the aqueous solution standard state, and in the isooctane standard state (Fig. 5) illustrates the effect of polar groups in a series of prostaglandin $F_{2\alpha}$ esters. An alkane solution of drug at infinite dilution was chosen as the reference state according to the convention proposed previously (9, 10). Thus, the free energies of various derivatives in isooctane arbitrarily were set equal to zero so that relative energies in the solid phase and in water could be compared. Polar substituents dramatically lowered the energy level of a drug in the solid phase and in water relative to an alkane solution; but the free energy difference between solid and aqueous solution, which is reflected by aqueous solubility, remained similar upon addition of increasingly polar substituents.

The conclusion is that polar, hydrogen-bonding substituents may not result in increased aqueous solubility. Even though lipophilicity as measured by partition coefficients may be decreased drastically, the effect of polar groups on the thermodynamic activity of a drug in the solid may tend to offset the change in lipophilicity (Fig. 5). Both thermodynamic components of aqueous solubility must be considered in predicting aqueous solubility.

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Preliminary Examination of Rabbit Conjunctival Mucins

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Abstract □ Conjunctival mucins from albino rabbits were fractionated using gel filtration and anion-exchange chromatography. Charge homogeneity of the various conjunctival mucin fractions was confirmed by polyacrylamide gel electrophoresis. The molecular weight characteristics of the isolated fractions varied with the analytical scheme employed. Two schemes yielded mucins with molecular weights of 10^4, 10^5, and 10^6. However, when anion-exchange chromatography was the first step in the fractionation scheme, the dominant mucin had a molecular weight of 1.7×10^5. In contrast, when gel filtration chromatography was the first step, the dominant mucin had a molecular weight of 5.7×10^4. It was postulated that during migration through the anion-exchange matrix, the low molecular weight conjunctival mucin underwent trimer formation. Comparison with the mucin fractions isolated from tear mucoid threads revealed that the scheme beginning with anion-exchange chromatography preserved the fractionation pattern seen in tear mucoid threads. This result implies that conjunctival mucins undergo an association prior to or after their entry into the tear film. The molecular event of interest in this process is self-association of the species with a molecular weight of 5.7×10^4, resulting in a trimer with a molecular weight of 1.7×10^5. This trimer appears to resist deaggregation on exposure to a medium of lesser ionic strength. Several explanations are offered for its formation as well as for its stability. The implication of multiple conjunctival (tear) mucins for tear film stability also is discussed.

Keyphrases □ Mucins—isolation from rabbit conjunctiva, fractionation by gel filtration and anion-exchange chromatography, determination of molecular weight, role in tear film stability □ Conjunctival mucins—isolation from rabbits, fractionation by gel filtration and anion-exchange chromatography, determination of molecular weight, role in tear film stability □ Ocular mucins—isolation from rabbit conjunctiva, fractionation by gel filtration and anion-exchange chromatography, determination of molecular weight, role in tear film stability

The strategic role assumed by conjunctival mucin in tear film stability has been recognized, but not understood, for many years. Alteration in either its chemical nature or quantity (probably both) has been implicated in keratoconjunctivitis sicca as well as other dry-eye syndromes (1–5), all of which are characterized by poor wetting of the corneal epithelium. It has been proposed that this fascinating substance derives its role from both the surface activity it is expected to exhibit at the air–tear and tear–corneal epithelium interfaces and the molecular events following its absorption at these two interfaces.

Principally because conjunctival mucins have never been isolated or identified, statements about their surface activity are based on experiments using bovine submaxillary mucin (6–9). In contrast, three tear mucins have been isolated (10), but the interrelationship between tear and conjunctival mucins is unknown. The tear mucins were shown to possess an amino acid and carbohydrate composition not shared by typical epithelial mucins (10, 11). Since the function it serves depends on its structure, considerable caution must be exercised in extrapolating the findings on surface activity of bovine submaxillary mucin to conjunctival and tear mucins. Successful isolation of conjunctival mucins, in reasonable quantity and purity, is important in understanding the mechanisms by which they confer stability to the tear film. This study is a first attempt in this direction; specifically, it concerns identifying conjunctival mucins present in a crude conjunctival extract.

The three tear mucins isolated by Iwata and Kabasawa (10) from tear mucoid threads on the conjunctival surface of albino rabbits possessed molecular weights of 4×10^5,

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5×10^4, and 1.4×10^4. Conspicuously absent was the mucin with a molecular weight of 10^6, a species commonly seen in epithelial mucins. However, this result probably is an artifact since the gel filtration medium employed, Sephadex G-150, does not fractionate proteins with molecular weights exceeding 4×10^5. Nevertheless, the isolation of multiple tear mucin fractions is intriguing because multiple fractions rarely have been isolated for simple proteins such as insulin and growth hormone. Moreover, since mucins in the precorneal area of the eye are derived from both the conjunctival (12) and lacrimal (13) glands and their composition varies with the source, it was of interest to see if the three tear mucin fractions were primarily of conjunctival origin. The results of the present study indicate that this is the case.

The three objectives of this study were: (*a*) to examine the type of mucins derived from the conjunctiva, as well as the interrelationship among them; (*b*) to learn how these mucins are related to those identified in the tear film; and (*c*) to gain further insight into tear film stability. The interrelationship between tear and conjunctival mucins will be reported later. The present paper concerns the interrelationship among conjunctival mucins and their probable roles in tear film stability.

EXPERIMENTAL

Materials—Male albino rabbits[1], 1.8–2.4 kg, were used.

The following chemicals and reagents were used as received: synthetic *N*-acetylneuraminic acid[2], 3,5-diaminobenzoic acid dihydrochloride[2], chromatographic gel[3], polyacrylamide gel[4], polyacrylamide gel basic buffer[5] (pH 8.9), protein assay kit[6], anion-exchange chromatographic gel[7], tracking dye solution[8] (acidic), trichloroacetic acid[9], Blue Dextran 2000[10], Sephadex G-25[10] (fine), rabbit albumin[11], γ-globulin[11], bovine submaxillary mucin[12] (type I), Coomassie brilliant blue G-250[12], and fumarase[13].

Analytical Methods—*Protein Assay*—In addition to its absorbance at 280 nm, A_{280}, the protein content of the column eluate was monitored using the protein assay kit. This assay is based on the differential color change of the dye in response to various protein concentrations (14). It was performed following the procedure outlined by the manufacturer (15).

Fluorometric Assay for Sialic Acid—A fluorometric assay (16) that can detect as little as 0.2 ng of sialic acid was adopted; the more widely used thiobarbituric acid assay (17) was found to underestimate sialic acid levels as a result of sialic acid degradation following its liberation from mucins (11).

The procedure was conducted as follows. To 10 μl of a sample containing sialic acid in a 1-ml ampul[14] was added 500 μl of 0.005 *M* 3,5-diaminobenzoic acid dihydrochloride in 0.125 *N* HCl. The ampul was sealed and placed in an oven at 100° for 16 hr. After cooling to room temperature, the contents of the ampul were transferred to a 5-ml volumetric flask and brought to volume with 0.05 *N* HCl. The fluorescence intensity was measured in a fluorescence spectrophotometer[15] with the excitation wavelength set at 425.8 nm and the emission wavelength at 507.5 nm. A standard curve was established daily using freshly prepared *N*-acetylneuraminic acid solutions.

Collection of Rabbit Conjunctivas—Rabbits were sacrificed with a rapid intravenous injection of pentobarbital sodium into a marginal ear vein. The corneal and conjunctival surfaces were washed thoroughly with 0.9% NaCl solution and blotted dry with tissue. A single incision was made at the upper nasal palpebral conjunctiva and was continued clockwise along the lid margin and terminated at the bulbar conjunctiva around the limbus. Each conjunctiva was rinsed carefully in saline, frozen, and stored frozen at −20° for a period not to exceed 3 months. Approximately 98 g of conjunctivas was collected from 150 rabbits.

