A PRELIMINARY EXAMINATION OF CONJUNCTIVAL MUCINS IN THE ALBINO RABBIT

A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BY

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Degree to be awarded: December 19_____ May 19_____ August 19 79

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June 25, 1979
Date of Examination

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VINCENT HON-LEUNG LEE

Under the supervision of Professor Joseph R. Robinson

The strategic role assumed by conjunctival mucin in tear film stability has been recognized, but not understood, for many years. Current research focus has been on its surface activity with respect to the tear/corneal epithelium interface. None of these studies as yet has employed conjunctival mucin; bovine submaxillary mucin is employed as a substitute. A comparison of bovine submaxillary mucin with tear mucins isolated by Iwata and Kabasawa about a decade ago reveals that tear mucins possess a unique composition not shared by typical epithelial mucins. The implication of this observation is obvious. So is the importance of successfully isolating conjunctival mucin in reasonable quantity and purity, and of achieving a thorough understanding of the mechanisms by which conjunctival mucins 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 two basic techniques employed in this study were chromatography -- gel filtration and anion exchange -- and

to resist dissociation upon exposure to a medium of lesser ionic strength. Several explanations are offered for its formation as well as stability.

APPROVED: Joseph R. Robinson
Joseph R. Robinson, Ph.D.

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(Pharmacy)

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Professor Joseph R. Robinson for leading me on an exciting journey into the world of scientific research. His enthusiasm about and creative insight into the physicochemical aspects of ophthalmology have been my constant source of intellectual stimulation. In addition to the unique research philosophy and experience he has afforded me, his interest in me as an individual, his friendship and our many discussions in matters other than science have been invaluable components of my graduate education and will forever be remembered. I thoroughly enjoyed every minute I spent in his laboratory.

I would like to thank my colleagues, Mr. Joseph M. Conrad and Mr. James W. Sieg, for their advice, help and friendship. I am grateful to the following individuals who perhaps unknowingly have contributed to the progress of this work: Mr. John Watkins, Mr. Manfred Brauer, Miss Joan Park, Mr. Ho-Wah Hui, Miss Janice Moloney, and the research group of Professor Dexter B. Northrop. A special note of thanks is due to my friend and colleague, Miss Janice Whaley, for her assistance in photography and related matters.

Most of all, I am indebted to my close friends, Mr. Hazen Stephans and the late Mrs. Grace Stephans, for their concerns and encouragement; to my two brothers who are

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ABBREVIATIONS

BSM = Bovine submaxillary mucin

DEAE = Diethylaminoethyl

KCS = Keratoconjunctivitis sicca

M.W. = Molecular weight

NH_4Ac = Ammonium acetate

OD_{280} = Optical density (absorbance) at 280 nm

PAGE = Polyacrylamide gel electrophoresis

TBA = Thiobarbituric acid assay

I. INTRODUCTION

Conjunctival mucin is a tear component believed to be vital to tear film stability. Alteration in either its chemical nature or quantity (probably both) has been implicated in a variety of dry eye syndromes. It has been proposed that this fascinating substance derives its strategic role in tear film stability 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 adsorption at these two interfaces. But these aspects have never been studied, principally because conjunctival mucin has not been isolated or identified. The intent of this work, therefore, is to identify conjunctival mucins and related proteins in the goblet cells of the albino rabbit's conjunctiva. Clearly, the successful isolation and purification of conjunctival mucins is crucial to future studies designed to understand the role(s) this molecule assumes in the precorneal area of the eye.

As information on conjunctival mucin is practically non-existent, much of the background material on mucin's biochemistry and surface chemistry presented here will draw on studies conducted with mucins found elsewhere in the body. Also included as background material for this work will be a short account on structure of the precorneal tear

in the cervical canal, depending on mucin's consistency, it either facilitates or impedes sperm transport.

1. Chemical Nature of Mucin

Mucin is a glycoprotein which is a subdivision of glycoconjugates. Glycoconjugates are carbohydrates covalently linked to proteins, lipids, or other non-carbohydrate substances excluding nucleic acid (4). Two types of carbohydrate-protein conjugates exist in mammals. They are glycoproteins and proteoglycans. The major differences between them are listed in Table I. Glycoproteins are comprised of two main types: mucous glycoproteins (mucins) and plasma globulins. The main differences between them are shown in Table II.

Mucins are a diverse group of biopolymers with molecular weights typically in the order of one million. As examples, the molecular weight of ovine submaxillary mucin is 1×10^6 (6) and that of bovine submaxillary mucin (BSM) and bovine cervical mucin is 4×10^6 (7,8). Bettelheim et al. (8) have reported molecular weight dispersity in BSM, Burton et al. (9) in progesterone-binding globulin, and Gibbons et al. (10) in intestinal glycoproteins. They did not elaborate on the cause underlying the polydispersity, but Schachter (11) felt that the existence of alternate biosynthetic pathways was one explanation.

TABLE II. Main differences between mucous glycoproteins (mucins) and plasma glycoproteins. (Adapted from Ref. 5.)

	Mucous Glycoproteins	Plasma Glycoproteins
Amino acid content	High levels of serine, threonine and proline; low levels of aromatic and sulfur-containing amino acids	Typical protein
Carbohydrate content	>50%	<25%
Monosaccharides:		
Fucose, galactose, N-acetylglucosamine, sialic acid	Present	Absent
Mannose	Low levels or absent	Present
N-Acetylgalactosamine	Present	Low levels or absent
Linkage	O-Glycosidic (N-acetylgalactosamine to serine/threonine)	N-Glycosidic (N-acetylglucosamine to asparagine)

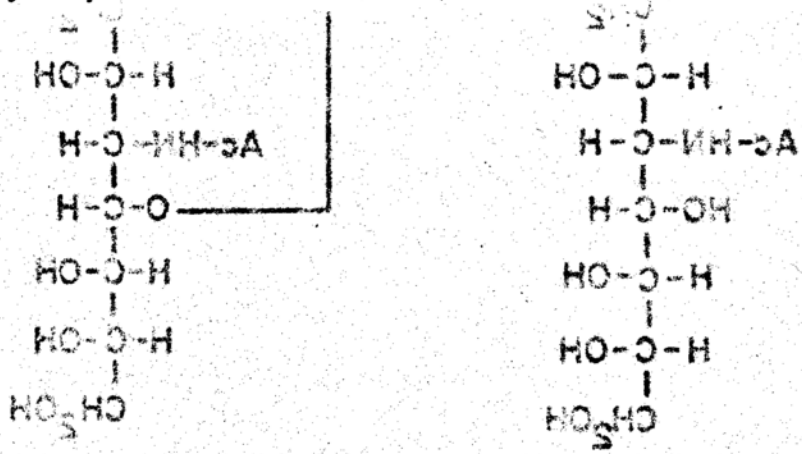
TABLE III. Proportion of protein, carbohydrate, and sialic acid in various mucins.

Mucin	Protein (g/100 g)	Carbohydrate (g/100 g)	Protein / Carbohydrate	Sialic acid (g/100 g)	Reference
Bovine cervical mucin	24.5	77.0	0.3	---	14
Bovine sublingual mucin	23.3	77.0	0.3	20.9	13
Bovine submaxillary mucin	35.8	60.8	0.6	30.8	13
Canine submaxillary mucin	36.4	62.0	0.6	9.2	13
Human intestinal mucin	83.0	15.6	5.3	---	15
Ovine submaxillary mucin	58.0	42.0	1.4	25.0	16
Rabbit tear mucins	70.1	10.9	6.4	---	17
Rat intestinal goblet cell mucin	12.0	88.0	0.14	10	18

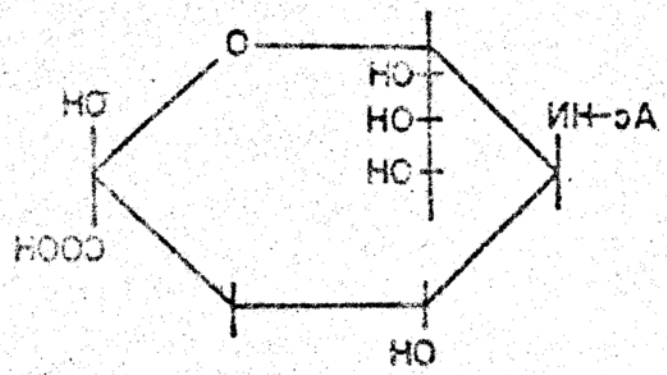
--- Data unavailable

PROTEIN CORE
OLIGOSACCHARIDE SIDE CHAINS
(Gal NAc, Glc NAc, Gal, sialic acids)

FIGURE 2. The keto form (I) and the pyranose ring forms (II, III, IV) of sialic acid. (From Ref. 32.)



II I



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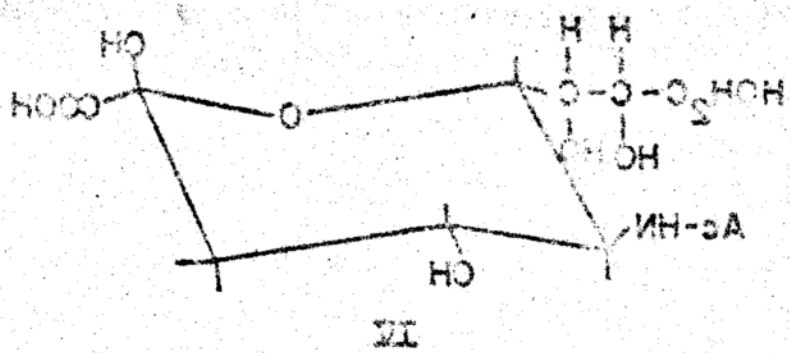


IV

a corresponding change in these properties. Indeed, Gottschalk and McKenzie (30) showed that when sialic acids are enzymatically removed or when the extent of ionization of sialic acid is suppressed, the viscosity of submaxillary mucin is markedly reduced. Similarly, Litt et al. (31) observed a small increase in the storage modulus of canine tracheal mucus under such conditions. On the other hand, Meyer et al. (33) detected no change in the shear modulus of bovine cervical mucin deprived of sialic acid. It is evident that more work on the role of sialic acid in mucus[1] function is necessary.

2. Biosynthesis of Mucin

The biosynthesis of mucin is a rather complicated process that is imperfectly understood at this time. Only highlights of the process will be presented here. For a detailed account the reader is referred to a recent review by Schachter et al. (34).

