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Two-Dimensional Gel Electrophoresis of Calf Aqueous Humor, Serum, and Filter-Bound Proteins

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Recent studies have demonstrated that bovine and primate aqueous humor (AH) obstruct flow when perfused through artificial membranes with pore sizes similar to those found in the aqueous outflow pathway. Proteinaceous AH components were implicated in this phenomenon, which is not observed with serum diluted to comparable protein concentrations. In this study, we used two-dimensional gel electrophoresis to characterize the protein composition of calf AH and to identify those proteins binding to the filters and presumably causing this obstruction. Comparison of AH and serum under denaturing conditions showed quantitative and qualitative differences in their protein content. Among the most important: AH was seen to possess two protein subunit trains (~28 kD and ~48 kD) not found in serum and two trains (~28 kD and ~80 kD) with additionally charged components not found in serum. Serum, on the other hand, possesses one train (~80–90 kD) not found in AH as well as a slightly greater relative amount of high-molecular weight protein subunits. The finding that hydrophobic filters retain more protein components than do hydrophilic filters suggests that the type and amount of protein adhering to them is determined largely by hydrophobic interactions. Whether such interactions occur in the outflow system, and if so, how they may relate to aqueous drainage remains to be determined. Invest Ophthalmol Vis Sci 30:731–738, 1989

The aqueous humor (AH) plays an integral role in the physiological homeostasis of the avascular tissues in the anterior segment of the eye. In addition to this biological function, the balance between its rate of formation and the outflow resistance controls the intraocular pressure (IOP).[1] There is, however, an incomplete understanding of the mechanism by which the outflow resistance is generated.[2] Nor is there a clear understanding of how the increased resistance of the outflow pathway develops in primary open-angle glaucoma (POAG).[3]

With few exceptions,[4,5] the composition of AH is not considered to play a role in the regulation of outflow resistance or in the pathogenesis of POAG. On the other hand, the finding that proteins not normally present in AH may be involved in some secondary glaucomas has been reported.[6–9] Recently, Johnson et al[10] demonstrated that bovine and primate AH obstruct microporous filters with pore dimensions similar to those found in the juxtacanalicular tissue (JCT) to a greater extent than does serum diluted to comparable protein levels. AH proteins were implicated in this phenomenon. They speculated that if such a blocking phenomenon occurs in vivo within the JCT, certain AH proteins may play a role in determining outflow resistance.

This study attempts to characterize further the protein composition of calf AH relative to serum by two-dimensional gel electrophoresis. This technique resolves complex mixtures of proteins by the independent parameters of isoelectric point (pI) and molecular size in the first and second dimensions, respectively. Our purpose is three-fold: (1) to develop a protein map of calf AH; (2) to identify any protein(s) that may be unique to AH; and (3) to identify those proteins that bind to the microporous filters used by Johnson et al[10] and Ethier et al[11–13] and presumably obstruct them. These three goals will better enable us to determine whether these proteins play a similar role in the AH outflow network of the eye.

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Materials and Methods

Reagents and Electrophoresis Supplies

All reagents were of analytical grade. All solutions were made with water purified to ≥14 MΩ (dH_2O) in a Barnstead Water I deionizer purchased from Barnstead Co. (Boston, MA).

Dulbecco's phosphate buffered saline (DPBS) was obtained from Gibco Laboratories (Grand Island, NY), and Sigma Chemical Co. (St. Louis, MO). Bovine albumin (Cohn fraction V), fibrinogen (type IV), and γ-globulins (Cohn fraction II) and urea (ACS reagent grade) were also obtained from Sigma.

Precast 10–20% polyacrylamide gradient (PAG) gels were obtained from Integrated Separation Systems (Newton, MA). Isoelectric focusing (IEF) was done with the MegaIso-40 IEF Tube Gel Apparatus from Health Products, Inc. (Rockford, IL). 2-D Pharmalyte 3-10 and the GE-2/4LS Gel Electrophoresis Apparatus were from Pharmacia, Inc. (Piscataway, NJ). Gels were silver-stained with the Rapid-Ag-Stain kit developed by ICN Radiochemicals (Irvine, CA). All other electrophoresis reagents were from Sigma.

Protein estimations were done by the Bradford method[14] with a reagent kit from BioRad Laboratories (Richmond, CA). For samples in Nonidet P-40 (NP-40, a nonionic detergent), the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce Chemical Co., Rockford, IL) was used. All protein concentration determinations were relative to bovine plasma γ-globulin (BioRad) standard dilutions.