Extraction of Conjunctival Mucins—The entire extraction was conducted in a cold room maintained at 4°. Approximately 90 g of conjunctivas was suspended in 450 ml of 0.01 *M* ammonium acetate buffer at pH 7.2 and homogenized in a blender for 10 min. The mixture was stirred constantly for 24 hr in the presence of toluene.

The homogenate was filtered through four layers of adsorbent cheesecloth, prerinsed in the buffer, and centrifuged at 15,000 rpm for 30 min at 4° in a centrifuge[16]. The supernate was filtered first through filter paper[17] and then through a 0.65-μm filter[18], both of which were placed in a buchner funnel connected to a water aspirator. The filtrate was lyophilized in a freeze-drying apparatus[19] at 65 μm Hg and −55° for 48 hr. The residue, denoted as conjunctival crude extract, was a yellowish powder and was stored in a desiccator placed in a freezer at −20°. Lyophilization was assumed to cause no changes in the mucin; this assumption was shown to be the case with bovine submaxillary mucin (18).

The conjunctival crude extract represented about 2.5% of the initial conjunctival weight.

Anion-Exchange Chromatography of Conjunctival Crude Extract—Approximately 70 mg of conjunctival crude extract was dissolved in 2.5 ml of 0.01 *M* ammonium acetate buffer at pH 7.2. Two milliliters of the resulting solution was layered onto an anion-exchange column (1.5 × 20 cm) that had been preequilibrated for 16 hr at 4° with the buffer. After the entire sample entered the gel, the column was washed with two bed volumes (100 ml) of the buffer at a constant flow rate of 58.5 ml/hr controlled by a peristaltic pump. The column then was eluted at the same flow rate with a linear gradient consisting of 200 ml each of 0.1 and 1 *M* NaCl, both dissolved in 0.01 *M* ammonium acetate at pH 7.2. Fractions of 1.5 ml were collected. The content of every third tube was measured for its absorbance at 280 nm and for sialic acid, protein, and sodium chloride concentrations.

Finally, the contents of several tubes were pooled, followed by desalting on a column (5 × 60 cm) of Sephadex G-25 (fine). The column was eluted with double-distilled water at a constant flow rate of 150 ml/hr. Fractions of 18.5 ml were collected. The proteins emerged with the void volume (~30% of total bed volume), while the salt emerged with ~80% of the total bed volume. Fractions containing protein were lyophilized at −55° and 65 μm Hg for 48 hr.

The lyophilizate was reconstituted with 0.01 *M* ammonium acetate buffer at pH 7.2. The behavior of the proteins contained therein was studied next on a chromatographic gel filtration column.

Gel Filtration of Conjunctival Crude Extract—Sample preparation and layering onto a column (1.5 × 60 cm) of chromatographic gel were essentially the same as described for anion-exchange chromatography. The column was eluted at a constant flow rate of 8.8 ml/hr with the buffer. Fractions of 1.5 ml were collected. The contents of appropriate tubes were examined at 280 nm for their protein and sialic acid content. Based on the results of these assays, certain tubes were selected for polyacrylamide gel electrophoresis. The contents of several tubes were pooled and lyophilized, and selected fractions were chromatographed further on an anion-exchange column (1.5 × 30 cm).

Molecular Weight Estimation—The molecular weight of various mucin fractions was estimated by calibrating a gel filtration column in the manner described previously (19–23). This procedure has been employed to estimate the molecular weight of tear mucins (10); consequently, a meaningful molecular weight comparison can be made between conjunctival and tear mucins.

A gel chromatography column (1.5 × 60 cm) was calibrated using the following proteins (or protein mixture): bovine submaxillary mucin (2.26 mg/ml) and rabbit γ-globulin (8.76 mg/ml), rabbit serum albumin (5.0 mg/ml), fumarase (5.0 mg/ml), and Blue Dextran 2000 (2.62 mg/ml). All solutions were prepared in 0.01 *M* ammonium acetate buffer at pH 7.2. To the Blue Dextran 2000 solution was added also 0.1 *M* NaCl. Two milliliters of sample was layered onto the column, which then was eluted

[1] Klubertanz, Edgerton, Wis.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Bio-Gel A 5m, Bio-Rad Laboratories, Richmond, Calif.
[4] Bio-Phore 4, 7.5, 12%, Bio-Rad Laboratories, Richmond, Calif.
[5] Bio-Phore, Bio-Rad Laboratories, Richmond, Calif.
[6] Bio-Rad Protein Assay Kit II, Bio-Rad Laboratories, Richmond, Calif.
[7] DEAE Bio-Gel A, Bio-Rad Laboratories, Richmond, Calif.
[8] Bio-Rad Laboratories, Richmond, Calif.
[9] Mallinckrodt, St. Louis, Mo.
[10] Pharmacia Fine Chemicals AB, Uppsala, Sweden.
[11] Schwarz/Mann, Orangeburg, N.Y.
[12] Sigma Chemical Co., St. Louis, Mo.
[13] United States Biochemical Corp., Cleveland, Ohio.
[14] Wheaton Scientific, Millville, N.J.
[15] Model MPF-4, Perkin-Elmer, Norwalk, Conn.
[16] Sorvall RC-5B refrigerated superspeed centrifuge using an SS-34 rotor, DuPont Instruments, Newtown, Conn.
[17] Whatman No. 2.
[18] Millipore.
[19] New Brunswick Scientific Co., New Brunswick, N.J.

1. Anion-Exchange Chromatography

conjunctival crude extract
anion-exchange chromatography
wash | gradient

peak	A	B	C	D E	F (0.032 *M* NaCl)	G (0.008 *M* NaCl)
fraction	W1	W2	W3	G1	G2	G3
AUC ratio[a]	1	18	—[b]	—[b]	1.1	1
sialic acid, μg[c]	0.03	0.22	0.24	0.04	0.13	0.04
percent	1	9.4	1.2	0.8	4.7	0.2[d]
polyacrylamide gel electrophoresis (bands)	—[b]	—[b]	—[b]	—[b]	5 (Fig. 1)	7

2. Gel Filtration Chromatography

	W2 gel chromatography			G2 gel chromatography			G3 gel chromatography		
peak	A	B	C	A	B	C	A	B	C
molecular weight	2.4×10^4	5.5×10^3	465 120	3×10^6	1.7×10^5	4.9×10^3	2.5×10^6	1.7×10^5	480
AUC ratio[a]	1	1	—[b]	1	16	—[b]	1	14	—[b]
sialic acid, μg[c]	—[b]	—[b]	—[b]	0.12	0.35	0.12, 0.05	0.05	0.07	0.04, 0.02
percent	—[b]	—[b]	—[b]	4.4	4.6	5.8, 1.6	1.2	0.3	0.3
polyacrylamide gel electrophoresis (bands)	—[b]	—[b]	—[b]	0	3	—[b]	1	1	—[b]

Scheme I—Fractionation of Conjunctival Crude Extract by Procedure A. [[a] Area under the curve (referred to A_{280} *versus* the tube number profile). [b] Not performed or determined. [c] Sialic acid in 10 μl of solution. [d] Insufficient data to give an accurate estimate.]

with 0.01 *M* ammonium acetate buffer at pH 7.2 at a flow rate of 8.8 ml/hr. Fractions of 1.5 ml were collected, and the absorbance of every third tube was measured at 280 nm.

