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Identification of Spectrin and Protein 4.1-like Proteins in Mammalian Lens

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Summary. Human, bovine, canine, and rabbit lenses were found to contain proteins which cross-react with anti-4.1 serum and which have molecular weights similar to erythrocyte proteins 4.1a and 4.1b (~ 80 kd). Additionally, bovine, canine, and rabbit lenses contain a 4.1-like protein of ~ 125 kd which is absent from human lens. Proteins which cross-react with antibody to human erythrocyte spectrin were also detected. The human lens showed weak cross-reaction of bands of 240 kd and 225 kd, and a more intense cross-reaction of a band of 235 kd. Canine and bovine lenses showed weak cross-reaction with only the bands at 240 kd and 235 kd. The lens 240 kd band of all species also demonstrated calcium-dependent binding of calmodulin. Our results indicate that proteins related to, but distinct from, erythrocyte protein 4.1 and spectrin are found in mammalian lens.

Spectrin and protein 4.1 are two of the major proteins which participate in the formation of the erythrocyte membrane skeleton (for review, see 1, 2). Mammalian erythrocyte spectrin is a long, filamentous dimer composed of two non-identical polypeptide chains which are called α-spectrin (molecular weight 240 kd) and β-spectrin (molecular weight 220 kd) (for review, see 3). One end of the spectrin dimer can self-associate in a head to head manner to form tetrameric spectrin (4-6). The tail of the dimer, in contrast, contains a binding site for F-actin (7-9) and protein 4.1 (10, 11).

Protein 4.1 can be resolved by discontinuous 1D-SDS-PAGE into polypeptides of 80 kd and 78 kd which have been designated 4.1a and 4.1b, respectively (12). These proteins are sequence-related phosphoproteins (13) which both bind to spectrin and may be functionally equivalent (2). Addition of 4.1 to mixtures of spectrin and F-actin results in enhanced binding of spectrin to actin (14, 15), and also gives spectrin-actin gels thixotropic properties (15).

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Several kinds of spectrin-like heterodimers have been detected in diverse non-erythroid cell types (for review, see 16). Characteristics shared by these proteins with erythrocyte spectrin include localization to the cytoplasmic surface of plasma membranes (17-24), the ability to self-associate to form tetramers (17, 25, 26), and binding of F-actin (17, 19, 25-27). All non-erythroid spectrins characterized thus far contain a calmodulin-binding subunit of 240 kd (28) that is partially homologous to mammalian erythrocyte α-spectrin and highly homologous to avian erythrocyte α-spectrin (17, 18, 29). In the majority of tissues which have been screened (22), including avian lens (29), non-erythroid α-spectrin is associated with a polypeptide of 235 kd (γ-spectrin) which is distinct from α and β-spectrin by peptide mapping (17).

Recently, proteins immunologically related to protein 4.1 have also been detected in non-erythroid cell types, such as fibroblasts (30), neutrophils, platelets, and monocytes (31). In this report, we present the first evidence for the presence of proteins related to protein 4.1 in mammalian lens. Additionally, we demonstrate that mammalian lens expresses proteins of 240 kd and 235 kd that cross-react with anti-human erythrocyte spectrin antibody. A minor human lens polypeptide of 225 kd also cross-reacts with this antibody. As in other tissues, we find that the polypeptide of 240 kd binds calmodulin in a calcium dependent manner.

Materials and Methods. Erythrocyte membranes were prepared by the method of Dodge (32), except that 5 mM Na PO_4, 1 mM EDTA, pH 7.6, was substituted for 5 mM Na PO_4, pH 8.0. Protein 4.1 was purified from membranes by the method of Tyler et al (10) as modified by Cohen and Foley (8). Spectrin was purified by the method of Gratzer (33).

Antibody to protein 4.1 was raised in New Zealand White Rabbits by the method of Cohen et al (29) as described by Aster et al (34). Antibody to spectrin was raised in sheep by injecting 5 mg of purified spectrin emulsified in Freund's complete adjuvant intramuscularly, followed by monthly booster injections of 1 mg of spectrin in Freund's incomplete adjuvant. Sera were screened for the presence of antibody using an immunoblot method (see below). An affinity column was produced by coupling purified spectrin to Bio Rad Affigel 15 in 50 mM HEPES, pH 7.6. Immune sera was then applied to this column, which was extensively washed with 5 mM Na PO_4, pH 7.6, containing 150 mM NaCl. Antibody was then eluted from the column with 0.2 M glycine, pH 2.7

Lenses were obtained from adult dog, rabbit, and cow immediately upon sacrifice of the animal. Human lenses were obtained from 15-year old and 60-year old individuals from the Kresge Eye Bank, Detroit, about 12 hours

after death. Each lens was decapsulated and the fiber mass was homogenized in a buffer containing 50 mM Tris, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.1% β-mercaptoethanol, pH 7.5. After centrifugation at 37,000xg, the water-insoluble fraction (WIF) was collected and solubilized as previously described (35).

