptide maps of the 50 000 molecular weight protein of cortical lens fiber membranes contained no identifiable δ-crystallin peptides, although it is possible that modified δ-crystallin peptides may be present. The level of cytoplasmic contamination of the membrane fraction was estimated by preparing lens membranes in the presence of added δ-[^{35}S]crystallin. The results indicated that cytoplasmic contamination contributes significantly to the presence of δ-crystallin in lens membrane preparations.

Introduction

Considerable interest exists in the protein composition of lens membranes. The major component of these membranes has been reported to be a 25 000–27 000 molecular weight protein [1–7], which has been implicated in gap junction formation [3,7]. The possibility has also been raised that crystallins are intrinsic membrane components [1–4].

In the present study we have investigated the protein composition of chicken lens membranes at different stages of development and maturation. Special attention has been given to the possibility that δ-crystallin, the principal protein of the developing chick lens [8,9], may contaminate lens membrane preparations. We show here that there are significant changes in the relative amounts of different proteins in the purified membranes at different stages of development, including an increase in the proportion of the 26 000 molecular weight protein, and provide direct evidence that δ-crystallin may indeed contaminate the membranes during their isolation. In view of the high concentrations of crystallins in lenses of all species, we suggest that such contamination may have led to an overestimate of the crystallin contribution to lens membranes.

Materials and Methods

Isolation of lenses. Adult, white Leghorn chickens (50–60 weeks old) and fertile, white leghorn eggs were obtained from Truslow Farms, Chestertown, MD, U.S.A. Adult chickens were decapitated; the lenses were removed, decapsulated, and separated into a 'cortical' fraction (the outer third of the lens) and a 'nuclear' fraction (the remainder of the lens). Fertile eggs were incubated 15 days; lenses were removed from the embryos but were not decapsulated. All lens tissues were frozen in liquid nitrogen within 10 min of the death of the animal, except in one control experiment described in the text.

Preparation of lens membranes. Each membrane preparation employed lenses from 6 to 12 adult chickens or 30 chick embryos. The lenses were allowed to thaw in approximately 20 vols. of 0.25 M sucrose in 25 mM KCl, 5 mM magnesium acetate, 50 mM Tris-HCl (pH 7.6), with 0.1% 2-mercaptoethanol. The tissues were then homogenized ten strokes in a tight-fitting Dounce homogenizer on ice. The homogenate was transferred to a cellulose nitrate tube (Beckman Corp.), underlayered with 2.4 M sucrose in the same buffer, and centrifuged at 75 000 × *g*, 4°C for 1 h in a Beckman SW 60 Ti rotor. This step, initially used to remove the cell nuclei of the embryonic lenses [10], was retained in preparing the adult chicken lens membranes in order to collect the membranes without packing them tightly into a pellet. The crude membrane layer which collected at the interface was removed with the tip of a wide-mouthed pipet and transferred to another cellulose nitrate tube, where it was suspended in 2 ml of 8 M urea in 15 mM sodium citrate/0.15 NaCl (pH 6.8). The membranes could be thoroughly dispersed by gentle vortex mixing. An equal volume of 15 mM sodium citrate, 0.15 M NaCl (pH 6.8) was added to dilute the urea to 4 M, the suspension was underlayered with 1.6 M sucrose in

the same buffer, and the membranes were collected at the interface by centrifuging at 75 000 × *g* at 20°C for 1 h. This washing procedure was carried out five times. Aliquots of the membrane fractions and the washing solutions were assayed for protein by a modification of the Lowry procedure which includes 1% sodium dodecyl sulfate [11,12]. The washed membranes were suspended in 0.25 M sucrose, 25 mM KCl, 5 mM magnesium acetate, 50 mM Tris-HCl (pH 7.6) and were pelleted at 100 000 × *g*, 4°C for 1 h. The final pellet was drained and dissolved in 10% glycerol, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 0.6% Tris-HCl (pH 6.8).

Polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and urea, was performed as previously described [13] by a modification of the method of Maizel et al. [14]. The samples were not boiled, since this may lead to the formation of insoluble complexes [5,15].