The position of the peak maximum in the elution diagram, *i.e.*, absorbance *versus* tube number, was taken as the elution volume, V_e. The correlation between molecular weight and elution behavior was analyzed by plotting K_{av} *versus* log molecular weight. The regression equation for the plot is:

$$K_{av} = 1.497 - 0.228 \log \text{molecular weight} \qquad \text{(Eq. 1)}$$

($r = -0.995$, $r^2 = 0.989$), where K_{av} is the partition coefficient of the solute between the liquid phase and the gel phase.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed at 4° using precast 4, 7.5, and 12% polyacrylamide gels and basic buffer at pH 8.9. The sample solution was mixed with tracking dye solution in a ratio of ~3:1. Thirty microliters of the resulting mixture was layered onto a gel using a microbiopet[6].

At the end of electrophoresis, each gel was carried through three steps leading to the visualization of protein bands:

1. The proteins were fixed onto the gel by immersing the gel for 90 min in a mixture of 12.5% trichloroacetic acid and 25% isopropanol.
2. The precipitated proteins were stained by immersing the gel for 6 hr in a Coomassie brilliant blue G-250 solution (the staining solution was 0.05% dye, 10% isopropanol, and 10% acetic acid).
3. The excess stain on the gel was removed in a diffusion destainer[20] filled with a mixture of 7% acetic acid and 25% ethanol.

The procedure outlined by Wardi and Michos (24) for staining glycoproteins on polyacrylamide gels was unsatisfactory for conjunctival mucins, probably due to the low sialic acid content of the glycoproteins. No further attempt was made to improve visualization of these glycoproteins on the gels.

RESULTS

Fractionation Procedure A—Scheme I summarizes the results obtained by chromatographing the crude conjunctival extract. The results were displayed as the ratio of area under each elution peak in a chromatogram (*AUC*) relative to an appropriately chosen reference peak; as the sialic acid content, in both amount and percent, corresponding to each peak; and, where appropriate, as the molecular weight corresponding to each peak as well as the number of protein bands yielded by each peak upon performing polyacrylamide gel electrophoresis.

Thus, anion-exchange chromatography on the crude extract yielded seven fractions, in close agreement with the seven bands observed in electrophoresis of the extract on a 12% polyacrylamide gel (Gel C in Fig. 1). The chromatogram displayed in Fig. 2 indicates that three peaks (A–C) emerged with the wash and that four peaks (D–G) emerged with the gradient. The major peaks were F and G, hereafter referred to as

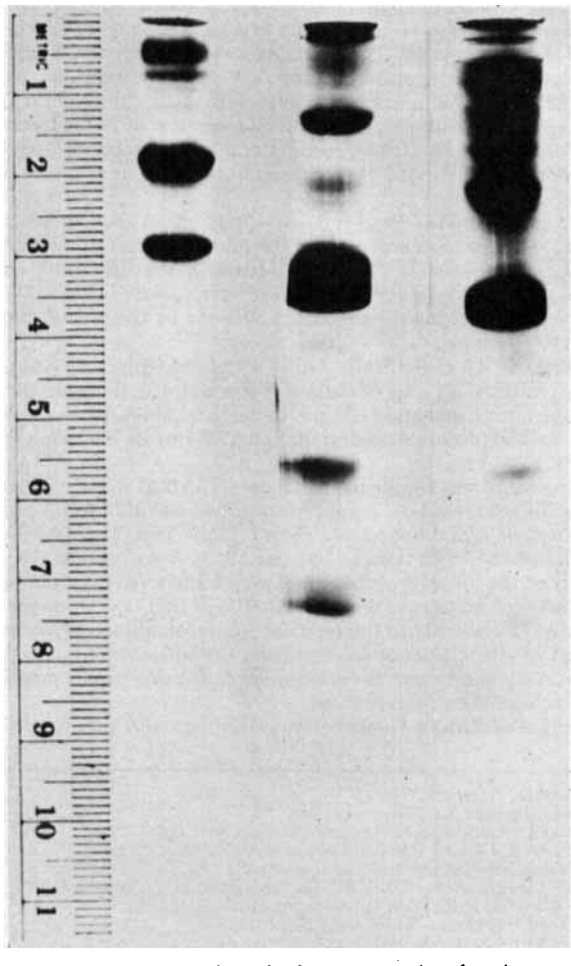


Figure 1—*Polyacrylamide gel electrophoresis of anion-exchange chromatography gradient Fractions 2 (G2) and 3 (G3) using 12% polyacrylamide gels. Key: Gel A, anion-exchange gel G2; Gel B, anion-exchange gel G3; and Gel C, conjunctival crude extract.*

[20] Model 172A, Bio-Rad Laboratories, Richmond, Calif.

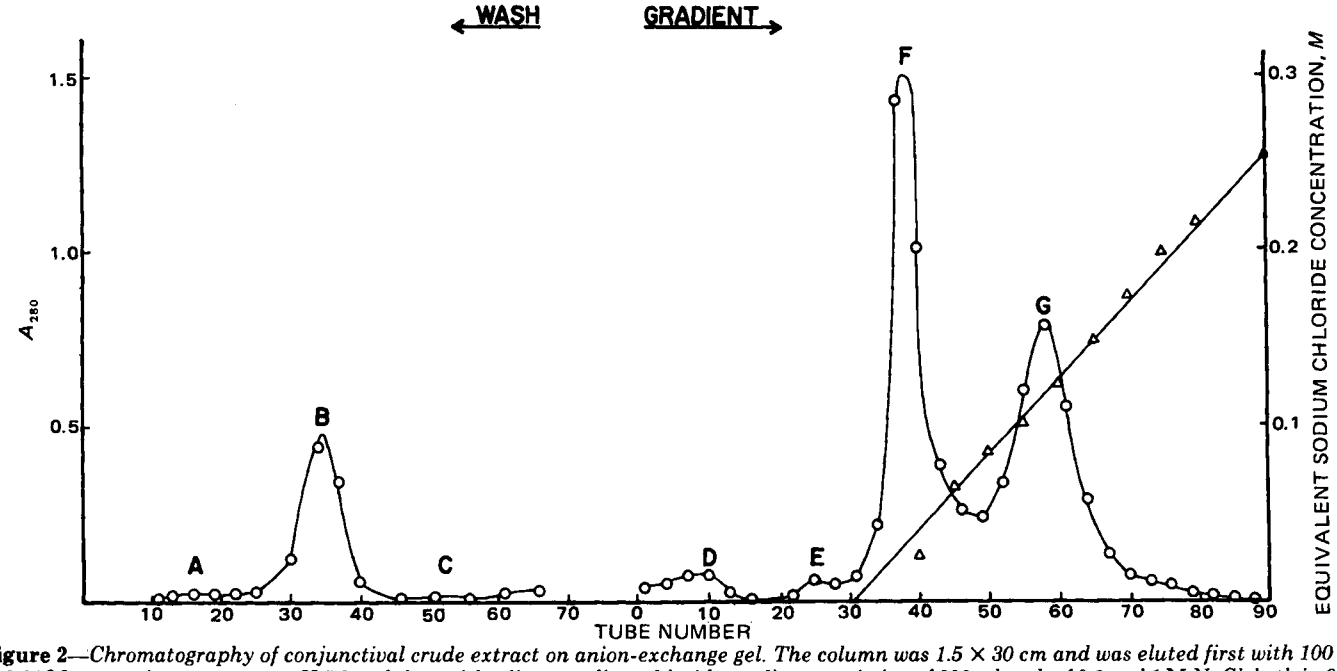


Figure 2—*Chromatography of conjunctival crude extract on anion-exchange gel. The column was 1.5 × 30 cm and was eluted first with 100 ml of 0.01* M *ammonium acetate at pH 7.2 and then with a linear sodium chloride gradient consisting of 200 ml each of 0.1 and 1* M *NaCl, both in 0.01* M *ammonium acetate at pH 7.2. The flow rate was 58.50 ml/hr. Fraction volumes were 1.5 ml.*

Fractions G2 and G3, respectively (Scheme I). Fraction G2 was eluted at 0.032 *M* NaCl, while Fraction G3 was eluted at 0.118 *M* NaCl. In contrast, under essentially the same chromatographic conditions, bovine submaxillary mucin eluted as a single peak at 0.146 *M* NaCl (Fig. 3).