The principal mucin-secreting cells are goblet cells. The biosynthetic process begins with the synthesis of activated peptides on ribosomes found in the rough endoplasmic reticulum (35). The next step is a controversial one (36) and it concerns whether the serine (threonine)-N-

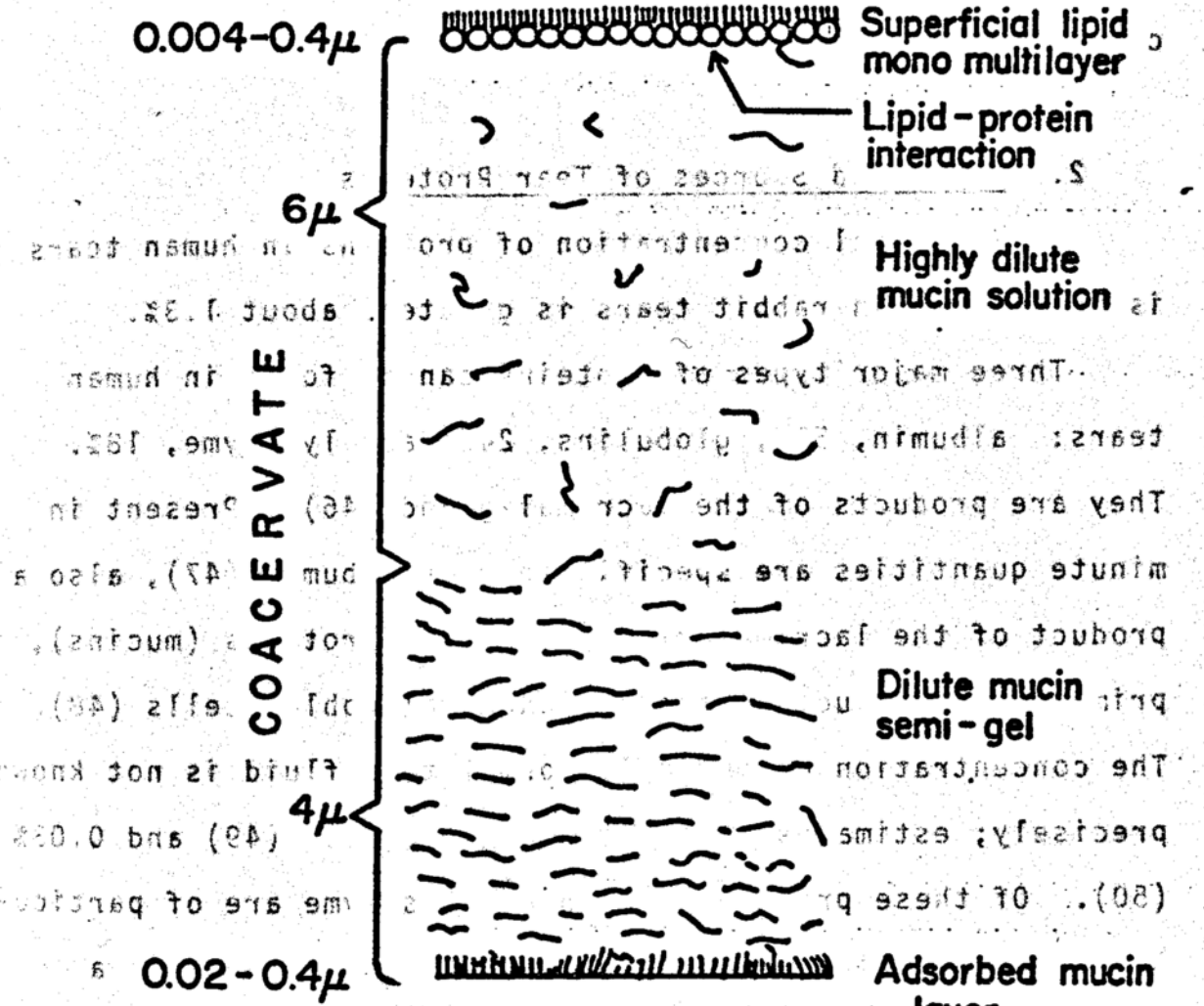
[1] Throughout this report the terms mucin, mucus, and glycoproteins will be used interchangeably.

precorneal fluid that covers the surface of the cornea; it is approximately 10 μm thick. In 1946 Wolff (37) proposed that the precorneal tear film consisted of three layers, as shown in Figure 3.

The superficial oily layer is extremely thin. It is composed of meibomian secretions including cholesterol esters and trace quantities of phospholipids such as lecithin and phosphatidylethanolamine (39,40). It serves to reduce the rate of evaporation of water from the tear film (41); removal of this oily layer increases evaporation rate by approximately four times (42). It also reduces the surface tension of tears so that the tear film spreads more evenly over the corneal surface.

The middle layer, or tear fluid, makes up the bulk of the tear film. It is a mixture of secretions from the lacrimal gland as well as the numerous glands of the conjunctiva including the accessory glands of Krause and Wolfring (see Figure 4). According to Ridley and Sorsby (43), tear contains 98.2% water and 1.8% solids, with proteins being the major constituent of the solids. Since proteins, like other asymmetric macromolecules, tend to separate spontaneously into two phases -- a more concentrated coacervate and a highly dilute one (44), the middle layer may contain two phases.

The innermost layer is the mucoid layer, which is as


0.004-0.4μ
Superficial lipid mono multilayer
Lipid-protein interaction
6μ
Highly dilute mucin solution
COACERVATE
Dilute mucin semi-gel
4μ
0.02-0.4μ
Adsorbed mucin layer
EPITHELIAL CELLS

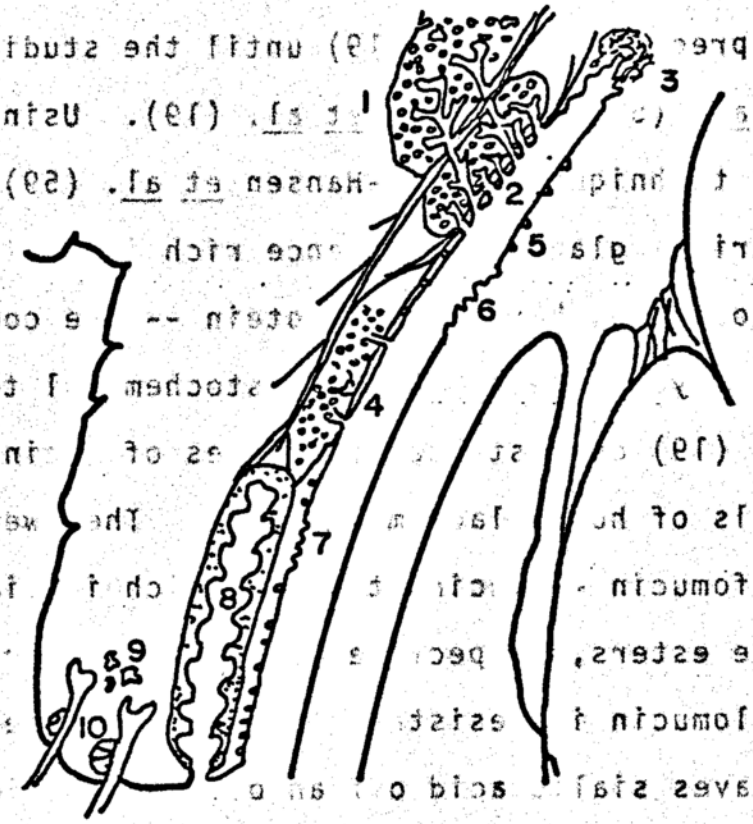
Tear lysozyme levels are reduced by as much as four times in KCS (52-54). As lysozyme is produced only by the lacrimal gland (55,56), the tear lysozyme level has been proposed as a test for lacrimal gland secretion. However, a recent finding (54) of no correlation between tear secretion rate and lysozyme titer in tear fluid casts doubts on the reliability of this test as a diagnostic aid. The significance of reduced lysozyme level in reduced tear film stability in dry eye patients is not known. Suffice it to say that rabbits with tear lysozyme levels constituting only 1% of total tear proteins (57) appear to have stable tear films.

3. Types of Mucins in the Precorneal Area

Mucins found in the precorneal area are derived from four sources (see Figure 4):

a. the conjunctival goblet cells that open independently into the surface,
b. the crypts of Henle lined with epithelium-containing goblet cells,
c. the glands of Manz, and
d. the lacrimal glands.

The relative contribution of mucins by each source in the precorneal area is unknown, although conjunctival goblet cells are generally thought of as the major source of mucin.



MAIN LACRIMAL GLAND

1 orbital lobe

2 palpebral lobe

ACCESSORY LACRIMAL GLANDS

3 glands of Krause

4 glands of Wolfring

MUCIN SECRETORS

5 goblet cells

6 glands of Manz

7 crypts of Henle

OIL SECRETORS

8 meibomian gland

9 glands of Moll

10 glands of Zeis

(62) using a simple biopsy technique. A preponderance of goblet cells is noted in regions of the conjunctival sac where pressure on the corneal epithelium is the slightest. Thus, as shown in Figure 5, the density of goblet cells is highest in the nasal palpebral conjunctiva; decreasing densities are found in the temporal palpebral conjunctiva, bulbar conjunctiva near the fornices, and bulbar conjunctiva near the corneoscleral limbus. Based on this observation, Kessing (63) speculated that a reciprocal relation existed between the degree of hydration and the density of goblet cells. This implies that mucin serves to maintain proper hydration in regions prone to desiccation.

Conjunctival goblet cells are quite similar in morphology to their counterparts elsewhere in the body (64). Among the similarities is the presence of microvilli on their surfaces (61,65,66). Their turnover rate is, however, unknown. Mice intestinal goblet cells have a turnover rate of two to three days (67). Pfister (61) postulated that conjunctival goblet cells had an indefinite life span, but this is possible only if goblet cells can anchor to the rejuvenating layers of the neighboring epithelial cells. It is interesting to note, nevertheless, that the density of goblet cells reaches a peak early in childhood (60), subsequently decreases and remains constant throughout adult life. It is reduced in dry eye syndromes, however (68,69).

Four histochemically distinct mucins -- three acidic

and one neutral[2] -- have been demonstrated in human conjunctival goblet cells. The acidic mucins are sulfomucin and sialidase (neuraminidase) resistant and sialidase sensitive sialomucins (70). An attempt was made to identify these mucins histochemically. Nevertheless, the pattern differs from that s ds in the presence of the additional idase sensitive mucin. The same fo en ed in bronchial mucins (71). In us ns only neutral mucin (ifated mucins (72).

Three = 1.4 x 10^4, 5 x 10^4 and 4 x ata and Kabasawa (17) from tear mu the conjunctival surface of rabbit ther epithelial mucins, these fractions c ively low percentage of sialic acid and total carbohydrate. The details of Iwata and Kabasawa's study will be presented in the Discussion.

4. Distribution of Conjunctival Mucin in the Pre

Area

The above description is, in essence, the current state of knowledge on conjunctival (tear) mucin. Little

[2] This mucin does not stain Alcian blue at pH 2.5.

known about the basal activity of the goblet cells and that in response to external stimuli. Nevertheless, Ehlers et al. (73) calculated a mucus flow rate of 2-3 µl every 24 hours. Similarly there are no definitive studies on the fate of mucin once it is discharged from the goblet cell or type B cell of the lacrimal gland.

Scheme I is a tentative scheme for the disposition of conjunctival mucin in the precorneal area. For simplicity consider a single mucin fraction from conjunctival goblet cells. One can imagine that a mucin molecule, upon secretion from the goblet cells, will find several destinations in the precorneal area, as shown in Scheme I. Thus, conjunctival mucin molecules can first be adsorbed at the conjunctival surface. From there a fraction of them enter the tear fluid, within which they distribute themselves among the three major layers of the tear film. Some mucin molecules may enter the tear film directly, without first being adsorbed onto the conjunctiva. Other mucin molecules may be adsorbed directly at the corneal surface, again, bypassing the M(conjunctiva) ⇄ M(tears) route. Indeed, prevalent thinking is that conjunctival mucin is rubbed onto the corneal surface through the shearing action of blinking, but this is possibly only if conjunctival mucin is first adsorbed onto the conjunctival surface.

A layer of mucus-like material has been shown to cover

the surfaces of both human and rabbit conjunctiva (61,74, 75). Examination of normal human conjunctival mucus by Millipore surface biopsy (62) reveals several distinct forms in the mucus layer; namely, clusters of granules, granular sheets, and strands, the latter often arranged as a network connecting adjacent conjunctival goblet cells. This amazing observation casts doubt on the notion of conjunctival mucin existing as a uniform, continuous layer at the conjunctival surface. In contrast to the conjunctiva, mucin at the corneal surface does not exist as clusters of granules and mucus network, principally because goblet cells are absent from the cornea (76). Since mucin is readily removed by touching the corneal or conjunctival surfaces with the smooth surface of a Millipore filter (77) as well as during increased tear secretion (63), the transfer of mucin from the conjunctival to corneal surface through the shearing action of blinking is a reasonable mechanism. As to which form of mucin is transferred has yet to be disclosed as it is unlikely to be the entire mucin network.

5. Surface Activity of Mucin

Like its precorneal disposition, the surface chemistry of conjunctival mucin is not known. Statements about it are based on experiments using bovine submaxillary mucin (BSM). They are largely the work of Holly and Lemp and

TABLE IV. Surface activity of macromolecular tear components or analogs at the air/solution and solution/solid interfaces. Each component was prepared in 0.154 M NaCl and measurements made at 25°C. (Adapted from Ref. 78.)