In vitro radioiodination. Radioiodination of proteins was performed by the method of Elder et al. [16]. Stained protein bands to be radioiodinated were cut from the gel, washed extensively with 10% methanol and lyophilized. The proteins were radioiodinated with ^{125}I (New England Nuclear), 17 mCi/μg while still in the polyacrylamide gel. After a single wash with 10% acetic acid, the gel slices were washed extensively with methanol and lyophilized.

Tryptic peptide mapping. Tryptic digestion of the radioiodinated proteins was carried out as described by Elder et al. [16]. The gel slices were placed in a solution of 50 μg/ml trypsin (Worthington, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)) in 50 mM NH_4HCO_3. After an overnight incubation at 37°C, the solution containing the tryptic peptides, was removed and lyophilized. The lyophilized samples were dissolved in 10 μl of acetic acid/formic acid/H_2O (15 : 5 : 80, v/v) and aliquots (2—4 μl) were spotted onto 10 × 10 cm cellulose-coated plates (EM Laboratories, Elmsford, NY). The peptides were separated in the first dimension by electrophoresis (30 min, 1 kV). The plates were dried and subjected to chromatography in a second dimension in butanol/pyridine/acetic acid/H_2O (32.5 : 25 : 5 : 20, v/v). The peptides were located by autoradiography using Kodak SB-5 X-ray film.

δ-[^{35}S]Crystallin mixing experiments. Embryonic lenses were homogenized in 2.0 ml of homogenization buffer, 0.25 M sucrose in 25 mM KCl, 5 mM magnesium acetate, Tris-HCl (pH 7.6) containing $4 \cdot 10^6$ dpm δ-[^{35}S]crystallin. δ-[^{35}S]Crystallin was labeled as described by Reszelbach et al. [13] and was purified by isoelectric focusing [17]: Nuclear fibers of adult chicken lenses were homogenized in 4.5 ml of the same buffer containing $4 \cdot 10^6$ dpm δ-[^{35}S]-crystallin to which had also been added the combined cytoplasmic proteins of embryonic lenses and adult cortical lens fibers to a final protein concentration of 19 mg/ml. The concentration of δ-crystallin in the homogenization buffer was 2 mg/ml.

Results

SDS/urea-polyacrylamide gel electrophoresis of lens membrane proteins

The proteins of the purified membranes prepared from 15-day-old embryonic chick lenses and from the nuclear and cortical regions of adult chicken lenses

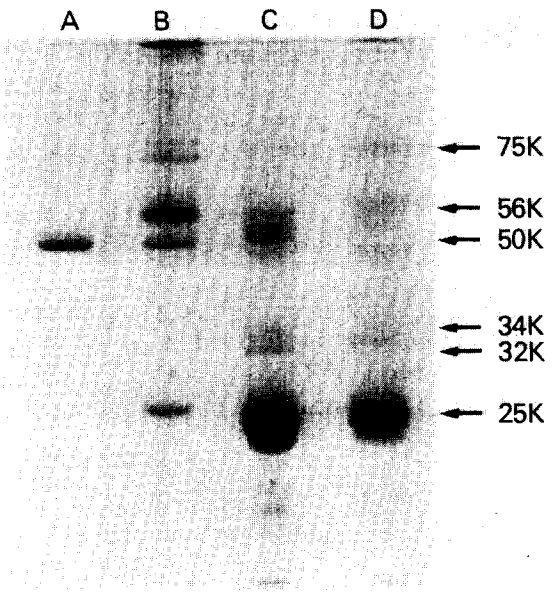

Fig. 1. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate and urea of (A) purified δ-crystallin; (B) membranes of 15-day-old embyronic chick lenses; (C) membranes of nuclear lens fibers of adult chickens, and (D) membranes of cortical lens fibers of adult chickens. Molecular weights were determined by comparison with marker proteins run in adjacent slots. Samples (C) and (D) have been intentially overloaded to make minor bands visible; the overloading has increased the migration of the 25 000 component.