The electrophoretic patterns due to Fractions G2 and G3 (Gels A and B) shown in Fig. 1 revealed that nearly all of the bands seen in the conjunctival crude extract (Gel C) represented these two fractions. At least three bands (0, 2, and 3 cm from the origin) due to Fractions G2 and G3 overlapped, reflecting, in part, the slight overlap between Fractions G2 and G3 as they were eluted from the anion-exchange column. Nevertheless, both fractions contained characteristic mucins. They were the two bands at 0.5 and 0.8 cm on Gel A, corresponding to Fraction G2, and the four bands at 1.3, 4, 5.5, and 7.4 cm on Gel B, corresponding to Fraction G3. The last three bands due to Fraction G3 were of higher charge density than the two bands due to Fraction G2. Together with the abundance of the band at 3 cm, this finding was consistent with the elution of Fraction G3 at a higher sodium chloride concentration. The major protein also was different for Fractions G2 and G3. In Fraction G2, the major protein migrated 1.8 cm into the gel; in Fraction G3, it migrated 3 cm into the gel.

The similarity in composition between Fractions G2 and G3 is apparent not only in their electrophoretic patterns (Fig. 4) but also in their chromatograms (Figs. 5 and 6). Moreover, according to Scheme I, both Fractions G2 and G3 contained mucins with molecular weights on the order of 10^5 and 10^6. The major fraction contained the lower molecular weight species; the area of the peak, B, containing it was approximately 15 times that of the peak containing the 10^6 species.

Two conclusions can be drawn from these observations. First, the elution of these two species, with a 10-fold difference in molecular weight, must be inversely related to their charge density since they eluted at the same sodium chloride concentration. Second, each species of a given molecular weight existed in two charge densities since it was eluted at sodium chloride concentrations differing by a factor of approximately four, *i.e.*, 0.032 and 0.118 *M*. These results indicate that four mucins, in suitable combinations of molecular weight and charge density, were present in Fractions G2 and G3.

In summary, Procedure A yielded mucins with molecular weights on the order of 10^4 (Fraction G2) and 10^5 and 10^6 (both from Fractions G2 and G3). Unlike other epithelial mucins, the major mucin was not the one with a molecular weight of 10^6. Rather, it was the one with a molecular weight of 10^5. This pattern is remarkedly consistent with that seen in tear mucins. The 10^5 and 10^6 species are heterogeneous with respect to charge.

Fractionation Procedure B—Scheme II summarizes the results obtained by chromatographing the conjunctival crude extract according to Procedure B.

As shown in Scheme II and Fig. 7, chromatography of the conjunctival crude extract on chromatographic gel yielded five peaks. Peak E did not contain proteins, as indicated by the protein assay. The molecular weight of the species contained in peak D was too low to be a protein. Therefore, proteins were detected only in peaks A, B, and C; this conclusion was confirmed by polyacrylamide gel electrophoresis (Fig. 8). Since all of the proteins reacted positively to the sialic acid assay, they were glycoproteins.

As with Procedure A, Procedure B did not yield the mucin with a molecular weight of ~10^6 as the major mucin. Unlike Procedure A, Procedure B yielded a major mucin with a molecular weight of ~10^4, not ~10^5. Clearly, the pattern seen in tear mucins was not preserved. The implication of this finding was elaborated upon previously. Briefly, trimer formation probably occurred as the mucin with a molecular weight of ~10^4 migrated through the anion-exchange matrix and a similar matrix may exist on the conjunctival surface.

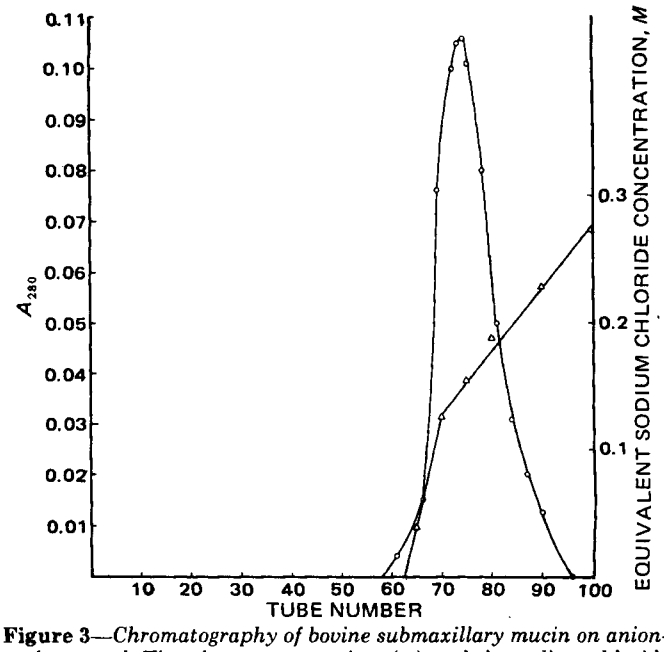


Figure 3—*Chromatography of bovine submaxillary mucin on anion-exchange gel. The plots represent* A_{280} (○) *and the sodium chloride concentration* (△). *The column was 1.5 × 30 cm and was eluted at 4° with a linear sodium chloride gradient at a flow rate of 58.5 ml/hr. Fraction volumes were 1.46 ml.*

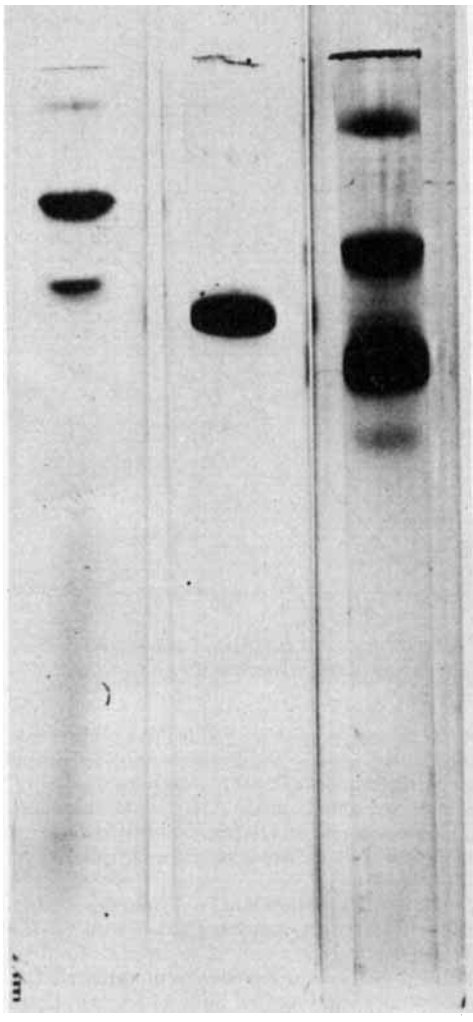


Figure 4—*Comparison of electrophoretic patterns of anion-exchange gel-chromatographic gel Fraction G2, peak B (Gel A); anion-exchange gel-chromatographic gel Fraction G3, peak B (Gel B); and chromatographic gel peak C (Gel C) on 12% polyacrylamide gels.*

The fact that the species with molecular weights of $\sim 10^4$ and $\sim 10^5$ were heterogeneous with respect to charge is evident in the electrophoretic patterns (Fig. 8) and the anion-exchange chromatograms (Figs. 9 and 10). The absence of multiple bands in the gel (A) layered with the 10^6 species should be expected from its inability to enter the gel. In contrast, anion-exchange chromatography resolved this species into three fractions, which eluted at 0.1, 0.105, and 0.135 *M* NaCl (Fig. 9). Similarly, four fractions were resolved for the mucins with a molecular weight of $\sim 10^4$. They eluted at 0, 0.02, 0.1, and 0.154 *M* NaCl (Fig. 10).