Component	%	M x 10^5	$\gamma^{a,b}$	$\pi_{LV}^{a,c}$	$\pi_{SL}^{a,d}$
Saline	0.9	0.154 M	72.1	--	--
BSM	0.22	0.05	37.4	34.6	30.9
Albumin	0.20	2.94	52.1	20.0	24.5
γ-Globulin	0.10	0.14	53.4	18.7	26.0
α_2-Macroglobulin	0.033	0.04	55.0	17.1	18.2
Lysozyme	0.08	0.56	56.9	15.2	16.5
Artificial tears[e]	--	--	38.8	33.2	31.5

[a] In dyne cm^{-1}.

[b] Surface tension.

[c] π_{LV} is the surface pressure exerted by the solute at the air/solution interface.

[d] π_{SL} is the surface pressure exerted by the solute at the solution/solid interface.

[e] Physiological saline containing 0.22% BSM, 0.2% albumin, 0.1% γ-globulin, 0.033% α_2-macroglobulin, and 0.08% lysozyme.

cause epithelial damage (82).

C. Wettability of the Corneal Epithelium

From the preceding discussion it is apparent that conjunctival mucin will be the protein preferentially adsorbed at the corneal epithelium. The corneal epithelium, with a low critical surface tension[3] value of 28 dyne cm^{-1} (45), is an intrinsically hydrophobic low energy surface and a mucin layer at its surface is thought to be necessary to achieve complete wetting by tears. Mishima (83) as early as 1965 demonstrated that wiping the corneal surface rendered it unwettable by tears. His finding was subsequently confirmed by Lemp et al. (84). Ehlers (85) offered an opposing view that meibomian lipids, not conjunctival mucin, were rubbed selectively onto the epithelial surface, thereby increasing its surface activity. But this was not supported by subsequent experiments (84).

To test the hypothesis that the wettability of the epithelium improved upon adsorption of mucin, Holly and Lemp (45) performed a series of experiments using surfaces of glass, polyethylene and bovine cornea coated with BSM solution. They found that as a result of this treatment, the

[3] Defined as that liquid surface tension above which all liquids show non-zero contact angles on a given surface (86).

corneal epithelium. Adams (77) showed that mucin at the corneal surface was readily removed upon touching its surface with a Millipore filter. In vitro studies (45,90) showed that BSM adsorbed to corneal epithelium did not resist rinsing as evidenced by an increase in contact angle post-rinsing. A recent study by Kiorpes et al. (91) showed that instillation of 10% N-acetylcysteine, a known mucolytic agent (92), in rabbit eyes resulted in a preferential mobilization of carbohydrate-rich protein, presumably mucin. The source of mucin, i.e., cornea or conjunctiva, was not known. In contrast, Pfister (87) reported the persistence of a "fuzzy coat", which he interpreted as mucin, at the microvilli surface of the corneal epithelium even after treatment of excised cornea for 20 minutes in 20% N-acetylcysteine. Similar observations were reported by Kaye and Pappas (93). Under scanning electron microscopy such a "fuzzy coat", also known as glycocalyx, was also observed on cell surfaces other than those of the corneal epithelium (94-106) and was thought to arise from membrane containing granules housed in them (107). Ito (100) argued that although this surface coat responded positively to staining reactions characteristic of glycoproteins, it could not be interpreted as a layer of adherent goblet cell mucus insofar as the cat intestinal microvilli were concerned. His reasoning was that this surface coat was remarkably resistant to washing in

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before the next blink (110-112). There is usually a marked decrease or even absence of conjunctival goblet cells (68,69, 76,113,114), resulting in a deficiency of mucin (115). There is also a reduced tear lysozyme level (54,116,117).

Although mucin deficiency is often implicated, the cause underlying tear film instability in dry eye patients remains obscure. Table VI shows that there is not a perfect correlation between tear hexosamine (a marker for mucin) levels and conjunctival goblet cell densities in the diseases surveyed, and the hexosamine level is reduced by a factor of only one-third to one-half. Nor is there a marked reduction in tear surface tension. These two observations can be rationalized by invoking a compensatory increase in mucin secretion from the lacrimal gland; indeed, Wright and Mackie (70) have noted a shift in the type of mucin from sialo to sulfomucin (primarily a product of the lacrimal gland). Another explanation may be an alteration in the ratio of conjunctival mucin fractions at constant total tear mucin concentration.

The significance of these two observations is that the root of tear film instability resides not in the gross chemical composition of tears, but at some molecular events at the tear/corneal epithelium or air/tear interface. Thus, even if there is a compensatory increase in mucin secreted from the lacrimal gland, this mucin may not be adsorbed to

the extent or at the rate that conjunctival mucin is. It is, of course, possible that conjunctival mucin itself is altered in its characteristics. In fact, KCS is often associated with dry tenacious mucus, which is only slowly rehydrated even when totally immersed in saline (70).

E. Statement of the Problem

The problem of dry eyes has plagued mankind throughout history. There are literally hundreds of scientific papers on the subject, and numerous attempts to duplicate normal tear flow in an effort to control the condition. To date, the problem remains far from solved.

The principal reasons underlying this dilemma are: (1) lack of information (understanding) about the chemical nature of conjunctival mucin, a glycoprotein that has been considered vital to tear film stability, and (2) the precise mechanisms by which it exercises its role(s).

According to Holly and Lemp, conjunctival mucin stabilizes the tear film by virtue of its affinity for the tear/corneal epithelium interface, resulting in the transformation of an inherently low energy, less wettable surface to a higher energy, more wettable one. This mechanism, while reasonable, cannot be the sole mechanism, for substances that wet surfaces readily, e.g., benzalkonium chloride, may in fact reduce tear film stability drastically.

II. EXPERIMENTAL

A. Materials

Male, albino rabbits, weighing between 1.8 and 2.4 kg, were obtained from Klubertanz Rabbitry (Edgerton, Wisconsin) and used throughout the studies. They were fed a regular diet with no restrictions on food or water consumed.

Chromatographic columns were from Ace Glass, Inc. (Vineland, New Jersey). Synthetic N-acetylneuraminic acid and 3,5-diaminobenzoic acid dihydrochloride were from Aldrich Chemical Co. (Milwaukee, Wisconsin). Alcian blue, Bio-Gel A 5m (200-400 wet mesh), Bio-Phore 4, 7.5 and 12% gels, Bio-Phore basic buffer pH 8.9, Bio-Rad protein assay kit II, DEAE Bio-Gel A, and tracking dye solution (acidic) were from Bio-Rad Laboratories (Richmond, California). Sodium arsenite was from J. T. Baker Chemical Co. (Phillipsburg, New Jersey). Periodic acid was from G. Frederick Smith Chemical Co. (Columbus, Ohio). Sodium bisulfite and trichloroacetic acid were from Mallinckrodt Inc. (St. Louis, Missouri). Blue Dextran 2000 and Sephadex G-25 Fine were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Rabbit albumin, α-globulin (fraction IV), and γ-globulin, as well as egg white lysozyme (specific activity 11,800 units/mg) were from Schwarz/Mann, Inc. (Orangeburg, New York). Bovine submaxillary mucin

TABLE VII. λ_{max} and $A_{1\ cm}^{1\%}$ values of selected proteins.

Protein	M.W. x 10^{-5}	pI[a]	λ_{max}	$A_{1\ cm}^{1\%}$[b]
Albumin	0.69	4.9	278	4.8
BSM[c]	40	--[d]	273	5.5
γ-Globulin	1.5	6.6	276	10.1
α-Globulin	2.5	--[d]	277	6.9
Lysozyme	0.14	11	281	19.4

[a]Isoelectric pH.

[b]At 280 nm.

[c]Bovine submaxillary mucin.

[d]Not reported.

FIGURE 6. Beer's law plots for Bio-Rad protein assay of selected proteins.

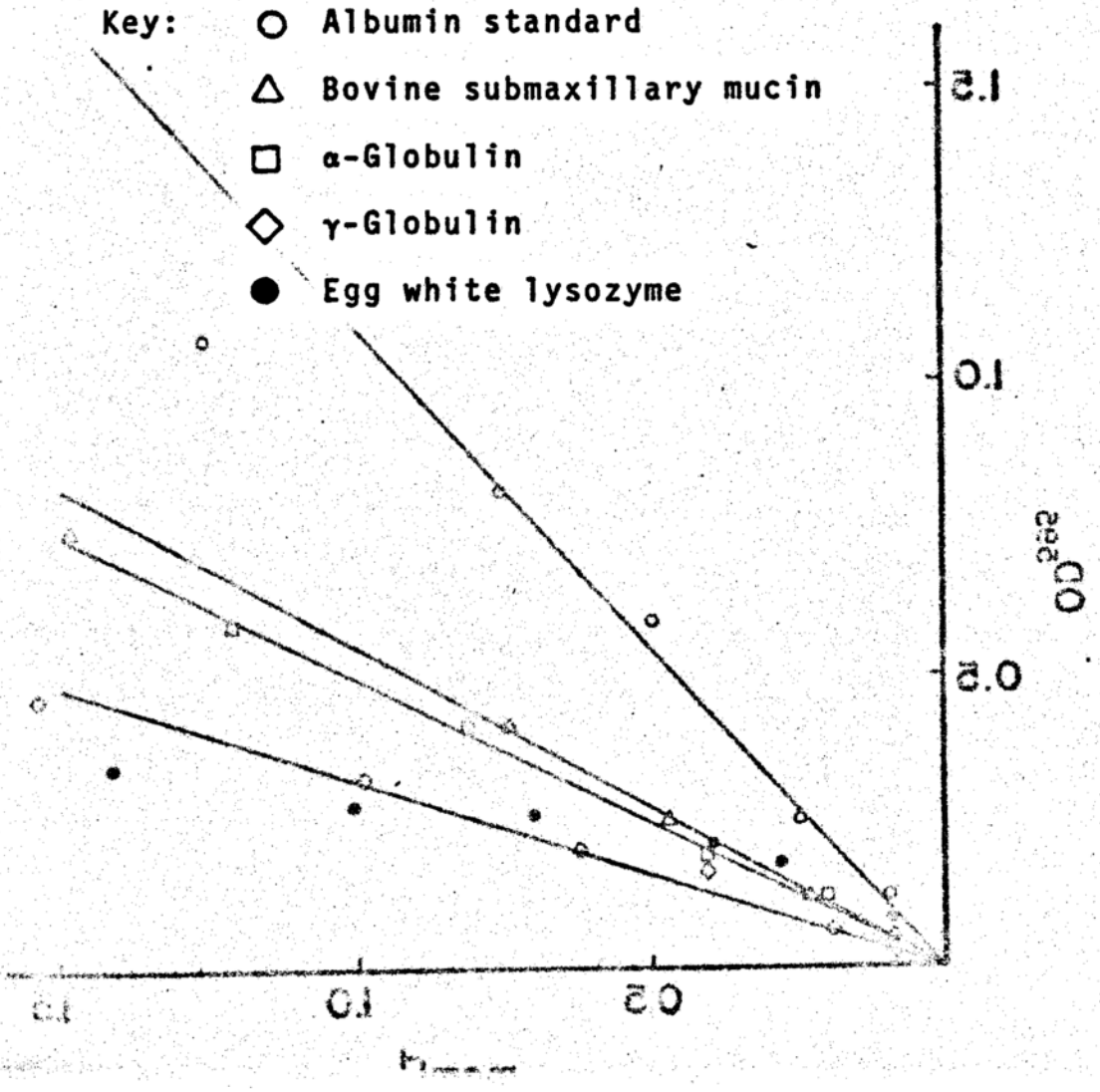


TABLE VIII. Slopes of OD_{595} vs. protein concentration ($mg\ ml^{-1}$) plots shown in Figure 6.

Protein	Slope (mg^{-1} ml)	Coefficient of Determination
Albumin standard	1.10	0.9939
BSM[a]	0.55	0.9992
α-Globulin	0.50	0.9986
γ-Globulin	0.33	0.9986
Lysozyme	---[b]	---[b]

[a]Bovine submaxillary mucin.

[b]Not determined.

Figure 7 displays the Beer's law plot obtained for a series of N-acetylneuraminic acid solutions. The molar absorptivity is 6.2×10^4 M^{-1} cm^{-1} (literature value -- 5.7×10^4 M^{-1} cm^{-1} (121)).