were separated by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and urea (Fig. 1). In each preparation, the fraction of the total membrane protein represented by the principal components was determined by scanning the stained gels and measuring the area under the peaks (Table I). A major component in all three membrane preparations was the protein of molecular weight 25 000. This protein represented almost half the total membrane protein in both the cortical and nuclear lens membrane preparations of the adult chickens. In the membranes of the embryonic chick lenses and of the nuclear region of the adult chicken lenses, two closely spaced bands, comigrating with the two subunits of δ-crystallin, were present in the 50 000 molec-

TABLE I

PERCENT OF TOTAL MEMBRANE PROTEIN

Percentage of total membrane protein represented by three selected proteins. Values are averages of three measurements for embryonic chick lens membranes or two for adult chicken lens membranes, plus or minus the range of observations. The remainder of the protein consisted of minor species, each contributing less than 5% of the total.

	Molecular weight		
	56 000	50 000	25 000
15-day-old embronic chick lens	14 ± 2	11 ± 3	12 ± 3
Adult chicken nuclear fibers	3 ± 1	2 ± 1	44 ± 2
Adult chicken cortical fibers	4 ± 1	<1	46 ± 4

ular weight region of the gel [13,17]. These bands were not visible in the membranes of the adult chicken lens cortex, although a faint band with a slightly greater apparent molecular weight was present. Three relatively small molecular weight proteins (18 000, 17 000 and 12 000) were present in the membranes of the cortical and nuclear lens fibers of adult chickens; the amounts of these proteins in the cortical fibers were so small, however, that they were only visible when the gels were greatly overloaded (data not shown). All the membrane preparations contained proteins with apparent molecular weights of 75 000, 56 000, 54 000, 48 000, 34 000, 32 000 and 22 000, although in different proportions. One embryonic protein, molecular weight 70 000, appeared to be lost entirely during development.

To test the possibility that freezing the lens tissues altered the protein composition of the membranes, a control experiment was performed in which membranes were prepared from frozen and unfrozen lenses of 1-year-old adult chickens, and directly compared by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea. The electrophoretic patterns of lens membrane proteins from frozen and unfrozen tissues were the same (data not shown).

Fingerprints of radioiodinated tryptic peptides

Individual protein bands were cut out of the gel and radioiodinated to label the tyrosine residues [18]. The labeled proteins were then digested with trypsin, and the labeled, tyrosine-containing tryptic peptides of the membrane proteins were compared with those of δ-crystallin. Initially, the two bands of membrane protein comigrating with δ-crystallin were examined separately; however, since the peptide maps of the two bands of membrane protein were indistinguishable, as are the two bands of δ-crystallin (Reszelbach, R. and Piatigorsky, J., unpublished data) both bands were analyzed together in later experiments. Fig. 2 presents the autoradiographs of four such tryptic maps. Many minor spots were always visible on the autoradiographs, even when highly purified δ-crystallin preparations were used. These minor spots may be artifacts resulting from incomplete tryptic digestion, radioautolysis of the iodinated proteins, iodination of amino acids other than tyrosine, or carbamylation of the proteins during their contact with urea; alternatively, they may result from the digestion of modified forms of native δ-crystallin. As indicated in Fig. 2, five major spots were consistently seen in fingerprints of purified δ-crystallin. This agrees well with reports that each δ-crystallin subunit contains 4—7 tyrosine residues [17,19,20]. Peptides comigrating with the major peptides of cytoplasmic δ-crystallin were present in the tryptic digests of the 50 000 molecular weight proteins from the embryonic lens membranes (Fig. 2B) and from the membranes of the nuclear regions of the adult chicken lens (Fig. 2C), consistent with the interpretation that these membrane preparations do, in fact, contain δ-crystallin. In contrast, there were no peptides which comigrated with the major peptides of cytoplasmic δ-crystallin in the fingerprints of the adult chicken lens cortex membranes (Fig. 2D). Of course, the possibility remains open that these peptides have been modified with respect to charge and therefore migrate to different locations on the fingerprint.