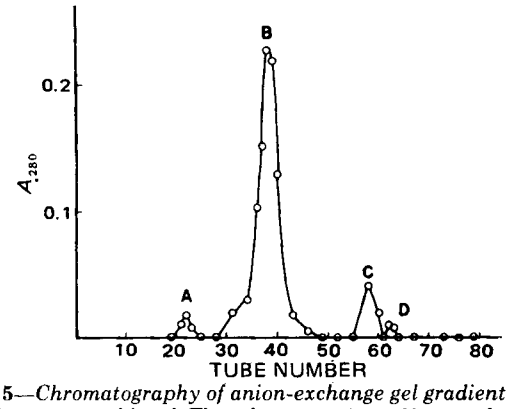


Figure 5—*Chromatography of anion-exchange gel gradient Fraction G2 on chromatographic gel. The column was 1.5 × 60 cm and was eluted with a flow rate of 8.8 ml/hr. Fraction volumes were 1.5 ml.*

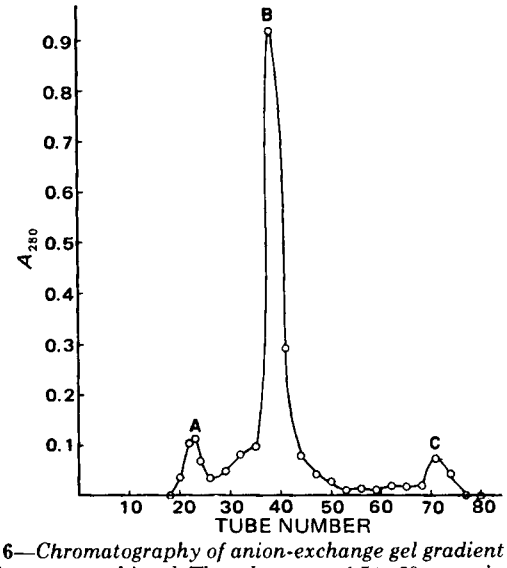


Figure 6—*Chromatography of anion-exchange gel gradient Fraction G3 on chromatographic gel. The column was 1.5 × 60 cm and was eluted with a flow rate of 8.8 ml/hr. Fraction volumes were 1.5 ml.*

DISCUSSION

This investigation showed that multiple conjunctival mucins exist, each differing in molecular weight and charge density. This finding is in accord with the isolation of multiple tear mucins in the precorneal area by Iwata and Kabasawa (10). There is evidence that the high molecular weight conjunctival mucins are aggregates of some monomer unit, and studies are underway to elucidate the mechanisms governing self-association and the factors contributing to stability of the resulting aggregates.

The remainder of this report will be devoted to an evaluation of the mucin extraction procedure, the conjunctival mucin fractionation schemes, postulated mechanisms of conjunctival mucin trimer formation, and the physiological significance of multiple conjunctival mucins in tear film stability.

Extraction of Mucin from Rabbit Conjunctivas—A caution exercised in this research was to isolate mucins from conjunctivas free from those in the precorneal area. To this end, each conjunctiva was rinsed with copious quantities of physiological saline following surgical removal from the eye. This rinsing should rid the surface of most of the adherent mucin and tear proteins since mucin was reported to be removed readily during increased tear secretion (25) and upon touching the conjunctival surface with a filter[18] (26, 27).

Another caution exercised in this research was to minimize denatur-

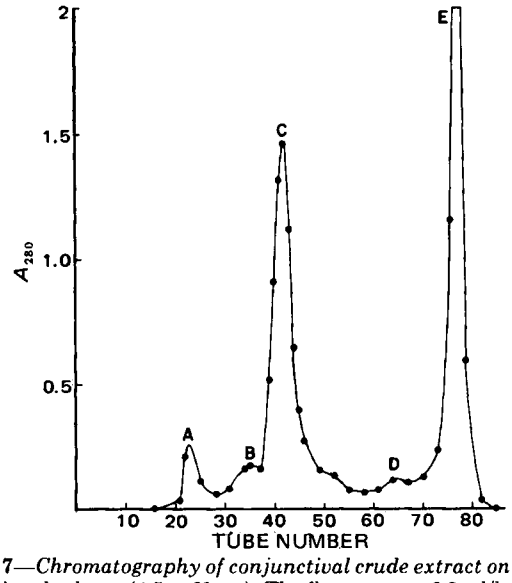


Figure 7—*Chromatography of conjunctival crude extract on chromatographic gel column (1.5 × 60 cm). The flow rate was 8.8 ml/hr, and the fraction volumes were 1.5 ml.*

1. Gel Filtration Chromatography

conjunctival crude extract

gel chromatography

	A	B	C	D	E
molecular weight	1.9×10^6	2.3×10^5	5.7×10^4	1.2×10^3	140
AUC ratio[a]	1	1	11	1	—[b]
sialic acid, %	16.4	2.4	2.4	10	5.6
polyacrylamide gel electrophoresis[c] (bands)	1	4	3	2	0

2. Anion-Exchange Chromatography

	Fraction A			Fraction C		
	anion-exchange gel			anion-exchange gel		
	wash	gradient		wash	gradient	
M NaCl	0.1	0.105	0.135	0	0.02	0.10	0.154
AUC ratio	1.2	1	1.3	0.8	1	2.9	14.5

Scheme II—Fractionation of Conjunctival Crude Extract by Procedure B. [[a] Ratio of area under the curve (referred to A_{280} *versus* tube number profile). [b] Not determined. [c] 12% gel.]

ation of conjunctival mucins during isolation. Gross fractionation of proteins from nonproteinaceous material by ammonium sulfate and ethanol precipitation is a step used in the fractionation schemes for many proteins (28). Such a treatment, while considered to be mild for most proteins, caused degradation of bovine and porcine submaxillary mucins (29–31). Because of this concern, the conjunctival crude extract was fractionated using chromatography as the first step.

In addition, since the slow process of denaturation would be delayed almost indefinitely if the temperature is low enough (32), all operations from tissue homogenization to chromatographic elution were conducted at 4° in a cold room. Similarly, to optimize stability of the glycoproteins on storage, the conjunctiva was frozen immediately following its removal at the temperature of a dry ice–acetone mixture. Thus, the ongoing glycoprotein synthesis is arrested and, depending on their quantities, such proteins may appear as mucin fractions when the crude extract is chromatographed. Similarly, unless they are membrane bound, the enzymes responsible for the biosynthesis (and, perhaps, degradation) of glycoproteins may be isolated. Neither possibility was vigorously investigated in this work.

An assumption made in this study was that little or none of the glycoproteins found in membranes (33) was isolated. Isolation of membrane glycoproteins often requires treatment of the cells with enzymes such as trypsin (34, 35) or chemicals such as lithium diiodosalicylate, phenol, aqueous pyridine, and sodium dodecylsulfate (36, 37). Since none of these agents was employed in the present study, the assumption seemed reasonable.