As the TBA assay is intended for quantitating free (unbound) sialic acid, it is necessary to first liberate sialic acids from a glycoprotein (mucin) by hydrolyzing it in 0.1 M H_2SO_4 at 80°C for 40 minutes, a commonly employed procedure. A kinetic study revealed that such treatment led to degradation of the liberated sialic acid. For BSM peak sialic acid level was reached at 1350 seconds. Figure 8 is a semilogarithmic plot of μg of sialic acid versus time. Its terminal slope yields an apparent first order rate constant of 0.01 min^{-1}, which is of the same order of magnitude as that (0.016 min^{-1}) governing sialic acid degradation under similar conditions (see Figure 9). Clearly sialic acid undergoes degradation as it is liberated from BSM, and this explains the low values given by TBA for bound sialic acids in BSM (122). Because of this concern, the thiobarbituric acid assay was abandoned.

A fluorometric assay due to Hess and Rolde (123) that is capable of detecting as little as 0.2 ng of sialic acid (total: free plus bound) will be adopted as the sialic acid assay in this work.

FIGURE 8 Hydrolysis of bovine submaxillary mucin in M H_2SO_4 to liberate sialic acid.

FIGURE 9. Degradation of sialic acid upon heating in 0.1 M H_2SO_4.

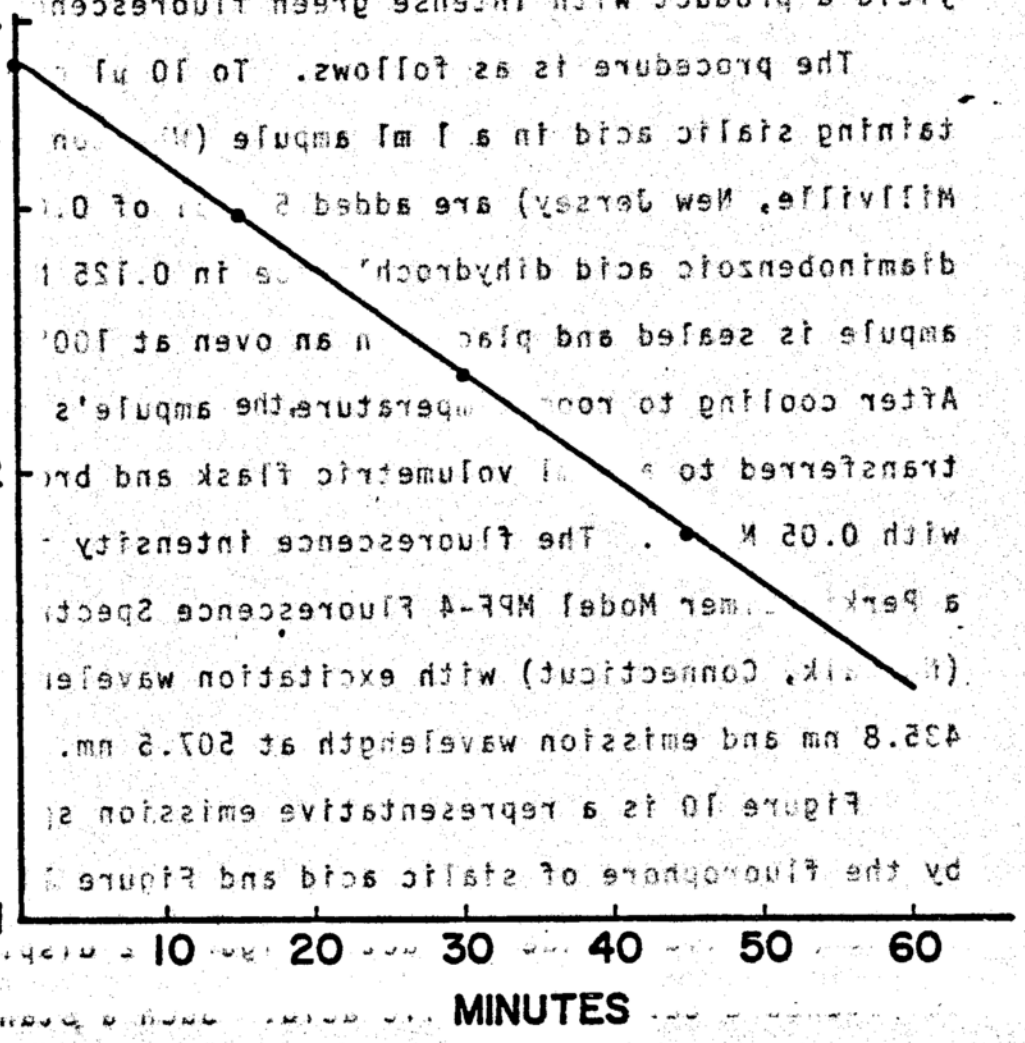

4
3
2
1
µg NANA
10
20
30
40
50
60
MINUTES

FIGURE 10. Emission spectrum of fluorophore of sialic acid.

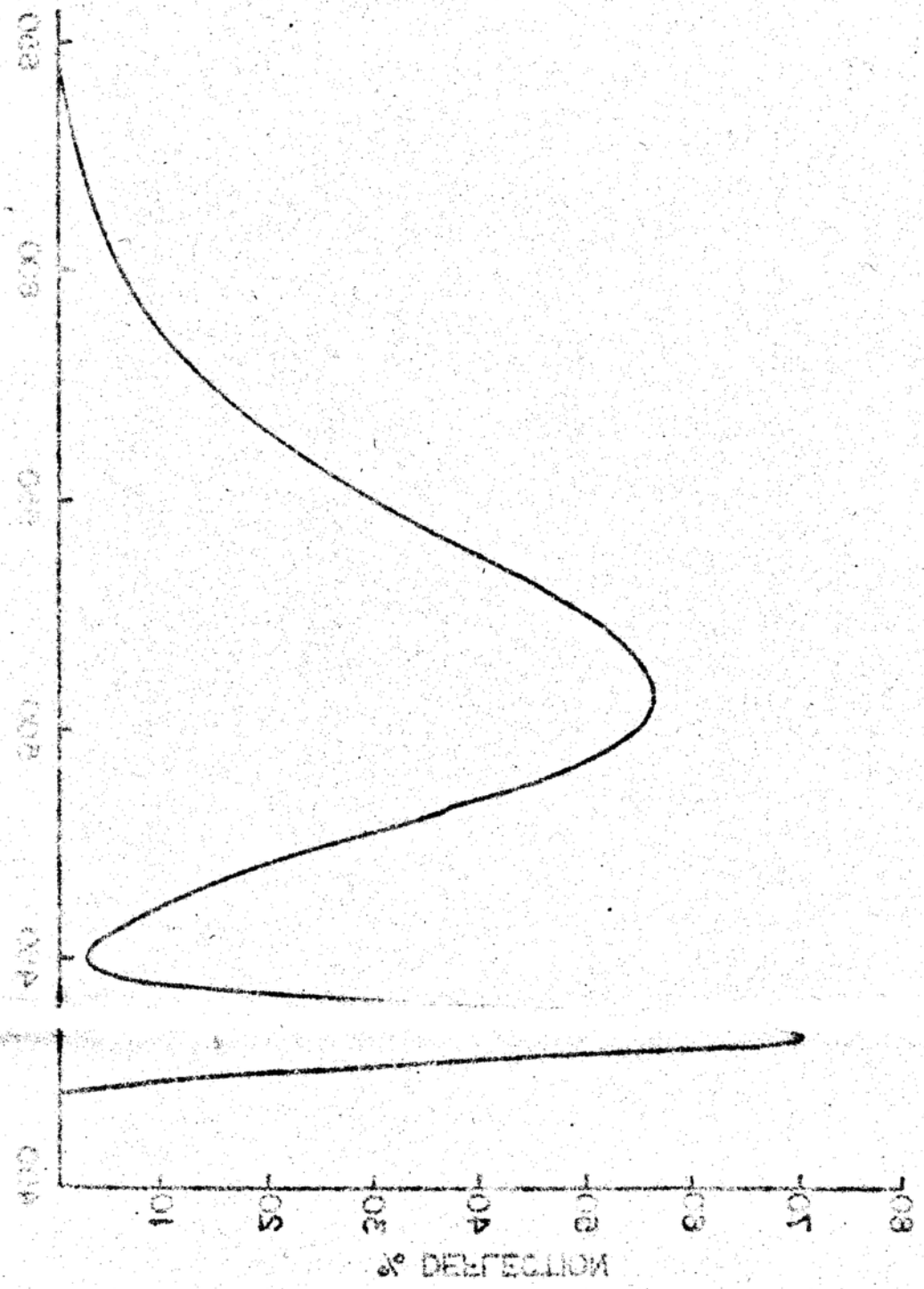


FIGURE 11. Emission spectrum of fluorophore of conjunctival crude extract.