Bovine Samples

Aqueous humor (AH) and serum samples were obtained from 2–4-week-old calves as previously described.[10] To control for postmortem or post-enucleation changes in composition, a batch of AH was collected in the slaughterhouse from eyes before enucleation. Calf lens proteins were obtained by homogenizing the lens in DPBS. All samples were left at room temperature for ½–1 hr (to remove clottable or aggregate-forming material) and then ultracentrifuged at 100,000 *g* for 1 hr. All samples were kept frozen at −80°C until use. When thawed for further work, they were recentrifuged for ¼ hr at 50,000 *g*.

Albumin Immunodeletion

AH and serum (diluted 1:100 with DPBS) were dealbuminated by immunoaffinity chromatography. One milliliter of rabbit anti-bovine albumin immunoglobulins containing approximately 10 mg protein capable of binding 0.9 mg of bovine albumin (Cat. No. Z229, Accurate Chemicals, Westbury, NY) was immobilized on 2.5 g of cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. This resulted in a 5 ml packed column which was stored at 5°C in 0.1 M NaCl containing 15 mM NaN_3.

The column was equilibrated with DPBS and 2 ml of sample was applied to the column and eluted with 5–10 ml of DPBS at 0.5–1.0 ml/min. The albumin-free sample was concentrated to approximately the original sample volume by centrifugal ultrafiltration in a Centricon-10 microconcentrator (Amicon, Danvers, MA). The column was regenerated by eluting the immunoadsorbed albumin with three successive cycles of 5 ml of 0.1 M sodium borate (pH 8.0) followed by 5 ml of 0.1 M sodium acetate (pH 4.0), both containing 0.5 M NaCl.

Filter Perfusions and Elutions

About 1–2 ml of AH or serum (diluted 1:100) were perfused through polycarbonate filters of 0.2 μm pore size and 13 mm diameter. These were of two types: polyvinylpyrrolidone (PVP)-free (hydrophobic) and PVP-coated (hydrophilic) filters (Nuclepore Corp., Pleasanton, CA). Filters were then washed by reperfusion with 4–12 ml of DPBS and the bound protein eluted with 0.1–1.0% NP-40 (100 μl/filter). The filtration assembly consisted of a 1 ml syringe acting as the sample reservoir, attached to the filter holder, which in turn was attached to a 23-gauge needle. The needle was forced through the stopper of a bottle connected to a vacuum source. Ten such assemblies were accommodated on two bottles run in parallel giving up to ten simultaneous filtrations. This is termed a perfusion set.

Two-Dimensional Gel Electrophoresis

A modification of the O'Farrell method was used.[15–17] To avoid induction of artifactual protein modifications, the samples were kept frozen at −80°C, lyophilized immediately before use and then solubilized in a mixture of 9 M urea, 1% NP-40, 2% β-mercaptoethanol (β-ME) and 4% 2-D Pharmalyte 3-10 for 1–2 hr at room temperature. The IEF separation (ISO-dimension) employed 15 cm × 1.5 mm gel rods consisting of 5% acrylamide, 3% cross-linked with bis-acrylamide (5%T, 3%C_{bis}) containing 9 M urea, 1% NP-40, and 4% 2-D Pharmalyte 3-10. These were cast and run with the MegaIso-40 apparatus. Desired amounts of protein samples were loaded in 40 μl per gel and focused at 200 V for the first hour and then at 500 V for 16 to 20 hr (8,000–10,000 V · Hrs) using 0.05 M NaOH as the catholyte and 0.05 M H_2SO_4 as the anolyte. Gels with focused samples were stored frozen at −80°C in 1 ml of equilibra-

tion buffer (62.5 mM Tris-HCl ⟨pH 6.8⟩, 1% SDS, 2% β-ME, 10% glycerol, and 0.002% bromophenol blue). For the second separation (DALT-dimension), the focused gel rods were thawed, equilibrated in 5 ml of the same buffer for 5–10 min and applied directly to 10–20% PAG slab gels (160 × 160 × 1.5 mm). They were sealed onto the slab gels with 1% agarose made in electrophoresis buffer. Gels were run in the GE-2/4 LS unit for 5–6 hr at 30 mA/gel and at 20–25°C using Laemmli's discontinuous electrophoresis buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3).[18] The gels were fixed in 40% methanol, 20% trichloroacetic acid, and 5% sulfosalicylic acid for 1 hr and then in 2.5% glutaraldehyde[19] for 1 hr to overnight. They were subsequently silver-stained with the Rapid-Ag-Stain.