Discontinuous one-dimensional-SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (36). Proteins were stained with Coomassie blue. Calmodulin-binding proteins were detected within gels by the method of Carlin et al (37). For immunoblot analysis, proteins were transferred to nitrocellulose paper by the method of Towbin (38). Blots were treated as described by Naz et al (39); purified spectrin antibody was used at a dilution of 1:5000, and anti-4.1 serum at 1:1000. Blots were stained using the immunoperoxidase method of Hawkes (40), except that after exposure to 4-chloro-1-naphthol blots were transferred to a small volume of distilled water and allowed to darken for 1-8 hours. Staining was then terminated by washing with distilled water, and blots were dried and stored in the dark.

Results. Figure 1a shows the Coomassie blue-staining pattern of human erythrocyte membranes and of the water-insoluble fractions (WIF) of human, bovine, and canine lenses which were electrophoresed in 5% polyacrylamide gels containing 0.1% SDS. Erythrocyte spectrin was seen as two bands of

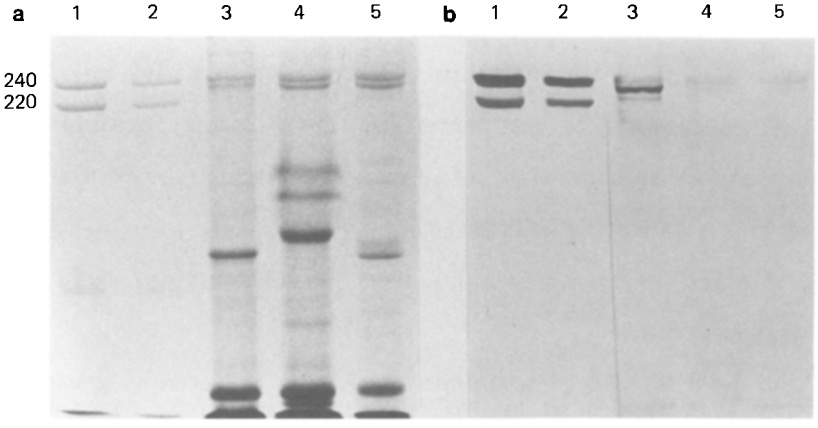

Figure 1. Identification of Spectrin-like Proteins of Mammalian Lens. Human erythrocyte membranes, and water-insoluble fractions of human, bovine, and canine lens were electrophoresed on 5% polyacrylamide gels. Gels were then split, and one half was stained with Coomassie blue and the other half was used for immunoblot analysis. Figure 1a shows the Coomassie blue-staining pattern of human erythrocyte membranes (lanes 1 and 2: 10 and 5 μg of protein, respectively), and human (lane 3), bovine (lane 4), and canine (lane 5) lens WIFs (65 μg protein/lane). Erythrocyte α and β-spectrin are seen as bands of approximately equal intensity at 240 kd and 220 kd; all lens fractions contain bands of approximately equal intensity at 240 kd and 235 kd. Figure 1b shows the corresponding anti-spectrin staining pattern. Intense staining of human erythrocyte α and β-spectrin is seen in lanes 1 and 2. Human lens (lane 3) shows weak staining of bands at 240 kd and 225 kd. A band at 235 kd is also stained; the leading edge of this band is more intensely stainined than the trailing edge. Bovine and canine lens fractions (lanes 4 and 5, respectively) show weakly staining bands at 240 kd and 235 kd. Staining of erythrocyte membrane proteins was terminated after 4 hours; staining of lens proteins was terminated after 8 hours.

approximately equal staining intensity at 240 kd (α-spectrin) and 220 kd (β-spectrin). The lens WIFs showed bands of 240 kd and 235 kd which also stained with near equal intensity. The corresponding immunoblot reactions with affinity purified sheep anti-human erythrocyte spectrin antibody are shown in figure 1b. The human erythrocyte α and β-spectrin were strongly stained. The human lens WIF showed weak staining of the 240 kd band and moderate staining of the 235 kd band. Close observation of the 235 kd band revealed that the leading edge of this band stained more intensely than did the trailing edge. Weak staining of a band of 225 kd was also observed. Bovine and canine lenses showed weak cross-reaction of the 240kd/235kd doublet with anti-spectrin. No cross-reaction at 225 kd was observed. In control experiments, all staining was abolished by incubation of the antibody with purified erythrocyte spectrin prior to exposure to the nitrocellulose blots (not shown).