Influence of Fractionation Procedure on Conjunctival Mucin Trimer Formation—Two major findings emerged from fractionation of conjunctival mucins by Procedures A and B. First, both procedures yielded multiple mucin fractions. This finding agrees with the observation that mucins, unlike simple proteins, are polydisperse and microheterogeneous (38) and tend to yield several fractions during chromatographic separation. In general, the number of mucin fractions for a given mucin ranges from two to five; they differ primarily in their threonine, serine, sialic acid, and carbohydrate content as well as on their molecular weights (11). Conjunctival mucins with molecular weights on the order of 10^4, 10^5, and 10^6 were isolated in this work.

Second, the molecular weight of the dominant fraction was affected by the isolation procedure. When anion-exchange chromatography was the first step in fractionation, the dominant mucin possessed a molecular weight of 1.7×10^5. In contrast, when gel filtration was the first step, the dominant mucin possessed a lower molecular weight (5.7×10^4). The two fractions behaved similarly in polyacrylamide gel electrophoresis (Fig. 4). According to these electrophoretic patterns, the proteins concerned had the same charge density, suggesting that the mucin with a molecular weight of 5.7×10^4 underwent trimer formation when placed in an electrostatic field, which also was present at the positively charged surface of the anion-exchange gel. The trimer thus formed resisted deaggregation when it was exposed subsequently to the lesser charged media, Sephadex G-25 and the chromatographic gel. This finding indicates that simple electrostatic interaction was not the major stabilizing factor; short-range interactions such as hydrophobic bonding may play a more important role.

Conjunctival Mucin Trimer: Postulated Mechanisms of Its Formation and Stability—Self-association has been observed in over 300 proteins (39, 40) and is particularly prevalent in proteins with high molecular weights. According to Reithel (39), proteins with molecular weights exceeding 6.6×10^4 should be considered as aggregates. Consequently, it is reasonable to consider the conjunctival mucin with a molecular weight of 1.7×10^5 as an aggregate of a monomer with a molecular weight of 5.7×10^4, a value that is at the upper monomer molecular weight limit. The same probably is true of the conjunctival mucin with a molecular weight of 1.9×10^6. In proteins, dimers and tetramers are common; multimers with an odd number of monomers are rare. On this basis, the conjunctival mucin trimer may be an oddity.

Several glycoproteins exist in an associated form. They include canine tracheal mucin (41), several submaxillary mucins (41, 42), erythrocyte membrane glycoprotein (43, 44), and sucrase (invertase)–isomaltase, a membrane-bound glycoprotein complex located on the brush border of the small intestine (45–48). The majority of these glycoproteins resist deaggregation by urea, guanidine hydrochloride, and sodium dodecylsulfate, agents known to disrupt hydrogen and hydrophobic bonds. As indicated previously, the conjunctival mucin trimer appears to be stable to deaggregation in a medium of low ionic strength.

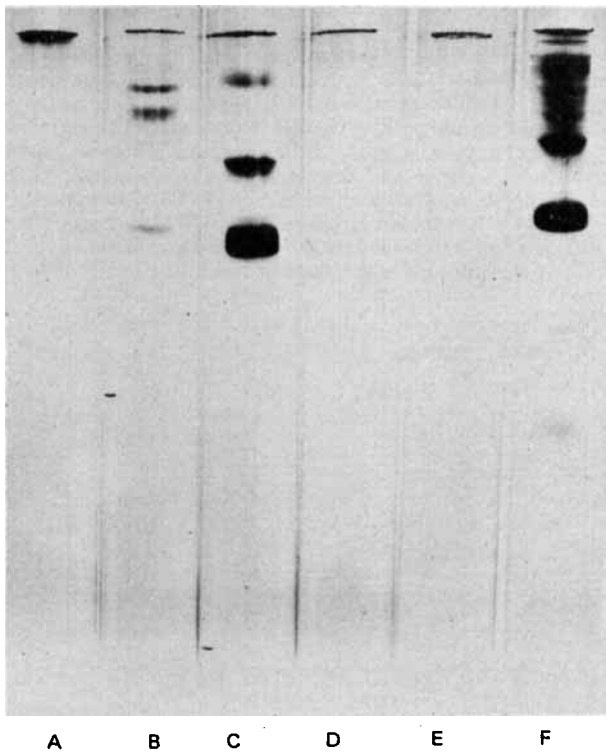


Figure 8—*Polyacrylamide gel electrophoresis on fractions obtained by chromatographing conjunctival crude extract on a chromatographic gel column (1.5 × 60 cm) using 12% polyacrylamide gels. Key: Gel A, peak A; Gel B, peak B; Gel C, peak C; Gel D, peak D; Gel E, peak E; and Gel F, conjunctival crude extract.*

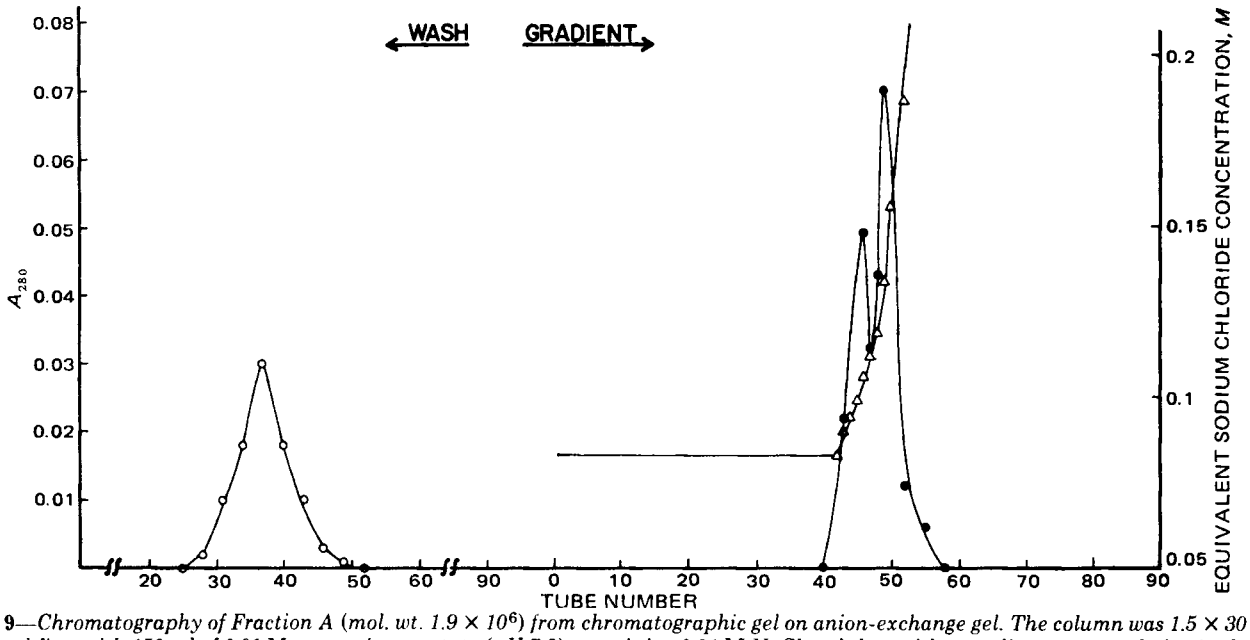


Figure 9—*Chromatography of Fraction A (mol. wt. 1.9 × 10^6) from chromatographic gel on anion-exchange gel. The column was 1.5 × 30 cm. It was eluted first with 150 ml of 0.01 M ammonium acetate (pH 7.2) containing 0.01 M NaCl and then with a gradient composed of 200 ml each of 0.1 and 1 M NaCl in the buffer. The flow rate was 62.4 ml/hr, and the fraction volumes were 1.6 ml.*

The mechanisms governing conjunctival mucin self-association, trimer formation in particular, have not been studied. However, two mechanisms can be postulated: the bulk mechanism and the surface mechanism. In the bulk mechanism, a monomer–trimer equilibrium already exists in the bulk. An ion-exchange gel promotes aggregation by preferentially adsorbing the trimers, thus effectively shifting the equilibrium in favor of the trimer.