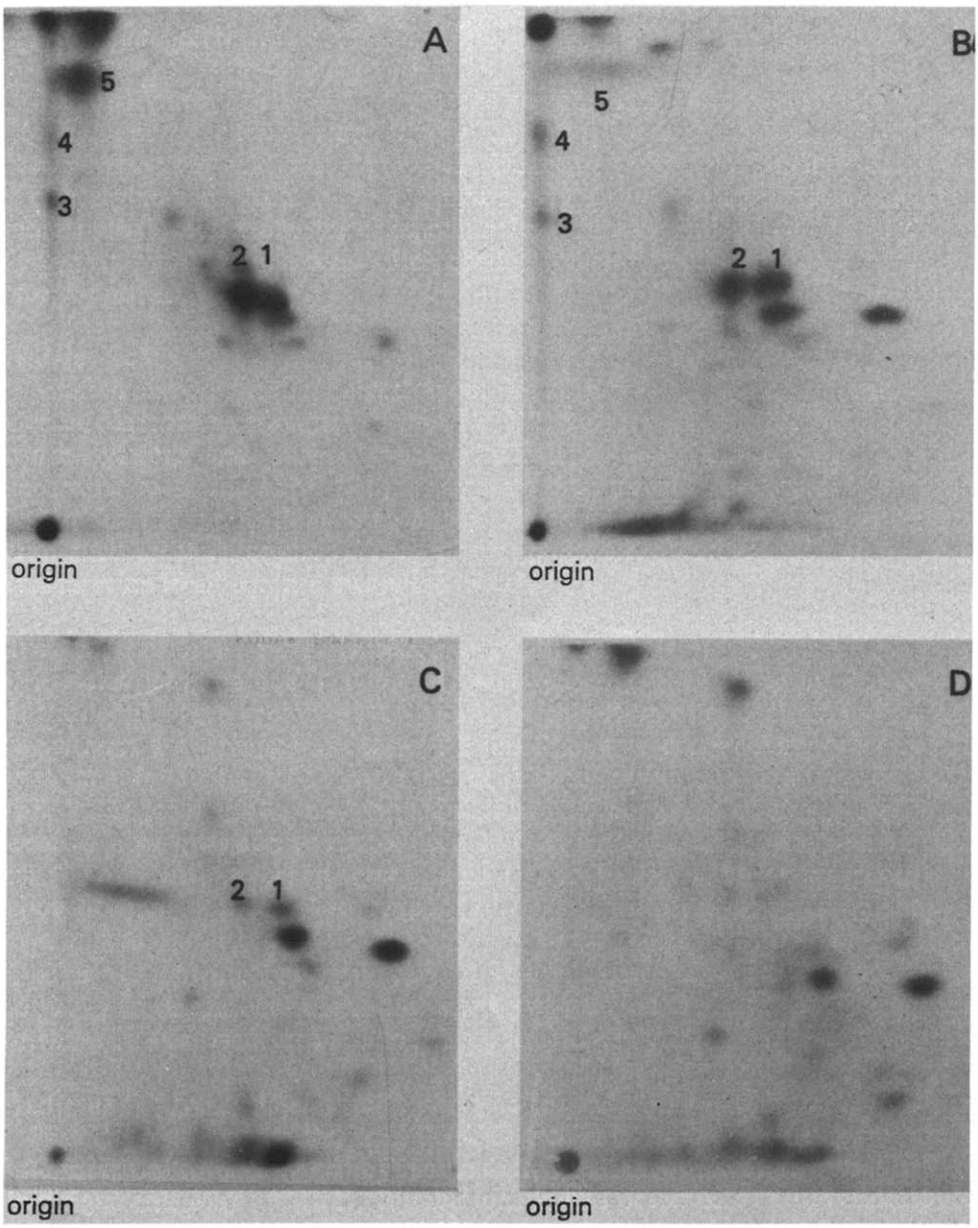

Fig. 2. Autoradiographs of radioiodinated tryptic peptide maps of (A) purified δ-crystallin; (B) 50 000 molecular weight protein of lens membranes of 15-day-old embryonic chicks, (C) 50 000 molecular weight protein of membranes from nuclear lens fibers of adult chickens, and (D) 50 000 molecular weight protein of membranes from cortical lens fibers of adult chickens. δ-Crystallin peptides are indicated by numbers.