The 240 kd polypeptide of lens WIF was tested for calmodulin binding using a gel overlay mehod (figure 2). As previously reported, no calmodulin binding to human erythrocyte spectrin is observed in the presence or absence of calcium. In contrast, the 240 kd polypeptide of human, bovine, and canine lenses showed binding of ^{125}I-calmodulin in the presence of calcium which was blocked by EGTA. A minor lens calmodulin-binding protein of 150 kd was also detected. Binding of calmodulin was also inhibited by cold calmodulin and by the calmodulin-binding drug trifluoperazine (not shown).

Figure 3a shows the Coomassie blue-staining pattern of erythrocyte membranes and bovine, human, canine, and rabbit WIFs electrophoresed on 7.5% polyacrylmide gels. Erythrocyte 4.1 was seen as a doublet of 80 kd and 78 kd; minor proteins of similar molecular weight were present in the lens WIFs. Figure 3b shows the corresponding immunoblot stained with a rabbit antiserum prepared against erythrocyte 4.1. Human erythrocyte membranes showed a complex pattern of cross-reacting bands similar to that previously reported by Cohen et al (30). All lens WIFs show protein doublets of ~80 kd which were stained by the anti-4.1 serum. The bovine doublet showed a relatively wide

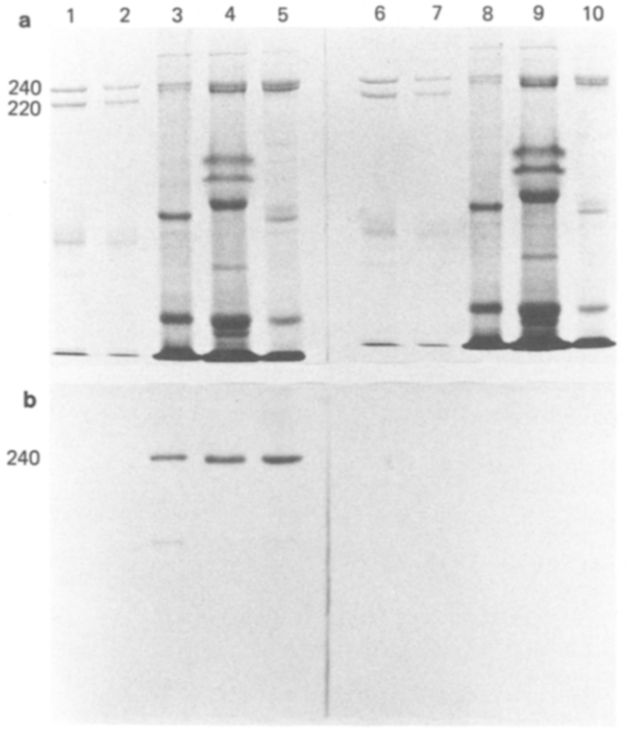

Figure 2. Calmodulin-binding Proteins of the Lens Detected by a Gel Overlay Method. Electrophoresis was in 5% polyacrylamide gels. Figure 2a shows the Coomassie blue-staining pattern of human erythrocyte membranes (lanes 1, 2, 6, 7) and human (lanes 3, 8), bovine (lanes 4, 9), and canine (lanes 5, 10) lens WIFs. A major protein of 240 kd is seen in figure 2b. Lanes 1-5 were treated with iodinated calmodulin and 1 mM calcium; lanes 6-10 with iodinated calmodulin and 1 mM EGTA. Binding of calmodulin to the 240 kd polypeptide is seen in all lens fractions (lanes 3-5). Binding is inhibited by 1 mM EGTA. No binding to human erythrocyte spectrin is seen in the presence or absence of calcium.

separation of its bands, which stained with approximately equal intensity and had molecular weights close to erythrocyte 4.1a and 4.1b. The components of the doublet in the other species were not as well resolved. In the human and the canine lenses the high molecular weight component predominated, while the reverse was true for the rabbit lens. A 4.1-like protein of ~125 kd was also observed in the bovine, canine, and the rabbit lens, but not in the human lens.