Standardization of System

The ISO-dimension was calibrated with Carbamylyte (Pharmacia). The carbamylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was dissolved in 1–1.2 ml of dH_2O and 5 μl were applied per IEF gel. The pH gradient range was also determined by checking the pH on the gel at 1 cm intervals using a flat membrane Ag/AgCl probe (Microelectrodes, Inc., Londonderry, NH) on a PHM62 pH meter (Radiometer, Copenhagen, Denmark). As the zero-adjustment of the PHM62 could not accommodate a Ag/AgCl reference, corrections to the readings were made from a standard curve.

The DALT-dimension was calibrated with SDS-PAGE Low Molecular Weight protein standards (Pharmacia). These were solubilized at a final concentration of 0.01 mg/ml/polypeptide subunit in the buffer used to equilibrate the IEF gel rods containing 1% agarose, heated at 95°C for ¼ hr, and aspirated into plastic tubing (~1.5 mm i.d.) which was then stored at 5°C. As needed, this preparation was expelled from the tubing, cut into ¼ cm pieces, and placed directly on top of both ends of the gel rods.

Results

In order to assess the degree of difference between individual samples and the effects of sample pooling, we analyzed the protein composition of individual and pooled calf AH and serum samples. Table 1 depicts the typically determined AH protein levels collected from pairs of eyes, pooled AH samples, and those of serum. Total protein levels of AH were between 0.5–1.0% that of serum. Comparisons of AH from individuals with their autologous serum samples revealed no clear correlation between protein levels in the AH and serum (data not shown). AH samples with higher than 600 μg/ml protein concentrations invariably contained lens proteins as shown by gel electrophoresis. Because of this finding we later selected samples with protein concentrations less than 600 μg/ml. The pooled samples of AH were made and used before this was recognized. Therefore pooled samples tended to have higher protein content and noticeable amounts of lens proteins.

Table 1. Protein concentrations of calf samples

	Individual samples (mg/ml)	*Pooled samples (mg/ml)*
Aqueous humor		
Range	0.22–0.78	0.45–1.80
Mean ± SE	0.57 ± 0.04	0.77 ± 0.09
No. of samples	(18)	(17)
Serum		
Range	73–106	73–102
Mean ± SE	87 ± 1	90 ± 2
No. of samples	(11)	(6)

Colorimetric assays were done by the Bradford method[10] with a bovine plasma γ-globulins standard.

For maximum resolution, all electrophoretically analyzed samples were resolved under protein denaturing conditions. Comparisons were made on equal amounts of protein loadings. Our reported findings are based on the analysis of five to seven different specimens of each sample category. An AH sample from an individual and a pooled serum sample are shown in Figure 1. Pooled samples of AH and serum with the albumin immunodeleted are shown in Figure 2. The electrophoretically resolved polypeptide patterns of both calf AH and serum closely resemble that of human plasma for which a 2-D electrophoretic map identifying many plasma protein subunits is available.[20] This reference map, in conjunction with the work reported by others and the use of bovine albumin, fibrinogen and γ-globulins standards, was used in an attempt to make tentative identifications of some similar components in calf samples. As expected, the 2-D electrophoretogram of AH resembles that of serum. Nevertheless, distinct quantitative and qualitative differences are observed.

In comparison with serum, AH appears to be relatively deficient in high molecular weight proteins (Figs. 1, 2). This is evident from the lower staining intensity of protein subunits greater than 94 kD and the lower levels of the heavy-chains of immunoglobulins (train I). Such an observation was evident in all AH samples including that which was collected shortly after death but prior to eye enucleation. This finding is also in agreement with reports by others.[21–23] The major protein component in both AH and serum is albumin. It seems to be present at a slightly higher relative concentration in AH. In addition, trains of protein subunits A (~28 kD) and B