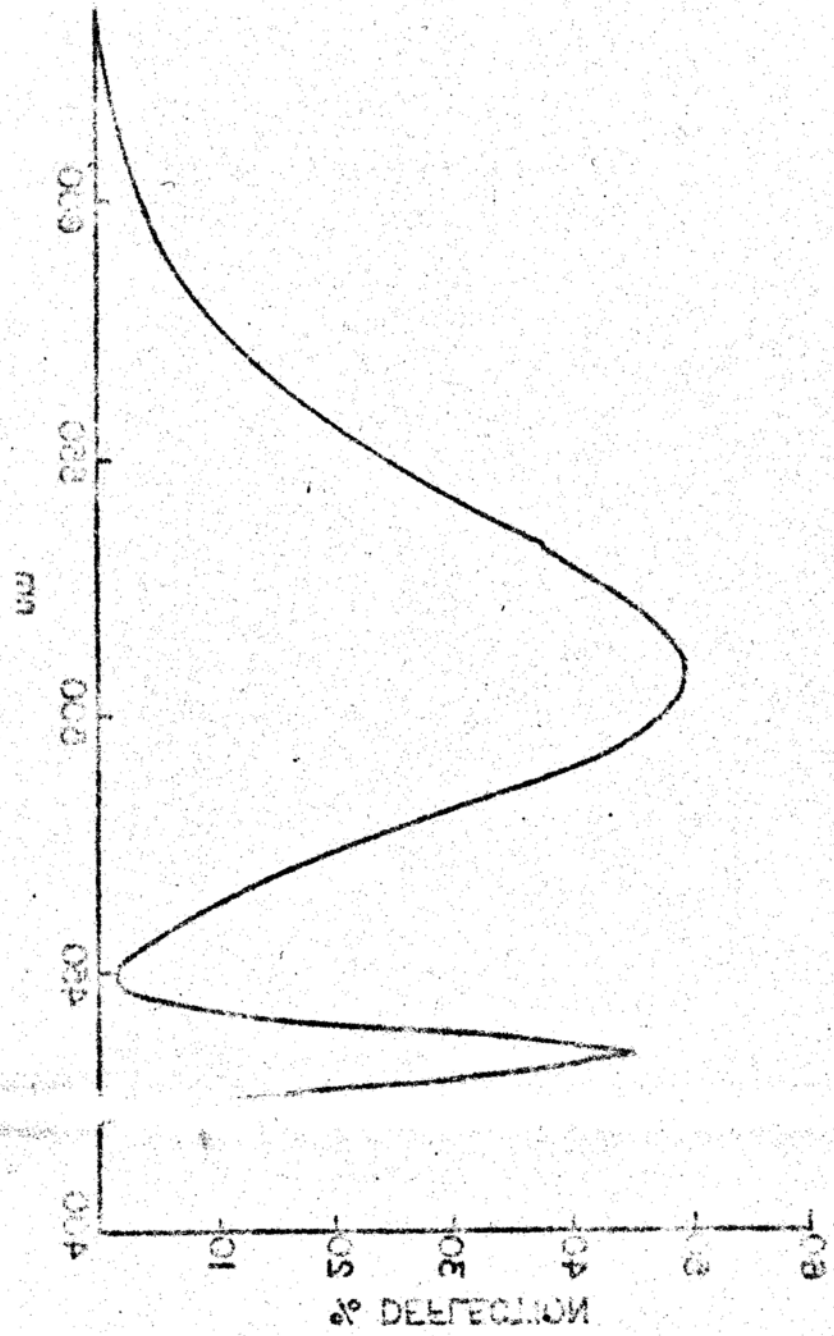
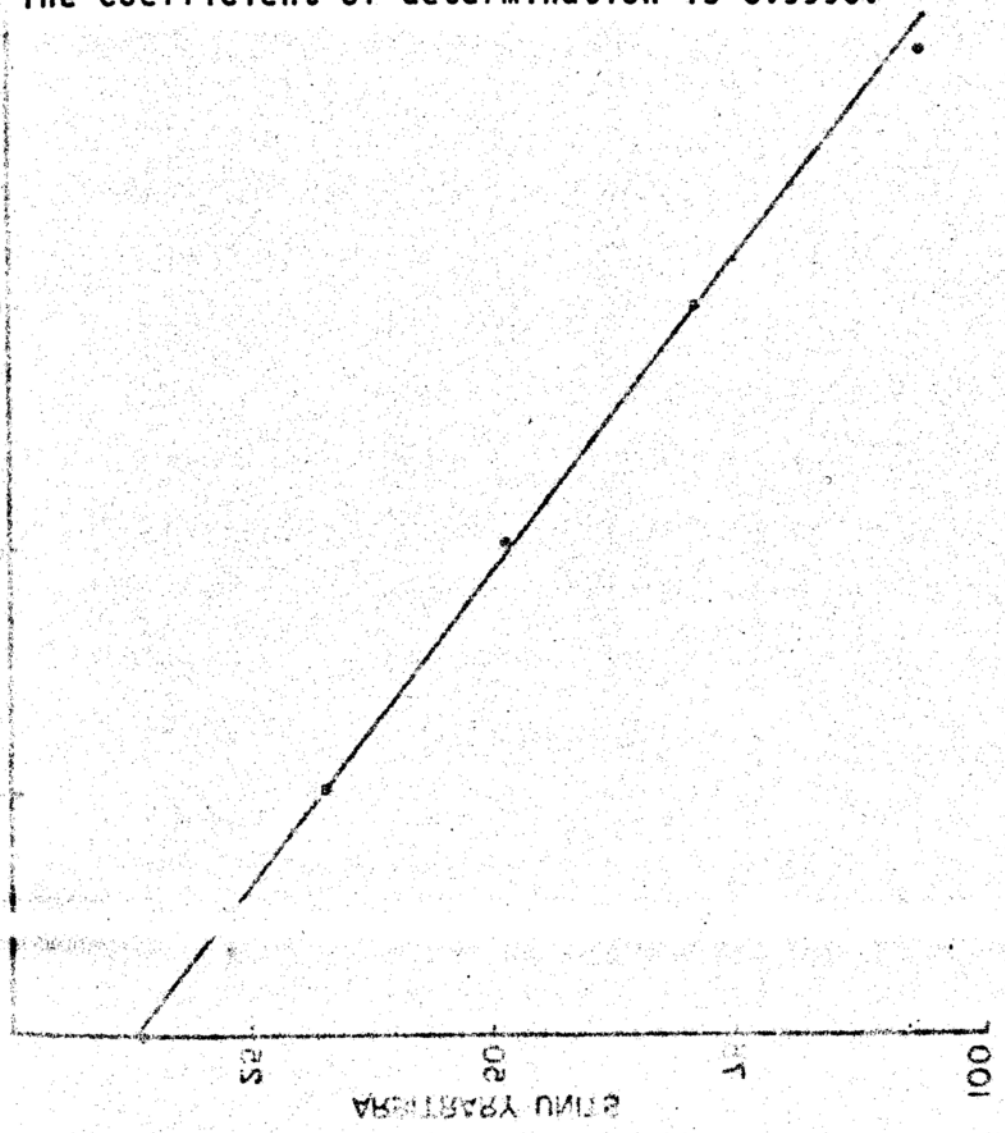


FIGURE 12. Calibration curve for fluorometric assay of sialic acid. The regression equation is:

$$A.U. = 12.7 + 1.99 \times 10^{8}[M]$$

where A.U. = arbitrary unit and [M] = molarity. The coefficient of determination is 0.9990.



interference was probably due to coupling of the amine groups on 3,5-diaminobenzoic acid with the free carboxylic groups in a protein.

2. Alcian Blue Staining for Conjunctival Mucin

The presence of mucin in the conjunctiva was ascertained by a characteristic blue color yielded by mucin in the presence of a 0.05% Alcian blue solution prepared in 3% acetic acid (pH 2.5) and 0.5 M $MgCl_2 \cdot 6H_2O$ (124). One hundred microliters of Alcian blue solution were instilled onto the cornea of several albino rabbits. Ten minutes post-instillation the rabbits were sacrificed by a rapid intravenous injection of sodium pentobarbital into a marginal ear vein. Both the conjunctiva and cornea were removed and examined, under 10x magnification, with a microscope (Spencer, Buffalo, New York). It was found that the cornea was free of a blue color whereas the conjunctiva was decorated with discrete blue spots, presumably corresponding to the location of goblet cells. Thus, mucin was present in the conjunctiva of the albino rabbit.

Scheme II summarizes the major operations performed in fractionating conjunctival mucins.

3. Collection of Rabbit Conjunctiva

Rabbits were sacrificed with a rapid intravenous

injection of sodium pentobarbital into a marginal ear vein. The corneal and conjunctival surfaces were thoroughly washed with 0.9% NaCl solution and blotted dry with tissue. A single incision was then made with a pair of scissors at the upper nasal palpebral conjunctiva. The incision was continued clockwise along the lid margin and terminated at the bulbar conjunctiva around the limbus. Each conjunctiva was carefully rinsed in saline, frozen in a jar surrounded by an acetone-dry ice mixture, and finally stored frozen at -20°C for a period not to exceed three months. Approximately 98 g of conjunctiva were collected from 150 rabbits.

4. Extraction of Conjunctival Mucins

The entire extraction process was conducted in a cold room maintained at 4°C. Approximately 90 g of conjunctivas were suspended in 450 ml of 0.01 M ammonium acetate (NH_4Ac) buffer at pH 7.2 and homogenized in a Waring blender for 10 minutes. The mixture was stirred constantly for 24 hours in the presence of toluene.

The homogenate was filtered through four layers of adsorbent cheesecloth prerinsed in the buffer. The filtrate was centrifuged at 15,000 rpm for 30 minutes at 4°C in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments, Newtown, Connecticut) using a SS-34 rotor. The supernate was first filtered through a Whatman #2 filter

electrophoresis, to be described below.

Disc Polyacrylamide Gel Electrophoresis.

Apparatus. The basic apparatus consisted of two transparent perspex buffer reservoirs which nested one above the other. The base of the upper reservoir was pierced with 10 holes fitted with neoprene grommets forming a seal with the gel tubes. Each reservoir was equipped with an electrode on its side. The electrode fitted to the upper reservoir served as the cathode while that fitted to the lower reservoir served as the anode. The electrodes were connected to a Bio-Rad Model 500 Power supply. Current flowed from upper to lower reservoir (i.e., cathode to anode). Electrophoresis was performed in a cold room at 4°C.

Buffer Introduction. Bio-Phore Basic Buffer, pH 8.9, was selected as the operating buffer. It consisted of 0.188 M each of TRIS and glycine. Prior to sample electrophoresis, it was necessary to electrophoretically introduce the operating buffer into precast polyacrylamide gels (Bio-Phore 4, 7.5, and 12%). This was accomplished by placing the gels in an electrophoresis cell containing the buffer and applying a potential of 40 V DC across the cell. The course of buffer introduction was monitored by applying 5 µl of a tracking dye solution (containing 0.5% bromophenol blue, 30% sucrose, and 0.04 M TRIS-glycine) to each gel.

with an observation with bovine submaxillary mucin (unpublished data).

Step 2 involved staining the proteins precipitated in Step 1. The gel was immersed, for 6 hours, in a Coomassie brilliant blue G-250 solution contained in another 18 x 100 mm test tube. The staining solution was 0.05% in dye, 10% in isopropyl alcohol and 10% in acetic acid.

Step 3 involved removing excess stain. The gel was transferred to a Bio-Rad Model 172A Diffusion Destainer filled with a mixture consisting of 7% acetic acid and 25% ethyl alcohol. The destaining process was continued until the background was clear. This took between 36 to 48 hours.

The procedure just described applies for staining proteins on polyacrylamide gels. The procedure to stain for glycoproteins is essentially the same, except Step 2 (126). After fixing the protein onto the gel, the gel was placed in a 1% periodic acid (in 3% acetic acid) for 60 minutes. Excess periodate was removed by repeated washing with distilled water for 15 minutes. Next the gel was placed in 0.5% potassium metabisulfite solution for 30 minutes. The gel was again washed with distilled water and immersed for 4 hours in 0.5% Alcian blue (in 3% acetic acid). This procedure was found to be unsatisfactory for staining the glycoproteins in the conjunctival crude extract, probably due to the low concentration of sialic acid on the

flow rate with a linear gradient consisting of 200 ml each of 0.1 M NaCl (starting buffer) and 1 M NaCl (limit buffer), both in 0.01 M NH_4Ac at pH 7.2. Figure 13 illustrates a linear gradient mixer. In addition to OD_{280} determination and fluorometric and Bio-Rad protein assays, the contents of appropriate tubes were measured for their NaCl concentration. This was performed in a Bionometer (Model No. 60, Lab-Line Instruments, Inc., Melrose Park, Illinois). The instrument was set at 100 arbitrary units with a 0.1 M KCl solution. A calibration curve was then established for a series of 0.01 M NH_4Ac buffers at pH 7.2 containing varying amounts of NaCl. A typical curve is shown in Figure 14.

The contents of several tubes were pooled, followed by desalting on a column (5 x 60 cm) of Sephadex G-25 Fine. The column was eluted with double-distilled water at a constant flow rate of 150 ml h^{-1}. Fractions of 18.5 ml were collected. The proteins emerged with the void volume (~30% of total bed volume), while the salt with ~80% of total bed volume. Those fractions containing protein were lyophilized at -55°C and 65 μm Hg for 48 hours.

The lyophilizate was reconstituted with 0.01 M NH_4Ac buffer at pH 7.2. The behavior of the proteins contained therein on a gel filtration column of Bio-Gel A 5m was studied next.

LIMIT
BUFFER
STARTING
BUFFER
TO COLUMN
STIRRER

M NaCl
1.0
0.8
0.6
0.4
0.2
ARBITRARY UNITS
700
600
500
400
300
200
100

III. RESULTS

A. Preliminary Experiments with the Conjunctival Crude Extract

1. Prior to lyophilization, protein content of the extract was estimated from its OD_{280} and response in the Bio-Rad protein assay, with albumin as the protein standard. It was found that the OD_{280} gave a protein content 7 times that given by the Bio-Rad protein assay. Granted the behavior of albumin in the two assays may not parallel one another, the above observation indicated that the extract contained non-proteinaceous material that also absorbed, relatively strongly, at 280 nm.

2. Information on the solubility of the conjunctival crude extract in 0.01 M NH_4Ac at pH 7.2 is helpful in setting the upper limit to the amount of crude extract that can be dissolved in the small sample volume (<2 ml) acceptable to the column. Its solubility was found to be 30 mg ml^{-1}. This translated to a protein content of 0.04% in the tear film, assuming that, of course, the crude extract contained purely protein (which was not the case).

The material that did not enter into solution (hereafter referred to as residue) appeared not to be cellular matter, since the OD_{280} increased proportionally with extract weight. (The OD_{280} was taken on a solution prepared

TABLE IX. Sialic acid content of bovine submaxillary mucin (BSM) and conjunctival crude extract (CCE).

Assay	BSM[a]	CCE[a]	BSM/CCE
Fluorometric	12.3	6.0	2.4
Thiobarbituric acid assay, TBA	4.4	1.8	2.1
Fluorometric/TBA	2.8	3.2	0.9

[a]Referred to an albumin standard.

FIGURE 15. Polyacrylamide gel electrophoresis (PAGE) of conjunctival crude extract. Gels A, B and C were polyacrylamide gels -- gel A, 4%; gel B, 7.5%; and gel C, 12%. Gel D was 0.5% agarose gel.