Figure 4 shows the cross-reactions of 15-year old and 60-year old human lens with anti-4.1 serum. No difference was observed in the staining pattern of these lenses of markedly different ages. This figure also shows clearly that the human lens 4.1 doublet is of slightly greater molecular weight than erythrocyte 4.1a and 4.1b. In control experiments, all staining of lens and

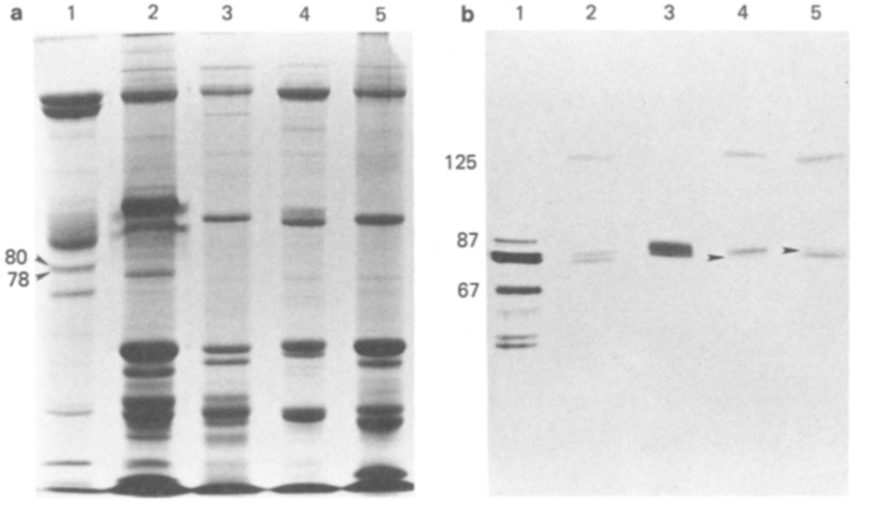

Figure 3. Identification of Protein 4.1-like Proteins of Mammalian Lens in 7.5% Gels Using a Rabbit Antiserum to Protein 4.1. Figure 3a shows the Coomassie blue-staining pattern of human erythrocyte membranes (lane 1; 40 µg protein), and bovine, human, canine, and rabbit lens WIFs (lanes 2-5, respectively: 65 µg protein/lane). Erythrocyte protein 4.1 is seen as a doublet of 80 kd and 78 kd. Minor proteins of approximately the same molecular weight are seen in all lens fractions. Figure 3b shows the anti-4.1 staining pattern of erythrocyte membrane and lens WIFs. Erythrocyte membranes (lane 1) show staining of the protein 4.1 doublet; bands at 87 kd, 85 kd, 67 kd, and at somewhat lower molecular weights are also observed. All lens fractions show a cross-reacting doublet at ~80 kd; the position of faint bands is denoted by an arrow. Additionally, bovine, canine, and rabbit lenses contain a high molecular weight cross-reacting band of ~125 kd which is not observed in the human lens.

erythrocyte was blocked by prior incubation of antiserum with purified 4.1, and no staining was observed when pre-immune serum was substituted for immune serum.

Discussion. We have presented data which indicate that there exists in mammalian lens a spectrin-like protein composed of polypeptides of 240 kd and 235 kd. Evidence for the spectrin-like nature of these two polypeptides includes: 1), the presence of approximately equal amounts of the polypeptides in lens WIFs, which is to be expected if they form heterodimers; 2), cross-reaction of both bands with an anti-spectrin antibody; and 3), the calcium-dependent binding of calmodulin to the 240 kd polypeptide. Preliminary work indicates that these polypeptides copurify, implying a tight association as is found between other spectrin subunits. The molecular weight of the subunits of this protein suggest that it is similar to fodrin, a