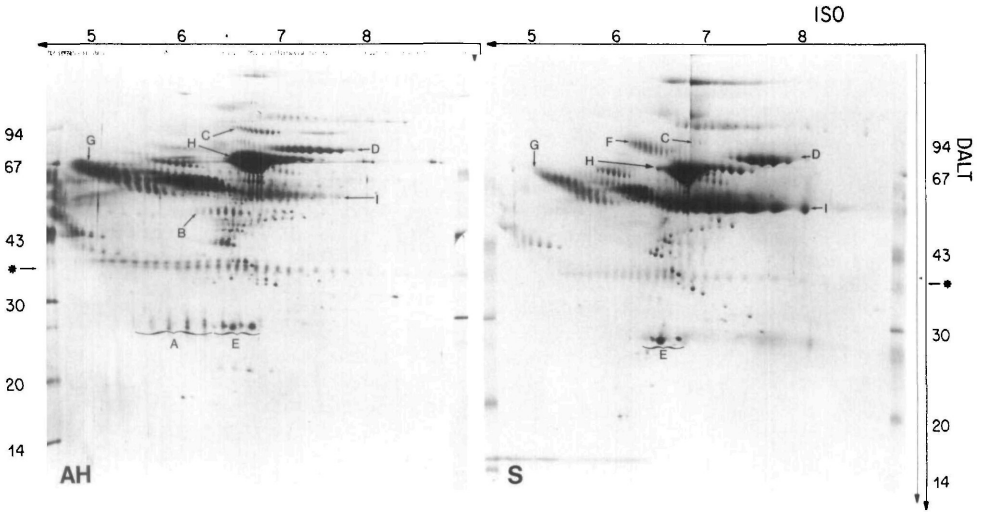

Fig. 1. Two-dimensional electrophoretograms (2-D maps) of calf aqueous humor (AH) collected from a pair of eyes and of pooled serum (S); (40 μg of protein in each). Samples were first lyophilized and then solubilized with 9 M urea, 1% NP-40, 4% 2-D Pharmalyte 3-10, and 2% β-ME. They were run in a pH 3-10 gradient in the ISO-dimension (horizontal axis) and then on 10–20% PAG gels with SDS in the DALT-dimension (vertical axis). (*) denotes the carbamylated standard (GAPDH) of ~36,000 daltons (kD) and having an apparent isoelectric point (pI) range of 4.7–8.3. SDS-PAGE protein standards ranging between 94–14 kD were used. See text for labels.

(~48 kD) in AH do not seem to be present in serum at these concentrations. However, the former train was later found to be present in some eluates of serum-perfused filters. Protein subunits with a similar apparent molecular weight to those of train B and also positioned to the anodic side of the albumin have

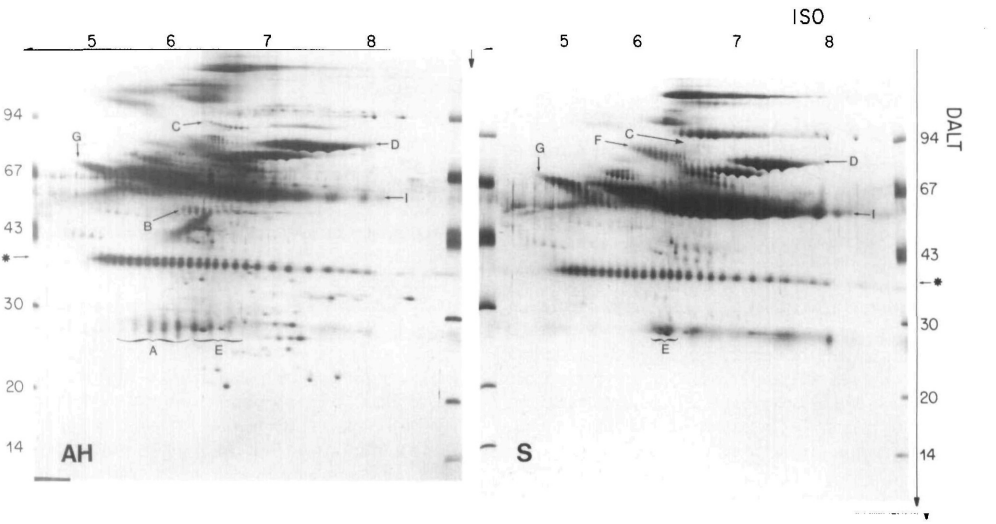

Fig. 2. 2-D maps of dealbuminated pooled calf aqueous humor (AH) and of pooled serum (S); (40 μg of protein in each). Albumin was immunodeleted by rabbit anti-bovine albumin immunoglobulins immobilized on a CNBr-activated Sepharose 4B column. Fractions were analyzed via SDS-PAGE to determine efficiency of albumin removal.