At least three possibilities can be explored for the surface mechanism. A pH effect cannot be a possibility because the microenvironmental pH is higher than the bulk pH due to repulsion of hydrogen ions from the positively charged gel surface. The higher pH favors ionization of sialic, glutamic, and aspartic acids, and the attendant electrostatic repulsion among the molecules concerned hinders aggregation.

On the other hand, the ion-exchange gel may promote aggregation by providing the proper matrix to immobilize a glycoprotein molecule through electrostatic interaction between negative charges on the glycoprotein molecule and positive charges in the matrix.This interaction conceivably can initiate unfolding of the glycoprotein, thereby exposing its hydrophobic moieties and allowing hydrophobic interaction with another glycoprotein molecule to occur (Scheme III). Alternatively, as a glycoprotein binds to the ion-exchange gel, the Ca^{2+} ions known (49–51) to be associated with its sialic acid are displaced and become available for bridging the unbound sialic acids of two glycoprotein molecules (Scheme IV).

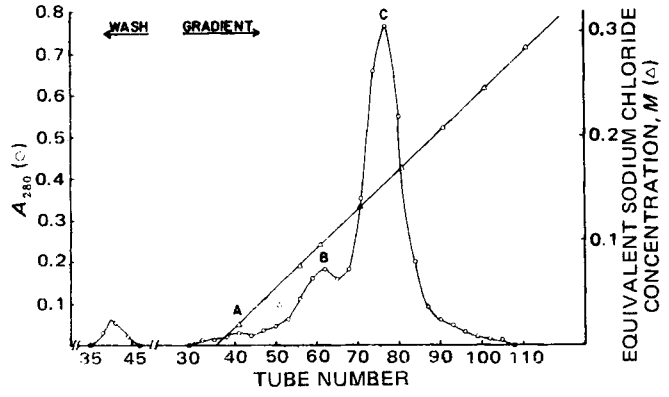
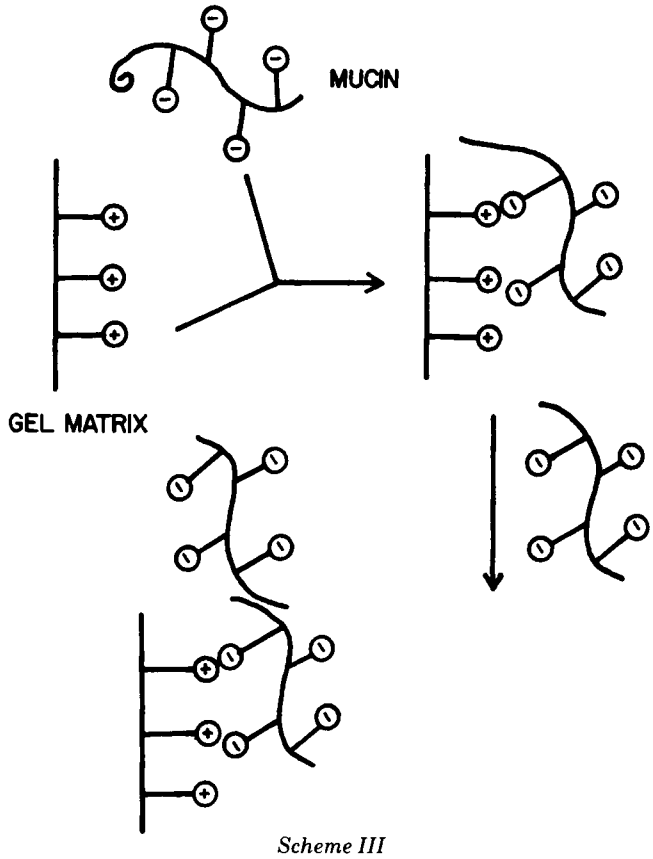


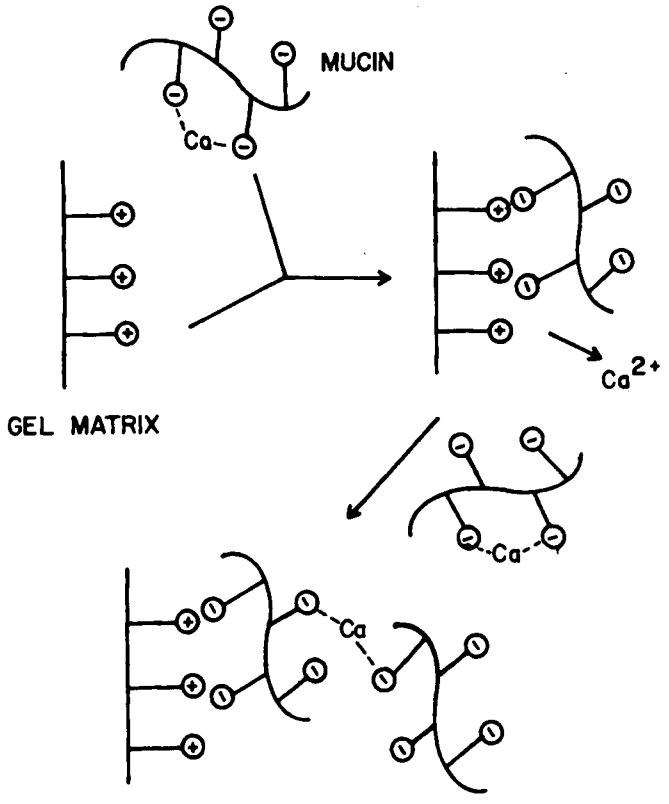
Figure 10—*Chromatography of Fraction C (mol. wt. 5.7 × 10^4) from chromatographic gel on anion-exchange gel. The column was 1.5 × 30 cm and was eluted with a 0.01 M ammonium acetate wash (100 ml) followed by a gradient composed of 200 ml each of 0.1 and 1 M NaCl in 0.01 M ammonium acetate buffer at pH 7.2. Fractions of 1.4 ml were collected. The flow rate was 56.0 ml/hr.*

The third possibility centers on elution of bound glycoprotein from the ion-exchange gel. As the ionic strength of the eluting buffer increases, the thickness of the electrical double layer about a glycoprotein molecule decreases. This effect allows two or more glycoprotein molecules to approach each other, thus favoring aggregation (Scheme V). However, in light of the stability of the aggregate in a medium whose ionic strength is 10 times less, this mechanism appears unlikely.

Like the mechanisms governing its formation, the apparent stability of the resulting aggregate has not been studied. Hill *et al.* (52) studied the aggregation of ovine submaxillary mucins and its desialylated (asialomucin) and deglycosylated (apomucin) variants. They found that the



Scheme III



Scheme IV

apomucin did not aggregate whereas the asialomucin and parent mucin did, indicating that the carbohydrate portion of the molecule, with many hydrogen bonding sites, was necessary for aggregation.