Contamination of lens membranes with δ-[^{35}S]crystallin in mixing experiments

A quantitative measure of the level of cytoplasmic contamination of the membrane preparations was needed to test the possibility that the δ-crystallin associated with the membranes of embryonic chick lenses and adult chicken nuclear lens fibers might be the result of contamination. To obtain such an esti-

mate, membranes were prepared from lenses homogenized in the presence of approximately $4 \cdot 10^6$ dpm of isoelectrically purified δ-[^{35}S]crystallin. Unlabeled, membrane-free, cytoplasmic lens extracts containing high concentrations of unlabeled δ-crystallin were also added to the buffer used for homogenizing the adult lens fibers. This avoided the possibility that small amounts of high specific activity δ-[^{35}S]crystallin might bind preferentially to the external membrane surfaces before homogenization. The radioactive environment to which the membranes were exposed was thus always similar to that which unavoidably occurs during homogenization. This precaution was not taken with the embyronic lenses, since their capsules were intact, preventing contact between the δ-[^{35}S]crystallin and the membranes until the cells were ruptured by homogenization. Following homogenization, the membranes were prepared in exactly the same way as in previous experiments, and the amount of radioactivity in the membrane fraction was determined after each wash (Fig. 3). After one centrifugation in the homogenization buffer and three urea washes, the radioactivity in the membrane fraction reached a plateau. The residual radioactivity in the membrane fractions of the embryonic chick lens and the cortical and nuclear regions of adult chickens lens after the usual five urea

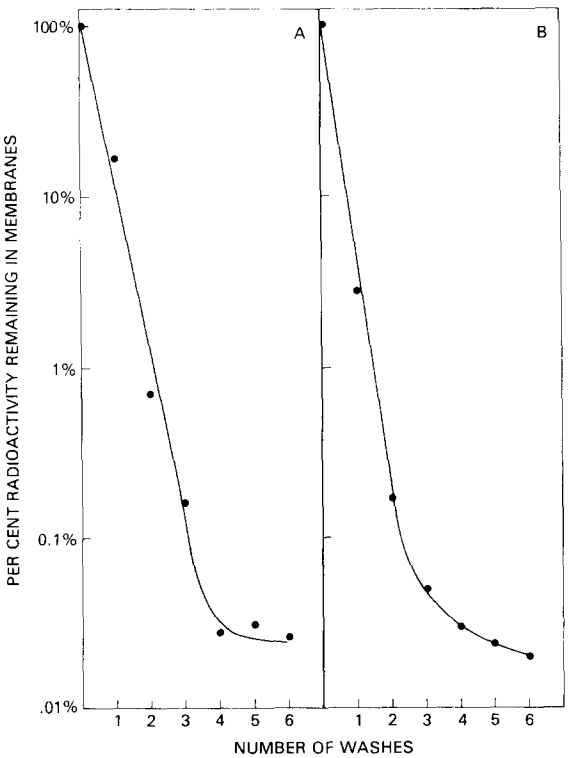

Fig. 3. Removal of δ-[^{35}S]crystallin from membranes of (A) 15-day-old chick embryo lenses, and (B) nuclear fibers of adult chicken lenses. Membranes were prepared as described in Materials and Methods. The first 'wash' refers to removal of the homogenization buffer; subsequent washes contained urea. In three independent experiments the percentage of initial radioactivity remaining after six washes (five urea washes) ranged between 0.02% and 0.03%. Similar results were also obtained using cortical lens fibers of adult chickens.

washes was about 0.03% of the original radioactivity. These results demonstrate that cytoplasmic δ-crystallin will contaminate lens membranes during isolation.