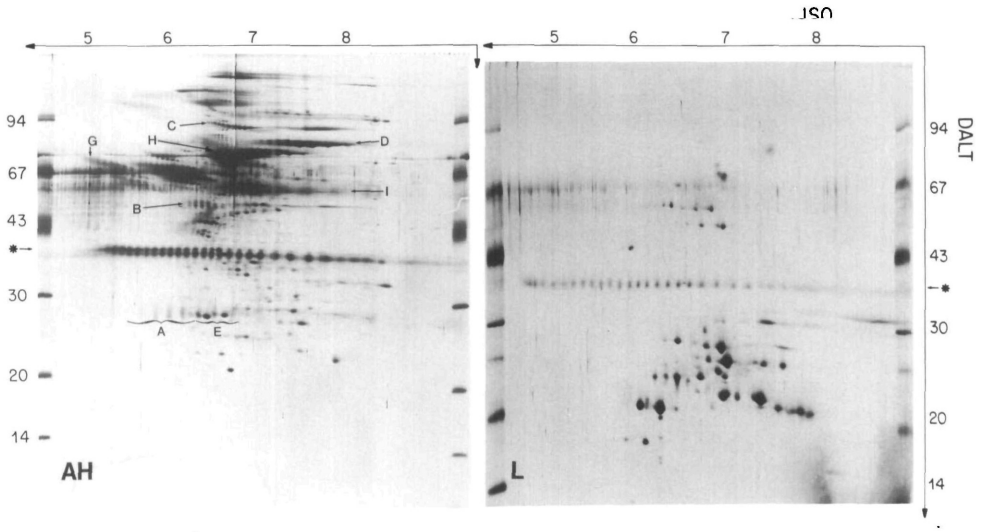

Fig. 3. 2-D maps of pooled calf aqueous humor (AH; 40 μg) protein subunits and of total calf lens proteins (L; 10 μg).

also been uniquely found in human AH.[24] Other protein subunits, such as train C (~94 kD), appear more concentrated in AH while train D (~80 kD; corresponding to transferrin in electrophoretic mobility) and train E (~28 kD), although also present in both specimens, show greater charge microheterogeneity in AH. Similar findings for transferrin in human AH have also been reported.[24,25] On the other hand, subunits F (~80–90 kD) are present in serum but not in AH.

Dealbuminated samples (Fig. 2) reveal additional components which electrophoretically comigrate with the albumin and are therefore normally obscured. For the most part, these newly visible spots consist of portions of trains that may be seen emerging from the albumin area in normal runs. For example, Figure 2 reveals in serum a six-spot train of which only two spots are visible in Figure 1. In Figure 3, the characteristic pattern of lens protein subunits is shown alongside a pooled AH sample containing some of these same components.

Filters were perfused with AH and with serum (diluted 1:100) as described in *Methods*. In order to maximize yield, the sample volumes were chosen on the basis of a trial experiment to saturate the filters. The filters were then thoroughly rinsed and eluted.

Table 2 summarizes the total amounts of protein perfused through and the quantities of protein eluted from each filter type. More filter-bound protein was eluted from the hydrophobic filters as opposed to their hydrophilic counterparts. Comparable amounts of protein were eluted from both AH- and serum-perfused filters. However, compared to the serum-perfused filter eluates, the quantities of eluted protein from the AH-perfused filters were more variable. The reasons are unknown and no correlation between these quantities and sample protein concentrations (or protein composition) was observed (data not shown). Also, 0.1% NP-40 was just as effective in eluting proteins from the filters as 1.0%. Figure 4 illustrates the proteins eluted from the hydrophobic filters. Similar AH and serum components adhered to the filters. The predominant proteins retained on them were the albumin, those designated G (~60–70

Table 2. Total amounts of protein perfused through and eluted from each filter

	N	*(n)*	*Amount of protein perfused/filter (μg ± SE)*	*Amount of protein eluted/filter (μg ± SE)*
Hydrophobic filters				
Aqueous humor	8	(5)	764 ± 85	3.3 ± 0.73
Serum	8	(4)	941 ± 49	2.6 ± 0.25
Hydrophilic filters				
Aqueous humor	5	(5)	898 ± 94	1.9 ± 0.45
Serum	6	(4)	1043 ± 77	1.5 ± 0.29

Filters were perfused with pooled batches (n) of aqueous humor or serum in sets (N) of five to ten filters simultaneously. The filters were washed by reperfusion with an excess volume of DPBS and the filter set pooled and extracted with 0.1–1.0% NP-40 with sonication for 30 min. As some batches were used twice the number of sets was somewhat greater than the number of batches.