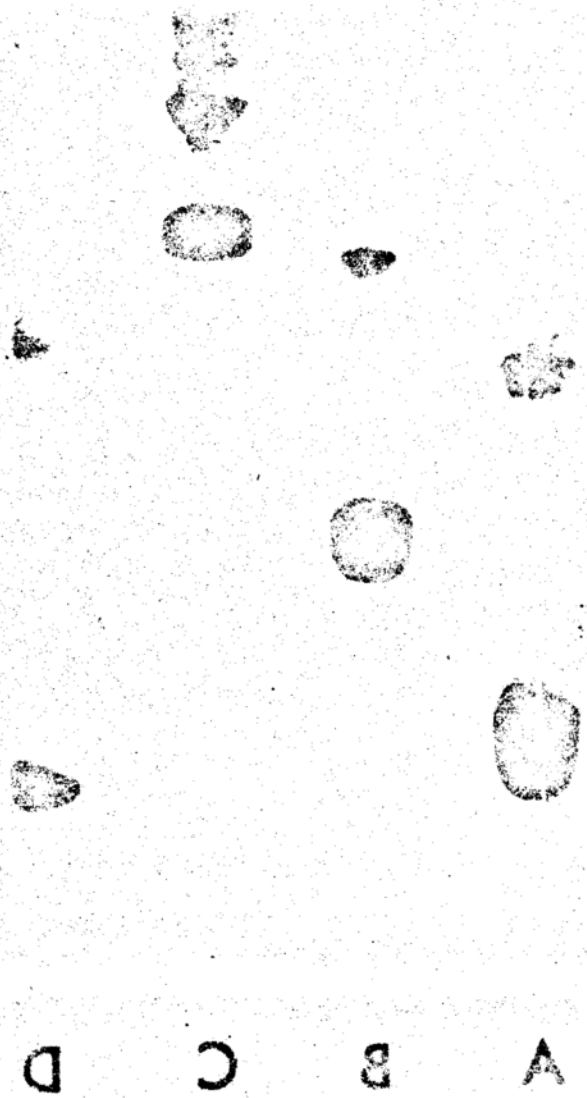


FIGURE 16. PAGE of conjunctival crude extract. 7.5% Bio-Phore gels. Gel A: conjunctival crude extract. Gel B: mixture. See text for details.

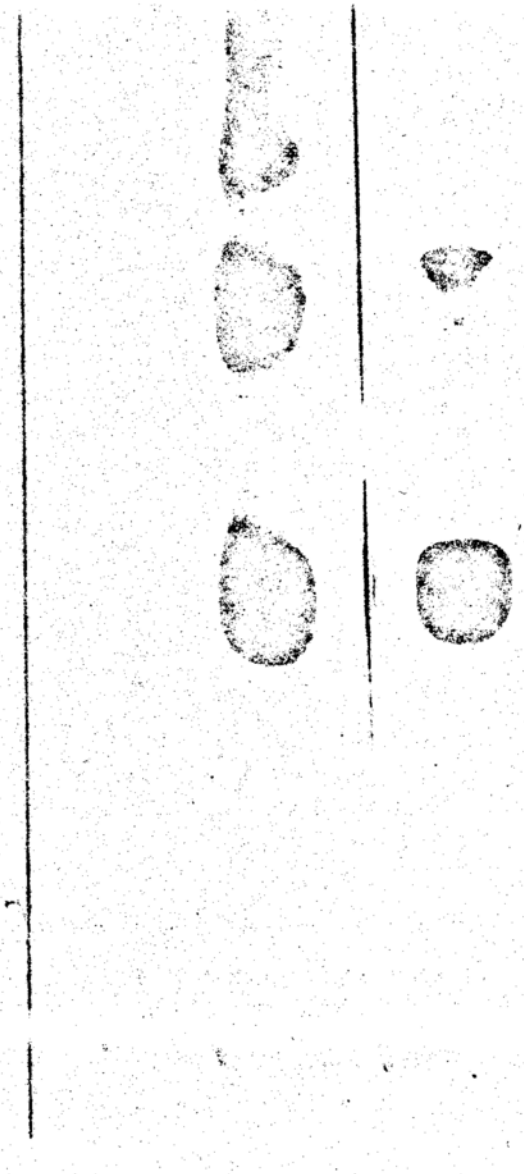
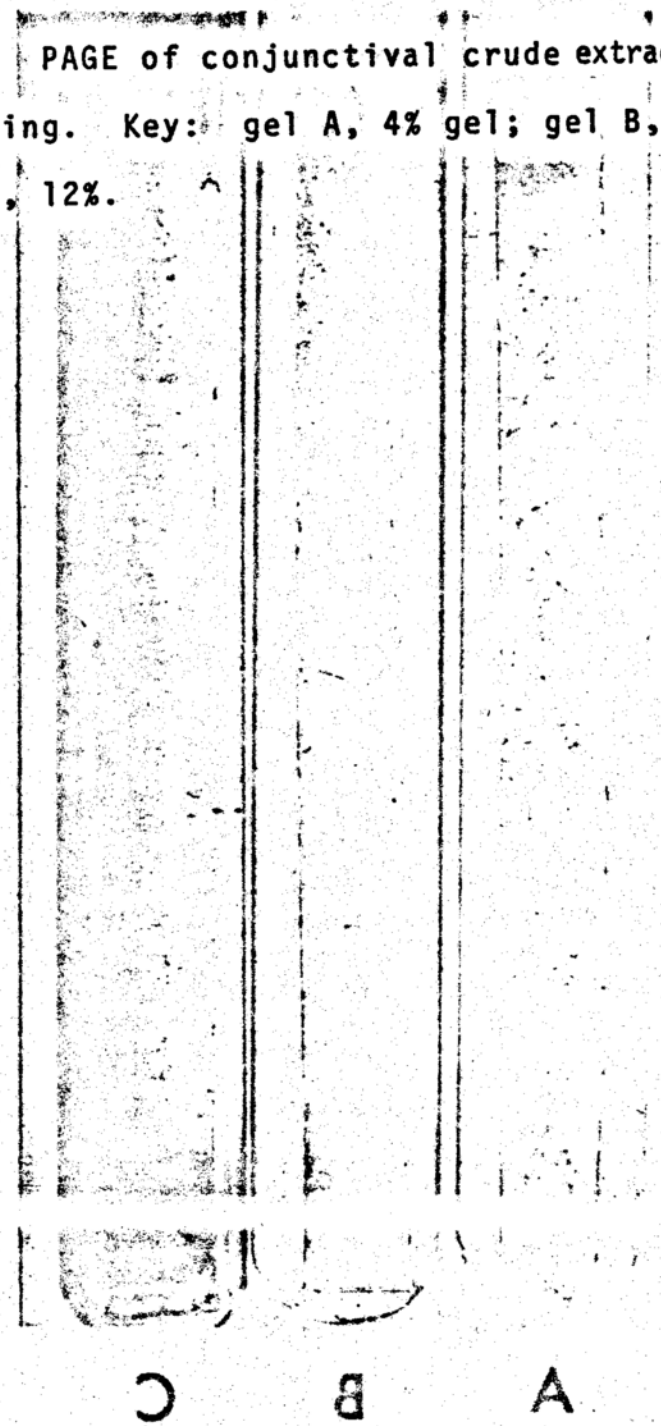


FIGURE 17. PAGE of conjunctival crude extract. Alcian blue staining. Key: gel A, 4% gel; gel B, 7.5% gel; and gel C, 12%.



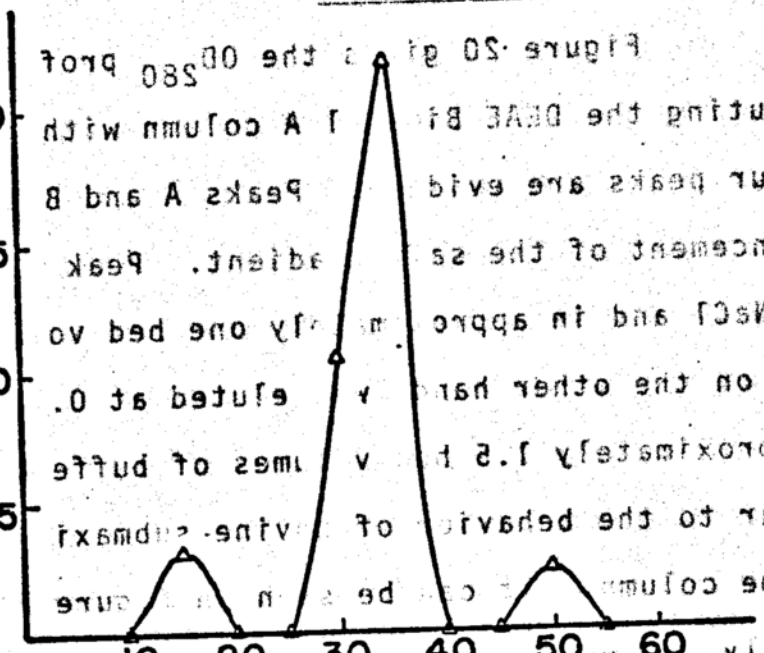
B. Chromatography of Conjunctival Crude Extract on DEAE Bio-Gel A

1. Wash. Figure 18 is an elution profile for washing the column with 0.01 M NH_4Ac buffer at pH 7.2 after layering a sample of conjunctival crude extract onto the gel. Three peaks are evident in the OD_{280} profile. The major peak emerged between tubes 25 and 45. The Bio-Rad protein assay indicated that only peaks A and B contained proteins. These proteins were eluted on the basis of total number of charges, but their molecular weight could influence the elution pattern also. They probably were not highly charged since no salt gradient was necessary to elute them.

Figure 19 shows the sialic acid content of the effluent. Three peaks can be distinguished and they coincided with the peaks seen in Figure 18. The maximum percent of sialic acid was contained in peak B.

The contents of tubes 10-22 were then pooled to yield Fraction (W1), while those of tubes 23-45 were pooled to yield Fraction (W2). Each fraction was desalted on a Sephadex G-25 Fine column (5 x 60 cm) at a flow rate of 150 ml h^{-1}, followed by lyophilization at -55°C and 65 μm Hg. The behavior of the proteins contained in the lyophilizate in a Bio-Gel A 5m column was next studied, to be presented in section III.B.3.

0.20
0.15
0.10
0.05
mgml⁻¹ Protein
10
20
30
40
50
60
70
TUBE NUMBER
A
B
C
1
2
0.5
0.4
0.3
0.2
0.1
OD280


0.20
0.15
0.10
0.05
µg NANA
10
20
30
40
50
60
TUBE NUMBER

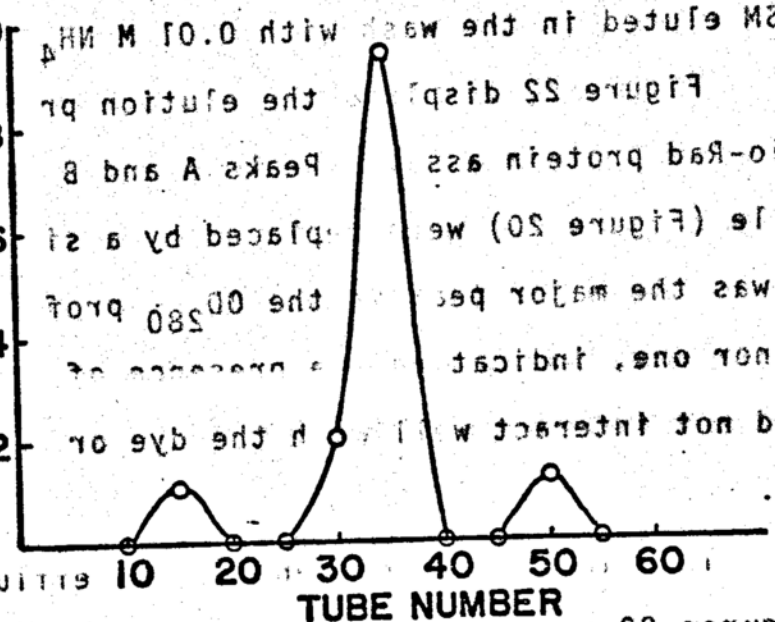

10
8
6
4
2
% NANA
10
20
30
40
50
60
TUBE NUMBER

FIGURE 20. Chromatography of conjunctival crude extract on DEAE Bio-Gel A. The plots represent OD_{280} (O) and M NaCl (Δ). The column was 1.5 x 30 cm and eluted with a linear NaCl gradient at 4°C at a flow rate of 58.5 ml h^{-1}. Fraction volumes were 1.5 ml.