Physiological Significance of Multiple Conjunctival Mucins in Tear Film Stability—A mucin layer adhering to the corneal surface has been considered an integral tear film component (53) since the tear film structure was proposed by Wolff (54) in 1946. The corneal epithelium is an intrinsically hydrophobic, low-energy surface, and a mucin layer at its surface generally is accepted as necessary to achieve complete wetting by tears. In fact, Mishima (55), as early as 1965, demonstrated that wiping the corneal surface rendered it unwettable by tears. This finding was confirmed subsequently by the *in vitro* experiments of Lemp *et al.* (8). Since then, the surface activity that mucin is expected to exhibit at the tear–corneal epithelium interface and, to a lesser extent, at the tear–lipid interface has captured the attention of researchers. To date, the mystery surrounding the driving force for tear film stability remains far from solved.

The principal reasons underlying this dilemma are the lack of information about the chemical nature of conjunctival mucins and the precise mechanisms by which they exercise their roles. Understanding the mechanisms by which each conjunctival mucin exercises its roles requires knowledge of factors contributing to thin film stability (or rupture).

Several factors contribute to rupture of thin liquid films (56). They include dissolution of the film liquid into the adjoining bulk phase, *e.g.*, evaporation, drainage of interlamellar liquid, gravitational convection, and marginal regeneration at the film borders. Although these factors originally were proposed for films that are at most 0.1 μm thick, they should also hold for a tear film that is 10 times thicker. Of course, some factors such as evaporation and drainage may predispose the tear film to rupture more readily than others.

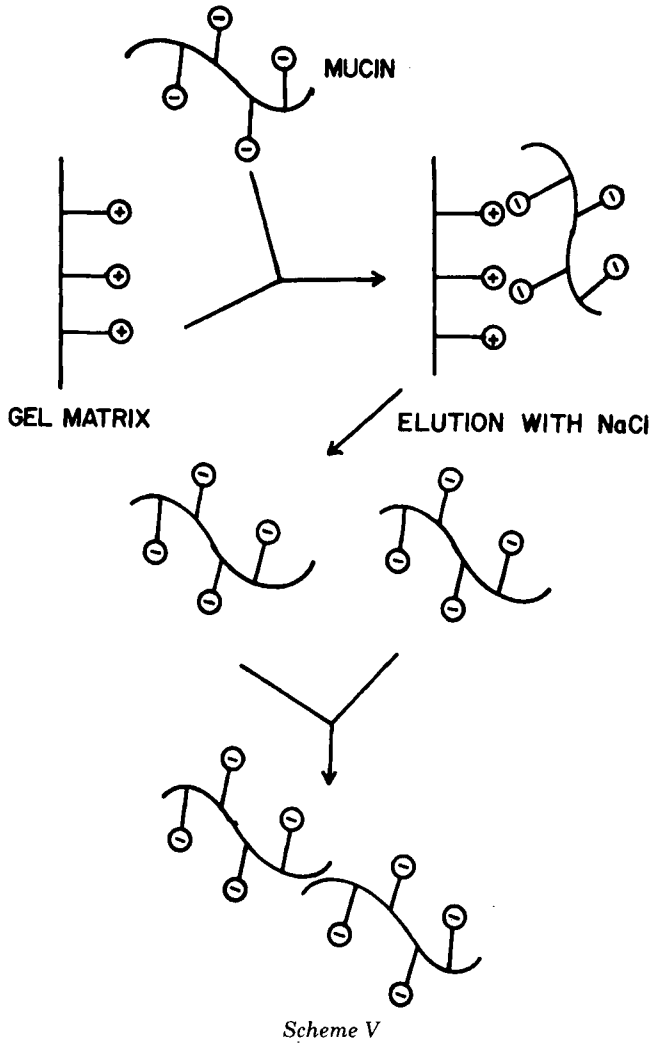
Thus, conjunctival mucin can stabilize the tear film in several ways. For instance, it may interact with the meibomian lipids, resulting in a mixed film that is surface rheologically stable. This film, when coupled with another surface rheologically stable layer at the corneal epithelial surface, can resist drainage. Drainage also can be reduced by increased bulk viscosity due to mucin. On the other hand, the evaporation tendency of the tear may be diminished due to the mixed mucin–lipid film and the diffusional barrier secondary to hydration of mucin in the bulk. Additionally, the shape and net charge of the mucin molecule can affect tear film stability through their influence on the surface activity, hydration, and surface and bulk viscosity of the mucin. It is clear that the surface activity of the mucin is just one of the many elements contributing to tear film stability.

It is useful to speculate about the functions performed by each conjunctival mucin. There are at least three conjunctival mucins with molecular weights of 1.9×10^6, 1.7×10^5, and 5.7×10^4. It is reasonable to expect each conjunctival mucin to possess a characteristic shape, degree of hydration, solubility, and surface activity, although each mucin has not been studied with regard to each property. Thus, the mucin with a molecular weight of 5.7×10^4, being a monomer and like other epithelial mucins, may have clearly delineated hydrophobic and hydrophilic regions and, therefore, may be well suited to adsorb at the superficial meibomian lipid layer as well as at the corneal epithelial surface.

The trimer, with its hydrophobic regions being buried by the prominent oligosaccharide mantle, may be the most soluble mucin despite its molecular weight. This characteristic helps to retain water of hydration, thus establishing a diffusional barrier to tear evaporation and eventually stabilizing the tear film. The trimer may be the least surface-active mucin and may interact minimally with either the air–tear or tear–cornea interface.

As the molecular weight exceeds 1.7×10^5, aqueous solubility diminishes, meaning that the mucin with a molecular weight of 1.9×10^6 prefers the interface to the bulk. Due to its molecular inflexibility compared to the mucin monomer, it is unlikely to be the species forming the monolayer at the corneal surface. However, it may exercise an important role in multilayer formation because less of it is necessary to achieve extensive surface coverage. Multilayer formation by the high molecular weight mucin at the corneal surface is roughly equivalent to viscoelastic film formation at the surface. This result not only allows lubrication of the surface but also hinders drainage of the liquid film.

This discussion implies that tear film stability hinges on an intricate balance among various conjunctival mucins. Therefore, a strategy to control dry-eye syndromes must involve selection of a polymer that behaves similarly to conjunctival mucins, *i.e.*, one that has the ability to self-associate to yield various fractions in the proper proportion and with the proper degree of hydration and affinity for interfaces. Selection of



Scheme V

the proper polymer alone may not be sufficient; it may be necessary to fabricate it into a unit possessing anion-exchange properties.

SUMMARY

Several mucins have been isolated from the albino rabbit conjunctiva. The preponderance of each mucin depends on whether gel filtration or anion-exchange chromatography is the first step in fractionation. As reported previously, the conjunctival mucins bear a closer resemblance to the tear mucins in their molecular weights when anion-exchange chromatography is the first step. The discrepancy centers on the anion-exchange fraction with a molecular weight of 1.7×10^5 and the gel filtration fraction with a molecular weight of 5.7×10^4. It is postulated that self-association (trimer formation) induced by the microenvironment of the ion-exchange gel occurs. It is postulated also that the same sequence of events exists during transit of conjunctival mucin from the goblet cell to the precorneal area of the eye. The resulting scenario is one in which the mucin with a molecular weight of 1.7×10^5 is a major tear mucin component.

The physiological advantage of having multiple rather than single fractions in the precorneal area has yet to be elucidated. Nevertheless, it seems reasonable to expect each mucin to perform a specific role in tear film stability. An evaluation of each conjunctival mucin with regard to its amino acid and carbohydrate composition, hydration, shape, and structure is desirable. Factors conferring stability to the mucin possessing a molecular weight of 1.7×10^5, a trimer of the one with a molecular weight of 5.7×10^4, should be identified. Studies are underway in these laboratories to investigate trimer formation and stability.

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