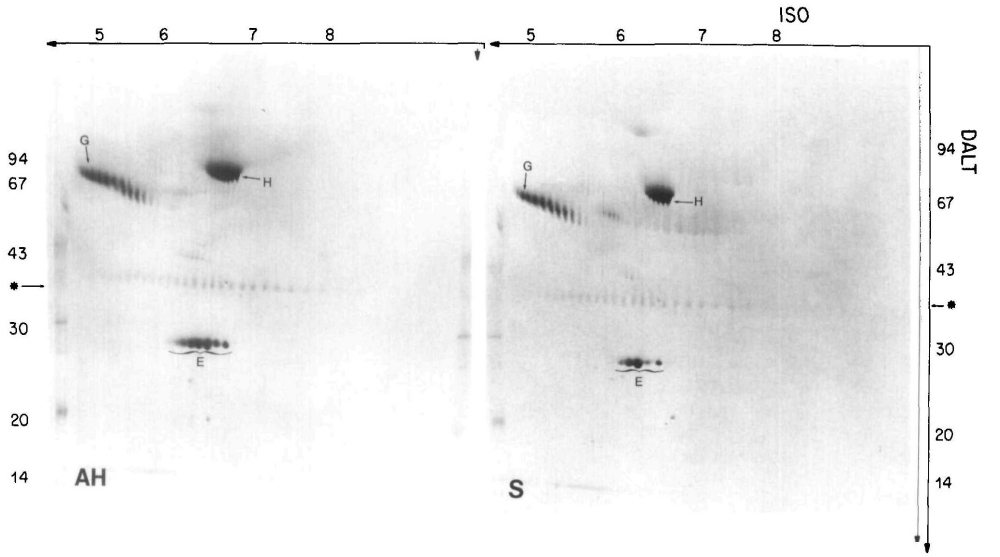

Fig. 4. 2-D maps of protein eluted from PVP-free (hydrophobic) 0.2 μm polycarbonate filters perfused with either 1 ml of pooled calf aqueous humor (AH) or diluted (1:100) serum (S); (5 μg of protein in each). Filter-bound proteins were eluted wtih 0.1% NP-40 and then treated as all other samples.

kD), and the subunits corresponding to train E (with the apparent extra subunits in AH more concentrated). Those proteinaceous components eluted from the hydrophilic filters are shown in Figure 5. Clearly, lesser amounts of protein are binding to these filters, notably, much less albumin. The major components eluted from these filters were albumin, train E, and other species identified as train J (~22 kD) in

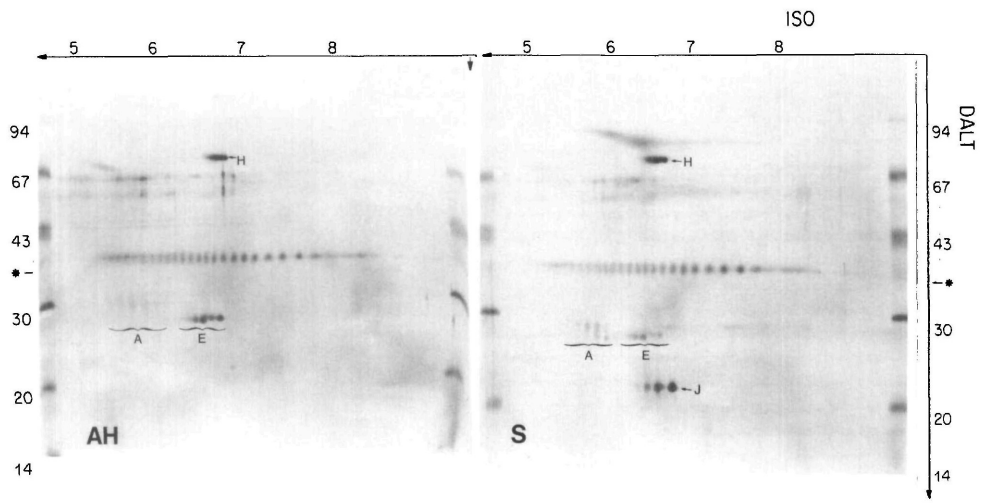

Fig. 5. 2-D maps of protein eluted from PVP-coated (hydrophilic) 0.2 μm polycarbonate filters perfused with either 1 ml of calf aqueous humor (AH) or diluted (1:100) serum (S); (5 μg of protein in each). Elution conditions were the same as in Figure 4.

serum. Interestingly, components resembling those of train A, which were previously undetectable in the serum 2-D maps, were retained and concentrated on these filters. Very little of these same serum components remained adsorbed on the hydrophobic filters. Furthermore, their AH counterparts did not behave in the same way; that is, they were not concentrated by either the hydrophilic or the hydrophobic filters.