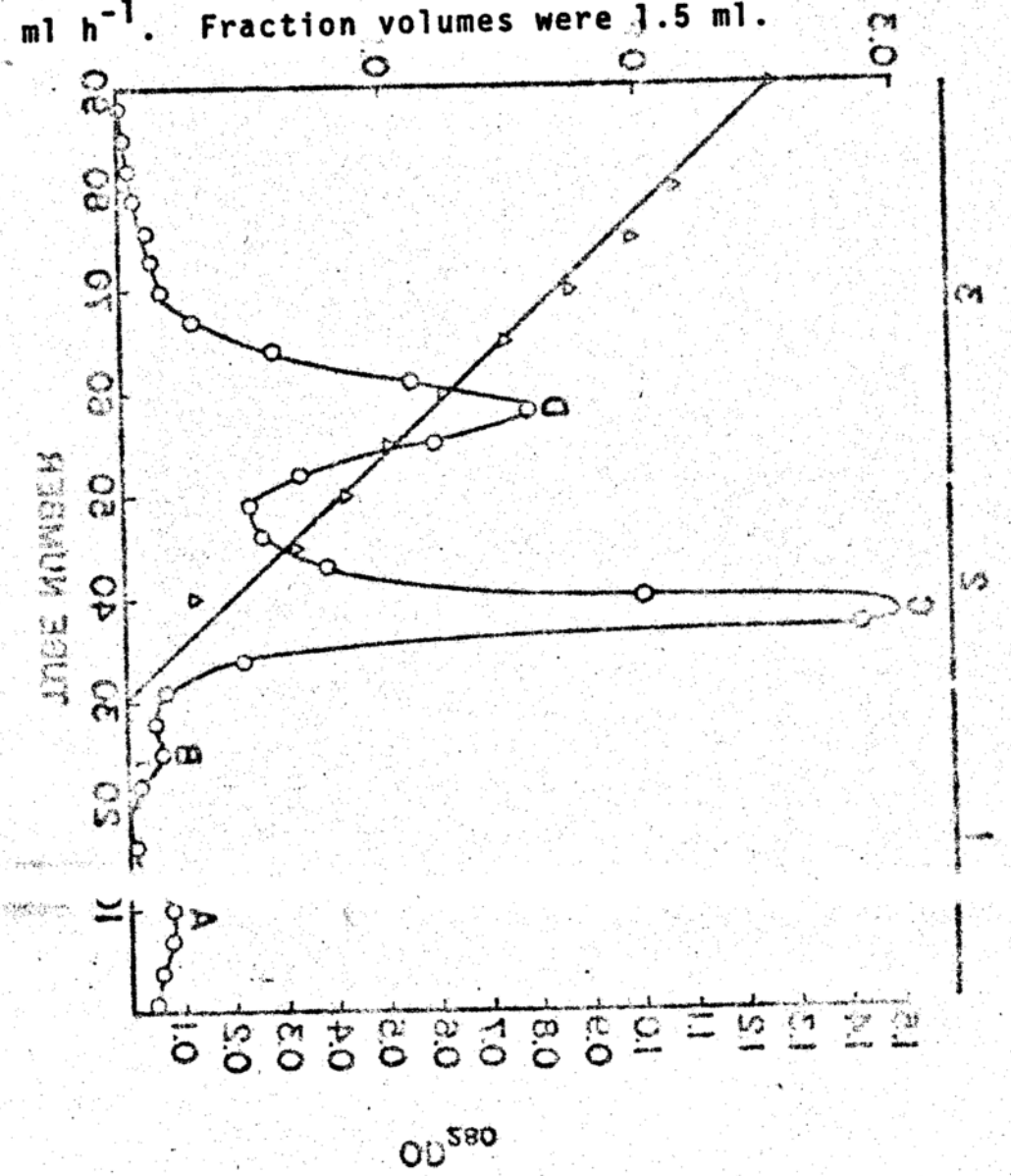


FIGURE 21. Chromatography of bovine submaxillary mucin on DEAE Bio-Gel A. The plots represent OD_{280} (○) and NaCl concentration (Δ). The column was 1.5 x 30 cm and eluted at 4°C with a linear NaCl gradient at a flow rate of 58.5 ml h^{-1}. Fraction volumes were 1.46 ml.

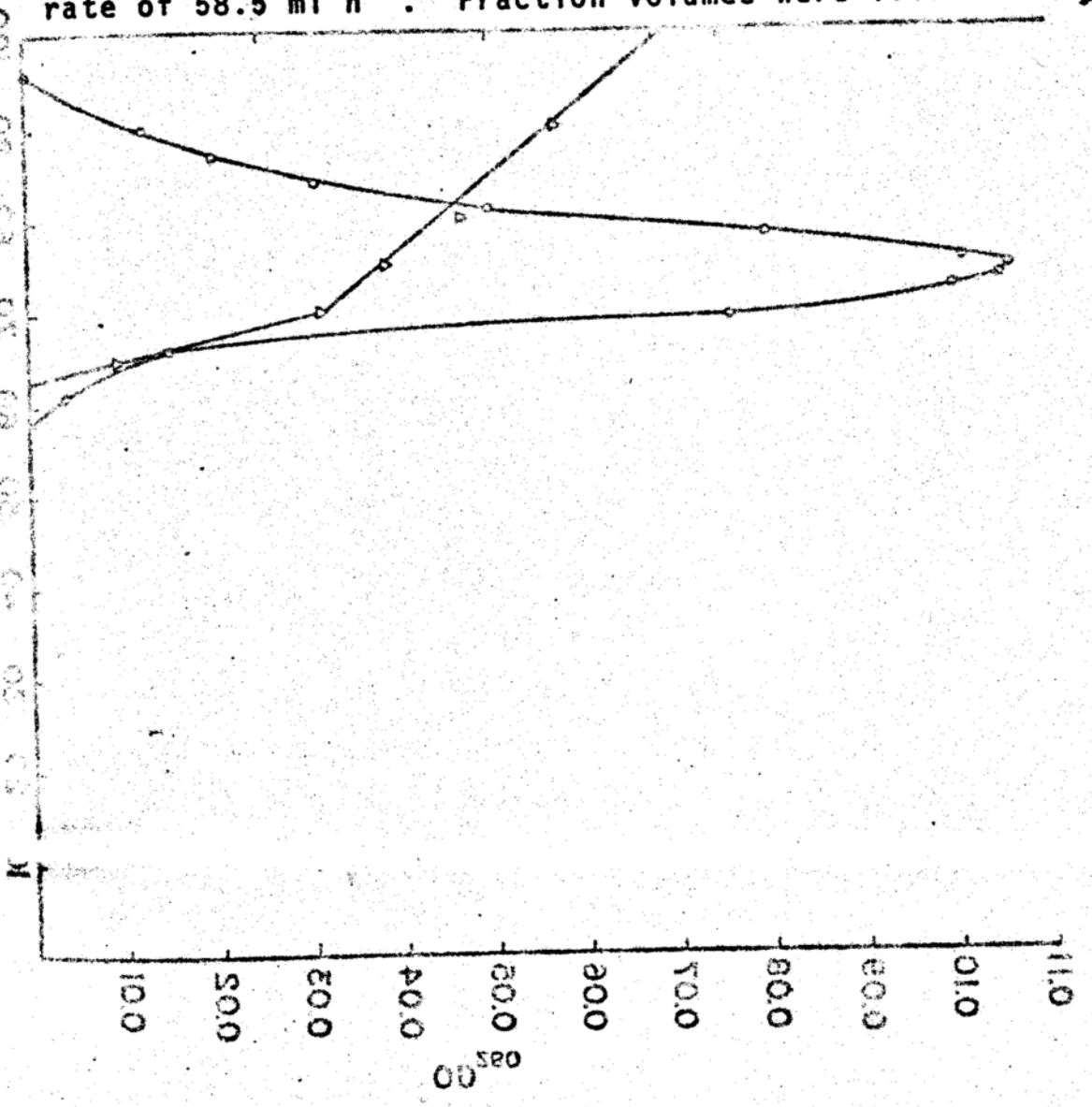


FIGURE 22. Chromatography of conjunctival crude extract on DEAE Bio-Gel A. The plot represents mg ml^{-1} protein (□) given by the Bio-Rad Protein Assay. The column was 1.5 x 30 cm and eluted with a linear NaCl gradient at 4°C at a flow rate of 58.5 ml h^{-1}. Fraction volumes were 1.5 ml.

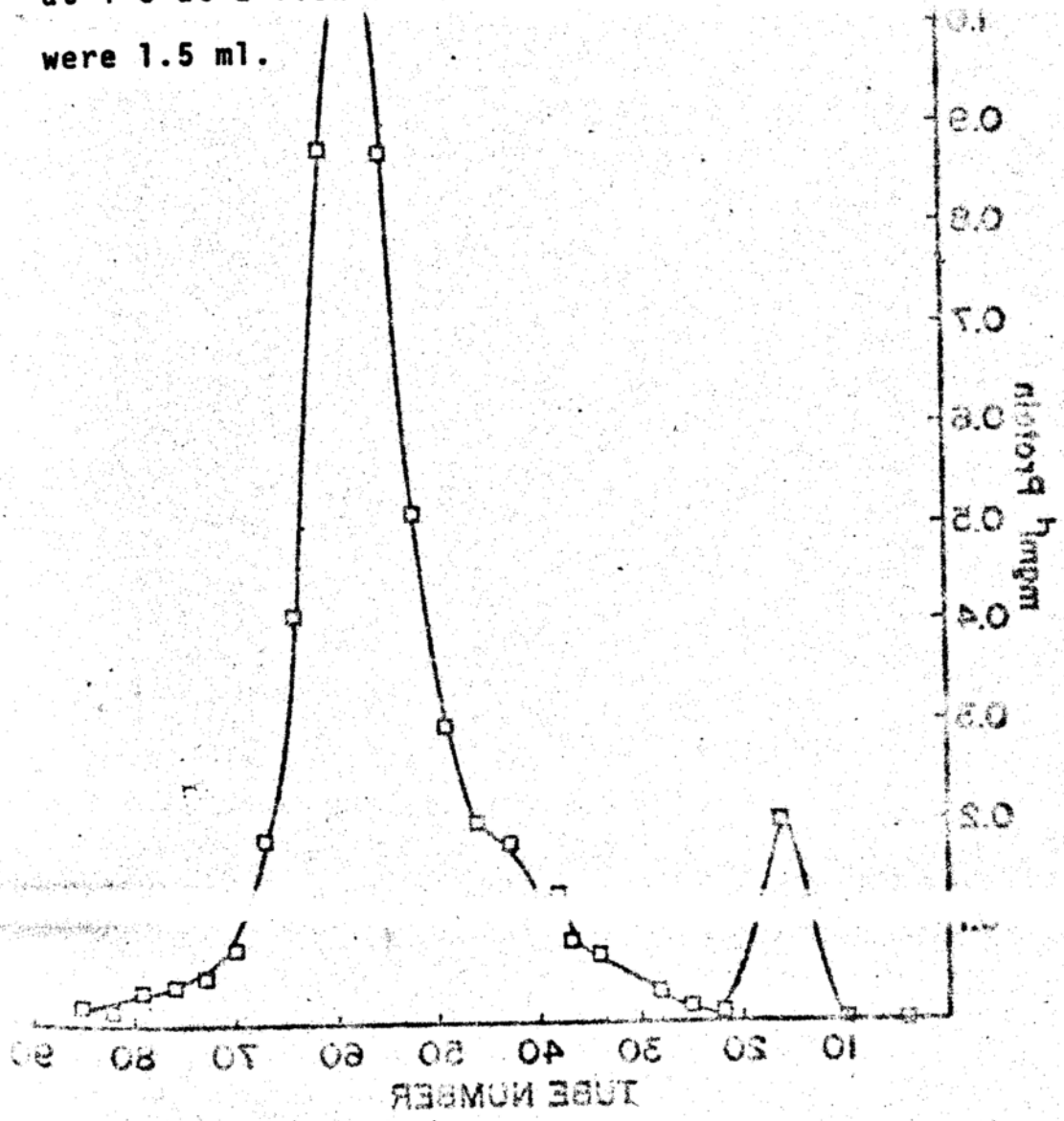


FIGURE 23. µg of sialic acid in effluent off DEAE Bio-Gel A column (1.5 x 30 cm) following gradient elution with 200 ml each of 0.1 M and 1 M NaCl in 0.01 M NH_4Ac buffer at pH 7.2.