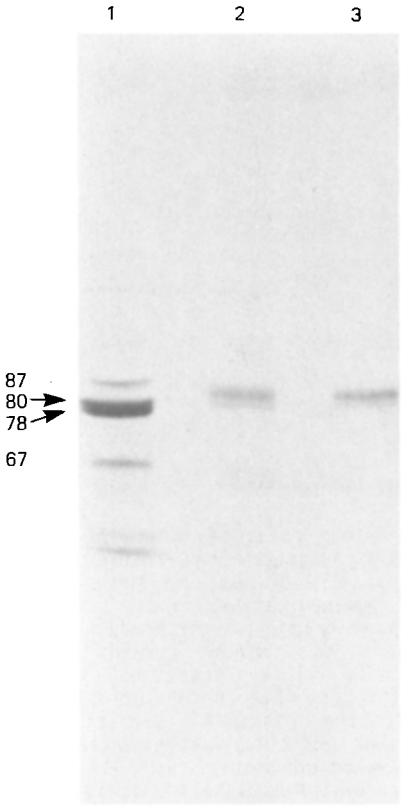

Figure 4. Comparison of the Protein 4.1-like Proteins of Young and Old Human Lenses. Lane 1 shows the typical anti-4.1 cross-reaction pattern of erythrocyte membranes (40 μg protein). Lanes 2 and 3 show the cross-reaction pattern of lens fractions prepared from a 15-year old and a 60-year old, respectively. A cross-reacting doublet of slightly greater molecular weight than protein 4.1a and 4.1b is seen in young and old lenses; no high molecular weight 4.1-like protein is detected in either sample.

protein composed of α and β -spectrin which has been identified in many cell types (22), including avian lens (29).

The strong cross-reaction of anti-spectrin with the human lens at 235 kd requires explanation, since γ-spectrin is not closely related serologically or by peptide mapping to α and β-spectrin (17, 18, 25). We hypothesize that this unusual cross-reaction was due to comigration of γ -spectrin with a β -spectrin-like polypeptide. Evidence supporting this explanation includes: 1), the presence in avian lens of minor β-spectrin-like polypeptides of 230 kd and 225 kd (29); 2), the presence of a minor cross-reactive polypeptide of 225 kd in the human lens, suggesting the presence of similar β -spectrin-like

polypeptides; 3), the failure of our anti-spectrin to cross-react strongly with γ-spectrin of the human lymphocyte (unpublished data); and 4), the consistent finding of more intense staining of the leading edge of the 235 kd band than of the trailing edge, which suggests heterogeneity in this band. Absence of cross-reaction at 225 kd and weaker cross-reaction at 235 kd in the bovine and the canine lens may have resulted from interspecies variation in β-spectrin-like polypeptides.

We have also screened mammalian lens for the presence of 4.1-like proteins. The lenses of all species screened contained two proteins with molecular weights similar to proteins 4.1a and 4.1b which cross-reacted with anti-4.1 serum. Additionally, bovine, canine and rabbit lenses, but not human lens, contained a 4.1-like protein of 125 kd. Since the initial experiments were performed with lenses obtained from adult animals and an adolescent human, we thought this variation might be explained by differences in the relative ages of the lenses, particularly since cross-linking of lens proteins occurs with aging (41). However, adult human lens showed no evidence of the 125 kd 4.1-like protein. This does not rule out the possibility that cross-linking produces the 125 kd 4.1-like protein in non-human lenses.

Although the function of non-erythroid spectrin is presently unclear, it is probably significant that all spectrins thus far characterized are F-actin-binding proteins. In avian (21) and bovine lenses (23), spectrin is localized to plasma membranes, so it is reasonable to suggest that one function of spectrin is to link F-actin to the membrane. Previous work demonstrating immunofluorescent localization of F-actin to the lens plasma membrane supports this idea (42, 43). In the erythrocyte, 4.1 is thought to enhance the intereaction between spectrin and F-actin (14, 15), possibly in a calcium dependent fashion (14). It is possible that 4.1-like proteins in the lens have a similar effect.