Discussion

Although the vast majority of AH proteins are blood-derived, this intraocular fluid is by no means a simple and dilute diffusate or filtrate of plasma. Its unique composition with respect to electrolytes, dissolved gases, amino acids and many other components has been established. Its protein composition also proves to be quantitatively and qualitatively different from that of serum, as noted in the literature[24–27] and as shown by our results.

Proteins observed in AH in greater relative concentration than in serum may be secreted preferentially by the ciliary processes during aqueous formation or secreted into AH by one or more of the other tissues of the eye. Similarly, proteins observed in lesser relative concentration may be discriminated against during aqueous formation or bound to or destroyed by one or more other ocular tissues. In addition, proteins being secreted into or already present in AH from whatever source, may be modified, either radically by cleavage or cross-linking, or slightly, by side-chain modification or, more likely, by glycosylation. Highly glycosylated proteins are less reactive with the silver stain,[28] and therefore some caution must be exercised in comparing relative intensity of spots. It may even be possible that we have failed to detect some significant proteins because of this masking effect. In the future, we will investigate stains specifically developed to detect glycosylated proteins.[29] There is a general impression, previously noted by Dernouchamps,[27] that in the process of aqueous formation smaller molecular weight proteins are favored over larger. The increased levels of albumin and other low molecular weight subunits relative to subunits of immunoglobulins and others of higher molecular weight would appear to be a good example of this mechanism of enhancement. Train B, undetectable in serum, may either be more enhanced in AH or possibly be locally produced. Local production of antibodies and transferrin has been previously suggested in the literature.[22,27,30,31] Trains D and E both show extra charged species in AH. These may arise from glycosylation of already existing proteins. The absence of train F from AH suggests that these proteins are being quite specifically excluded during AH formation. The consistency with which these differences were maintained in every AH specimen reassures us that little or no contamination with plasma proteins occurred prior to or during AH collection. For reasons given above, maps produced from pooled samples contain lens proteins while most of those from individual samples do not. The lens proteins may be an artifact of the collection procedure, but given the care exercised during collection, this does not seem likely. We suspect that some of these neonatal calves may simply have leaky lenses. Comparison of maps from pooled and from individual samples shows that lens proteins are readily identified and do not interfere with our interpretations.

The work of Johnson et al[10] showed that filter obstruction by calf AH was eliminated by proteases but not by hyaluronidase. Subsequent studies done by Ethier[11] and Ethier et al[12,13] indicated that filter surface chemistry played an important role in the degree of filter blockage in that hydrophobic membranes blocked to a much greater extent than hydrophilic ones. Furthermore, the nonionic detergent Triton X-100 completely reversed this filter obstruction. Therefore, hydrophobicity was determined to be a significant factor in these protein filter-binding interactions and thereby, filter flow obstruction. Our findings of more hydrophobically adsorbed protein on the filters are consistent with the observation that the hydrophobic filters are blocked more severely than the hydrophilic filters. These and other factors and types of interactions that may be involved in the protein filter adsorption process are discussed in greater detail by Ethier et al.[13]

Albumin and trains G and E were the major components retained on the hydrophobic filters. Train E showed a relatively enhanced binding of those subunits peculiar to AH. Interestingly, the hydrophilic filters retained very little albumin following their reperfusion with DPBS. Train E was again the one consistently found in these eluates, again with enhanced relative binding of the AH-specific subunits. The filters perfused with AH showed 5- to 10-fold greater resistance than those perfused with matched, diluted serum but much less than 5- to 10-fold the amount of protein was eluted from the more resistant filter. Either some nonproteinaceous component is also taking part in the blocking process or the pattern of protein deposition within the filter must be very different in the two cases. The role of albumin will be further investigated by studying the capacity of albumin-free AH to block the filters. This may also show if any components that comigrate with albumin are involved, as these would not be visible in Figures 4 and 5.

What is most important now is to determine whether these proteins are in fact responsible for the filter obstruction exhibited by AH, and furthermore, to investigate whether a similar binding of AH proteins occurs in the outflow pathway of the calf eye, and if so, to evaluate how it relates to outflow resistance. The isolation of our current suspects is underway in order to further investigate this question.