The amount of δ-crystallin typically found in the membrane preparations was calculated and compared with the level of contamination. The embryonic lens membranes contained about 2 μg of protein/lens; 11% of this was in the 50 000 molecular weight band (Table I), although some of this may not be δ-crystallin (Fig. 2). Thus, the membrane fraction contained not more than 0.2 μg of δ-crystallin/lens. Since there is about 300 μg of cytoplasmic δ-crystallin/lens in the 15-day-old chick embryo (Ref. 2; Coulombre, A.J., personal communication, and Shinohara, T., personal communication) the δ-crystallin in the membrane fraction corresponds to about 0.08% of the cytoplasmic δ-crystallin. Contamination, estimated from the mixing experiments to represent 0.03% of the cytoplasmic δ-crystallin, clearly contributes significantly to the δ-crystallin found in the membrane fraction. If only a part of the 50 000 molecular weight protein is actually δ-crystallin, as suggested by the fingerprints (Fig. 2B), contamination could conceivably account for all the membrane δ-crystallin. Similarly, the nuclear fibers of the adult chicken lens contain about 15 mg of cytoplasmic δ-crystallin (Ref. 2; Coulombre, A.J., personal communication); the membranes contained 29 μg of protein/lens nucleus, 3% of which was in the 50 000 molecular weight band (Table I). This corresponds to 0.9 μg/lens nucleus or about 0.01% of the cytoplasmic δ-crystallin. Again this value is comparable to the estimated level of cytoplasmic contamination of 0.03%. These calculations should be considered only rough estimates, since neither the amount of cytoplasmic δ-crystallin nor the proportion of 50 000 molecular weight protein represented by δ-crystallin is known precisely. Nevertheless, the approximate correspondence between the amount of δ-crystallin found in the membrane fraction and the amount expected from contamination demonstrates the magnitude of the problem of cytoplasmic contamination.

Discussion

The protein components of adult chicken lens membranes reported here are very similar to those of calf lens membranes reported by others [3—5,21]. Our data agree particularly well with those of Broekhuyse and Kuhlmann [5], indicating that lens membranes of chickens as well as calves contain proteins with molecular weights of about 56 000, 54 000, 48 000, 34 000, 32 000 and 17 000 and have a major intrinsic protein with a molecular weight of 25 000—27 000. The 68 000 molecular weight component of calf lens membranes is absent from chicken lens membranes, but is replaced by a 75 000 molecular weight component, also observed by Alcala et al. [1].

We find that the major component of adult chicken lens membranes is a protein with a molecular weight of about 25 000. This protein is probably identical to the 27 500 molecular weight protein found by Alcala et al. [1]. A similar protein in calf lens membranes has been reported to have a molecular weight of 25 500—27 500 [3—7,21]. In membranes of both the cortical and nuclear regions of the adult chicken lens this component represents almost half of the total protein. In the embyronic chick lens membranes, however, it

represents only about 13% of the total, even when a correction is made for the presence of cytoplasmic δ-crystallin in these preparations. A developmental increase in this protein has also been shown by Waggoner and Maisel [22] using immunofluorescence. Since this protein is thought to be associated with the numerous gap junctions of the lens [3,7], its accumulation during lens development may reflect a corresponding increase in the number of gap junctions.

Previous studies of purified urea-washed membranes of adult chicken lenses have indicated that δ-crystallin comprises almost 40% of the lens membrane protein [1]. Even the cortical lens fibers which do not contain cytoplasmic δ-crystallin [8], have been reported to contain δ-crystallin in the membrane fraction [1]. In calf lenses varying amounts of α-crystallin have been found in the membrane fraction [2—5], although it has been suggested that this may represent contamination of the membrane preparations by cytoplasmic material [4—6]. Our results demonstrate that cytoplasmic δ-crystallin does indeed contaminate chicken lens membrane preparations under the present conditions. A possible explanation of the ineffectiveness of the usual washing procedures in eliminating this contamination is that cytoplasmic material may be trapped in vesicles formed during the preparation of the membranes [5]. Vesicles have been observed in lens membrane preparations [2,4,7,23]. These experiments demonstrate the difficulty of distinguishing between δ-crystallin which is present as a contaminant and that which is truly a membrane component when δ-crystallin is present in high concentrations in the cytoplasm. The results also show that the small amount of 50 000 molecular weight protein associated with the cortical lens fibers does not have the same tryptic peptide map as cytoplasmic δ-crystallin. Thus, it appears that if δ-crystallin is an intrinsic component of these membranes, it has been highly modified.

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