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REFERENCES

1. Cohen, C.M. (1983) Semin. Hemat. 20, 141-158.
2. Goodman, S.R., and Schiffer, K. (1983) Am. J. Physiol. 244, C121-C141.
3. Knowles, W., Marchesi, S.L., and Marchesi, V.T. (1983) Semin. Hemat. 20, 154-179.
4. Ralston, G. (1976) Biochim. Biophys. Acta 443, 387-393.
5. Shotton, D., Burke, B., and Branton, D. (1979) J. Mol. Biol. 131, 303-329.
6. Ungewickell, E., and Gratzer, W. (1978) Eur. J. Biochem. 88, 379-385.
7. Brenner, S. and Korn, E. (1979) J. Cell Biol. 86, 694-698.
8. Cohen, C.M., and Foley, S. (1980) J. Cell Biol. 86, 694-698.
9. Cohen, C.M., Tyler, J., and Branton, D. (1980) Cell 21, 875-883.
10. Tyler, J., Hargreaves, W., and Branton, D. (1979) Proc. Natl. Acad. Sci. USA 76, 5192-5196.
11. Tyler, J., Reinhardt, B., and Branton, D. (1980) J. Biol. Chem. 255, 7034-7039.
12. Mueller, T., and Morrison, M. (1977) J. Biol. Chem. 252, 6573-6576.
13. Goodman, S.R., Yu, J., Whitfield, C.F., Culp, E.N., and Posnak, E.J. (1982) J. Biol. Chem. 257, 4564-4569.
14. Fowler, V., and Taylor, D. (1980) J. Cell Biol. 85, 361-376.
15. Cohen, C.M., and Kosgren, C. (1980) Biochem. Biophys. Res. Comm. 97, 1429-1435.
16. Lazarides, E., and Nelson, W.J. (1982) Cell 31, 505-508.
17. Bennett, V., Davis, J., and Fowler, W.A. (1982) Nature 299, 126-131.
18. Burridge, K., Kelly, T., and Mangeat, P. (1982) J. Cell Biol. 90, 478-486.
19. Levine, J., and Willard, M. (1982) J. Cell Biol. 631-643.
20. Nelson, W.J., and Lazarides, E. (1983) Proc. Natl. Acad. Sci. USA 80, 363-367.
21. Repasky, E.A., Granger, B.L., and Lazarides, E. (1982) Cell 29, 821-833.
22. Glenney, J.R., Jr., and Glenney, P. (1983) Cell 34, 503-512.
23. Lehto, V.P., and Virtanen, I. (1983) J. Cell Biol. 96, 703-716.
24. Goodman, S.R., Zagon, I.S., and Kulikowski, R.R. (1981) Proc. Natl. Acad. Sci. USA 78, 7570-7574.
25. Glenney, J.R., Jr., Glenney, P., and Weber, K. (1982) Cell 28, 843-854.
26. Glenney, J.R., Jr., Glenney, P., and Weber, K. (1982) J. Biol. Chem. 257, 9781-9787.
27. Burns, N.R., Ohanian, V., and Gratzer, W.B. (1983) FEBS Lett. 153, 165-168.
28. Glenney, J.R., Jr., Glenney, P., and Weber, K. (1982) Proc. Natl. Acad. Sci. USA 79, 4002-4005.
29. Nelson, W.J., Granger, B.L., and Lazarides, E. (1983) J. Cell Biol. 97, 1271-1276.
30. Cohen, C.M., Foley, S., and Korsgren, C. (1982) Nature 299, 648-650.
31. Spiegel, J., Beardsley, D., and Lux, S. (1982) Fed. Proc. 41, 657.
32. Dodge, J., Mitchell, C., and Hanahan, D. (1963) Arch. Biochem. Biophys. 100, 119-130.
33. Gratzer, W.B. (1981) Methods In Enzymol. 85, 475-480.
34. Aster, J.C., Rosenblum, B.B., Brewer, G.J., and Hanash, S.M. (1983) Erythrocyte Membranes, Alan R. Liss, New York, in press.
35. Maisel, H., Alcala, J., Lieska, N., and Rafferty, N. (1977) Ophthal. Res. 9, 147-154.
36. Laemmli, U.K. (1970) Nature 227, 680-682.
37. Carlin, R.K., Grab, D.J., and Siekovitz, P. (1981) J. Cell Biol. 89, 449-455.
38. Towbin, H., Staehelin, R., and Fordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
39. Naz, R.K., Saxe, J.M., and Menge, A.C. (1983) Biol. Reprod. 28, 249-257.
40. Hawkes, R. (1982) Anal. Biochem. 123, 143-146.
41. Garner, W.H., and Spector, A. (1978) Proc. Natl. Acad. Sci. USA 75, 3618-3620.
42. Ireland, M., and Maisel, H. (1983) Exp. Eye Res., in press.
43. Kibbelaar, M.A., Selten-Versteegen, A.E., Dunia, I., Benedetti, E.L., and Bloemendal, M. (1979) Eur. J. Biochem. 95, 543-549.