Key words: aqueous humor proteins, bovine, microporous filters, perfusions and elutions, two-dimensional electrophoresis

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References

1. Bill A: Blood circulation and fluid dynamics in the eye. Physiol Rev 55:383, 1975.
2. Ethier CR, Kamm RD, Palaszewski BA, Johnson M, and Richardson TM: Calculations of flow resistance in the juxtacanalicular meshwork. Invest Ophthalmol Vis Sci 27:1741, 1986.
3. Epstein DL: Open angle glaucoma—Why not a cure? (Editorial). Arch Otolaryngol 113:1187, 1987.
4. Troncoso MU: Pathogenie du glaucoma—recherches cliniques et experimentales. Ann Oculiste 126:401, 1901.
5. Herschler J and Litin BS: Biochemical abnormalities in the aqueous in chronic open-angle glaucoma. Ophthalmic Surg 18:792, 1987.
6. Epstein DL, Hashimoto JM, and Grant WM: Serum obstruction of aqueous outflow in enucleated eyes. Am J Ophthalmol 86:101, 1978.
7. Epstein DL, Jedziniak JA, and Grant WM: Obstruction of aqueous outflow by lens particles and heavy-molecular-weight soluble lens proteins. Invest Ophthalmol Vis Sci 17:272, 1978.
8. Epstein DL, Jedziniak JA, and Grant WM: Identification of heavy-molecular-weight soluble protein in aqueous humor in human phacolytic glaucoma. Invest Ophthalmol Vis Sci 17:398, 1978.
9. Zirm M: Protein glaucoma—Overtaxing of flow mechanisms? (A preliminary report). Ophthalmologica 184:155, 1982.
10. Johnson M, Ethier CR, Kamm RD, Grant WM, Epstein DL, and Gaasterland D: The flow of aqueous humor through micro-porous filters. Invest Ophthalmol Vis Sci 27:92, 1986.
11. Ethier CR: The flow of aqueous humor in microporous materials. PhD Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1986.
12. Ethier CR, Kamm RD, Johnson M, Pavao AF, and Anderson PJ: Further studies on aqueous humor flow in microporous filters. ARVO Abstracts. Invest Ophthalmol Vis Sci 28(Suppl):284, 1987.
13. Ethier CR, Kamm RD, Johnson M, Pavao AF, and Anderson PJ: Further studies on the flow of aqueous humor through microporous filters. Invest Ophthalmol Vis Sci 30:739, 1989.
14. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976.
15. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007, 1975.
16. Tollaksen SL, Anderson NL, and Anderson NG: Operation of the ISO-DALT system, (7th ed.). Argonne National Laboratory Report, ANL-BIM-84-1, May 1984.
17. Marshall T, Williams KM, and Vesterberg O: Two-dimensional electrophoresis of proteins in human serum: Improved resolution by use of narrow pH gradients and prolonged electrophoresis. Clin Chem 30:2008, 1984.
18. Laemmli U: Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680, 1970.
19. Dion AS and Pomenti AA: Ammoniacal silver staining of proteins: Mechanism of glutaraldehyde enhancement. Anal Biochem 129:490, 1983.
20. Anderson L and Anderson N: Some perspectives on two-dimensional protein mapping. Clin Chem 30:1898, 1984.
21. Saari KM and Parviainen MT: Determination of protein content in aqueous humour by high-performance gel filtration chromatography. Acta Ophthalmol 61:611, 1983.
22. Grabner G, Zehetbauer G, Bettelheim H, Hönigsmann C, and Dorda W: The blood–aqueous barrier and its permeability for proteins of different molecular weight. Graefes Arch Klin Exp Ophthalmol 207:137, 1978.
23. Fielder AR and Rahi AHS: Immunoglobulins of normal aqueous humour. Trans Ophthalmol Soc UK 99:120, 1979.
24. Segers J, Rabaey M, and Van Oye R: Analysis of normal human aqueous humour proteins by two-dimensional gel electrophoresis. Electrophoresis 5:48, 1984.
25. Yu TC and Okamura R: Comparative study of native proteins in aqueous humor and serum—detection of characteristic aqueous humor proteins. Jpn J Ophthalmol 31:235, 1987.
26. Zirm M: Proteins in aqueous humor. Adv Ophthalmol 40:100, 1980.
27. Dernouchamps JP: The proteins of the aqueous humour. Doc Ophthalmol 53:193, 1982.
28. Dubray G and Bezard G: A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal Biochem 119:325, 1982.
29. Dzandu JK, Deh ME, Barratt DL, and Wise GE: Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double-staining technique. Proc Natl Acad Sci USA 81:1733, 1984.
30. Dernouchamps JP, Vaerman JP, Michiels J, and Masson PL: Immune complexes in the aqueous humor and serum. Am J Ophthalmol 84:24, 1977.
31. Rahi AHS, Kanski JJ, and Fielder AR: Immunoglobulins and antinuclear antibodies in aqueous humour from patients with juvenile "rheumatoid" arthritis (Still's disease). Trans Ophthalmol Soc UK 97:217, 1977.