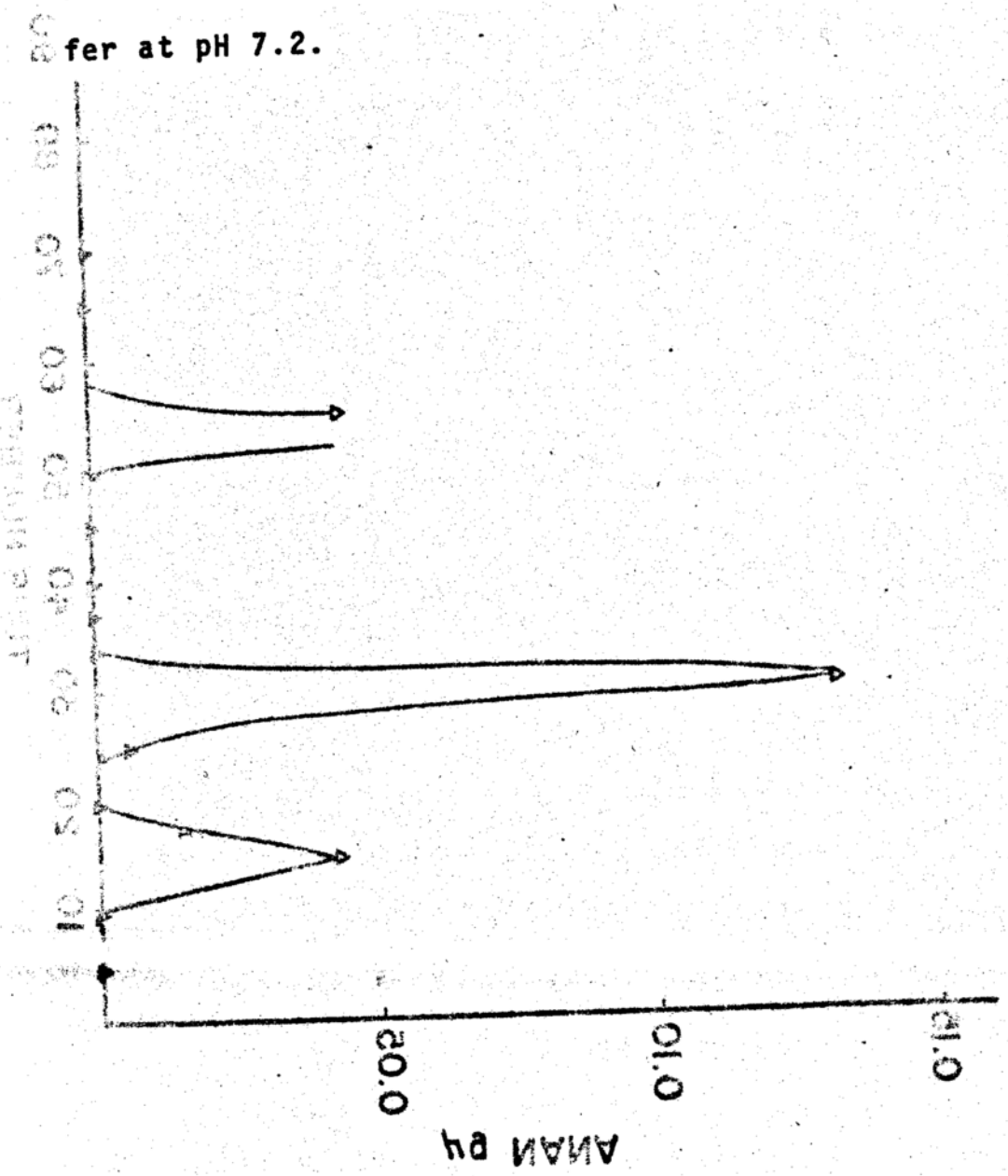
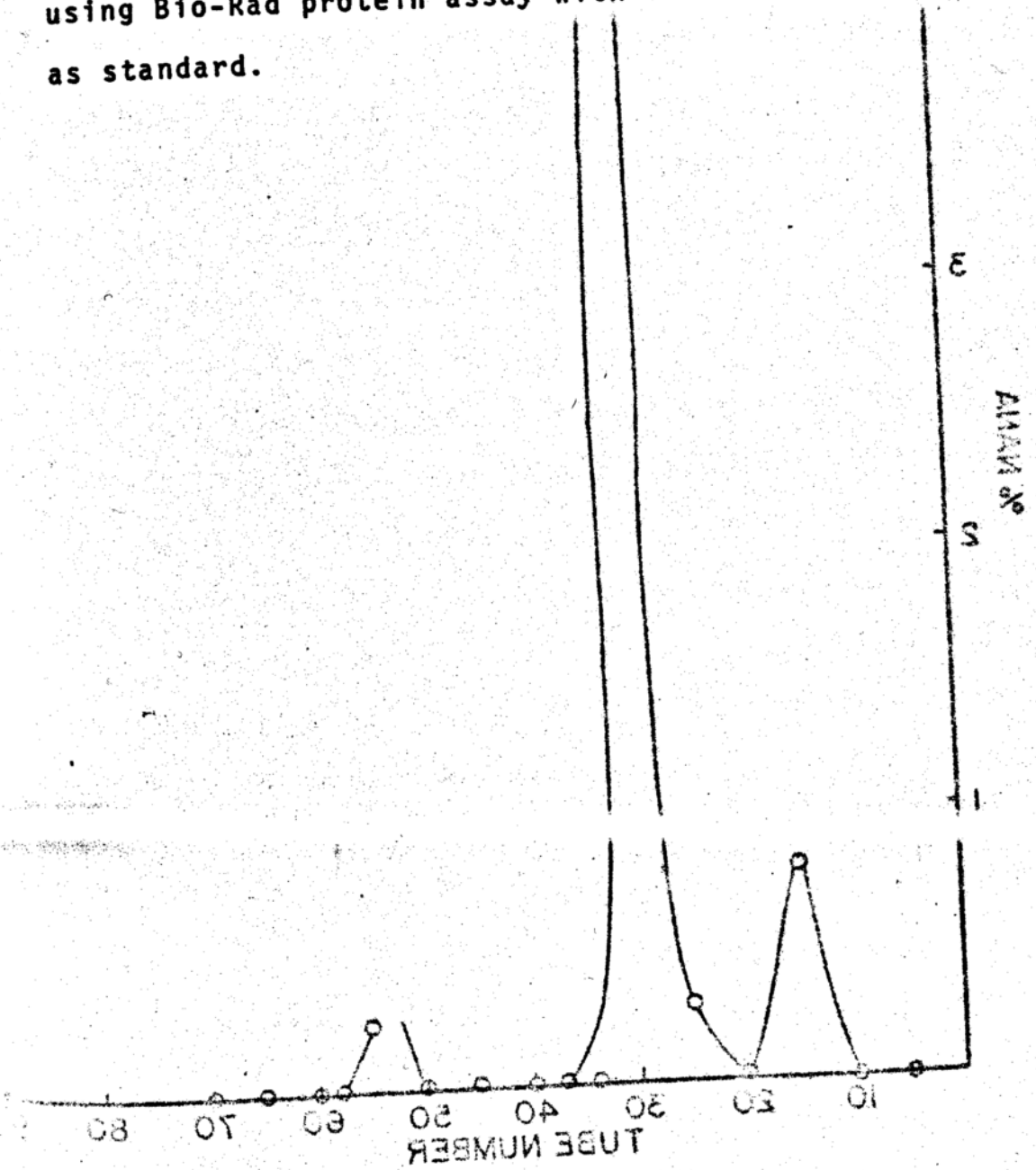


FIGURE 24. Percent w/w of sialic acid in effluent off DEAE Bio-Gel A column (1.5 x 30 cm) following gradient elution with 200 ml each of 0.1 M and 1 M NaCl in 0.01 M NH_4Ac at pH 7.2. Protein concentration was determined using Bio-Rad protein assay with bovine serum albumin as standard.



The effluent following gradient elution was pooled to yield three fractions. Fraction G1 consisted of the contents in tubes 1-30; fraction G2, tubes 31-50; and fraction G3, tubes 51-90. Each fraction was desalted and lyophilized as described earlier.

The lyophilizates for fractions G2 and G3 were reconstituted in 0.01 M NH_4Ac buffer (pH 7.2). Ninety microliters of the resulting solution were mixed with 30 µl of tracking dye solution. Thirty microliters of the mixture were layered onto 4, 7.5 and 12% polyacrylamide gels and subjected to electrophoresis.

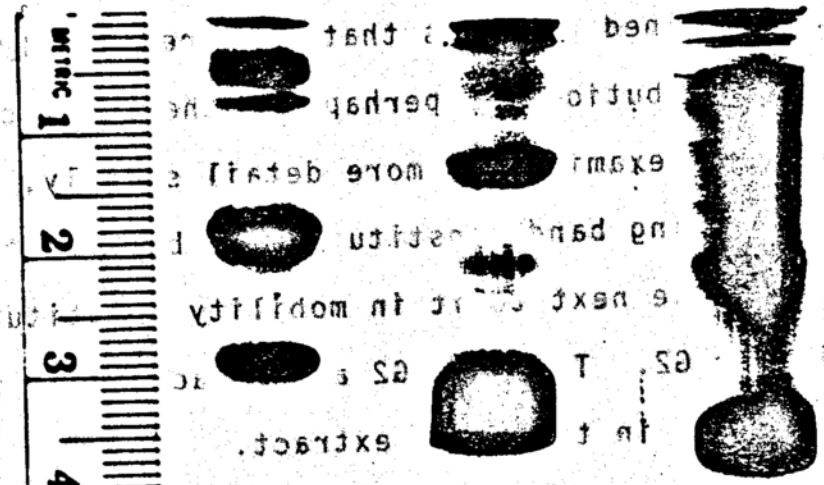
The electrophoretic patterns are shown in Figures 25, 26 and 27. Two protein bands were identified for both G2 and G3 in the 4% gels (Figure 25). G3 contained most of the fast moving band, a result that was consistent with the relatively high salt concentration (0.118 M) needed to elute it from a DEAE Bio-Gel A column. More protein bands were resolved in the 7.5 and 12% gels (Figures 26 and 27). In 12% gels (Figure 27), bands that moved even faster than the fast moving (heavily stained) band were detected for G3, and two bands that stayed close to the origin were detected for G2. A similar comment can be made for the 7.5% gels (Figure 26).

The significance of these electrophoretic patterns is two fold. First, overlap of the fractions aside, both G2

FIG 26. [illegible] DEAE [illegible] dient fraction 2 (G2) and fraction 3 (G3). 7.5% Bio-Phore gels. Key: gel A, DEAE G2; gel B, DEAE G3; and gel C, conjunctival crude extract.

FIGURE 7. [illegible] DEAE [illegible] t fra[illegible]S (G2) and frac-
n 3 (G3). 12% Bio-Phore gels. Key: gel A, DEAE
gel B, DEAE G3; and gel C, conjunctival crude
ract.

A B C



A B C

FIGURE 28. Chromatography of fraction W2 on Bio-Gel A 5m. The plot represents OD_{280}. The column was 1.5 x 60 cm and eluted at 4°C at a flow rate of 9.4 ml h^{-1}. Fraction volumes were 1.6 ml.

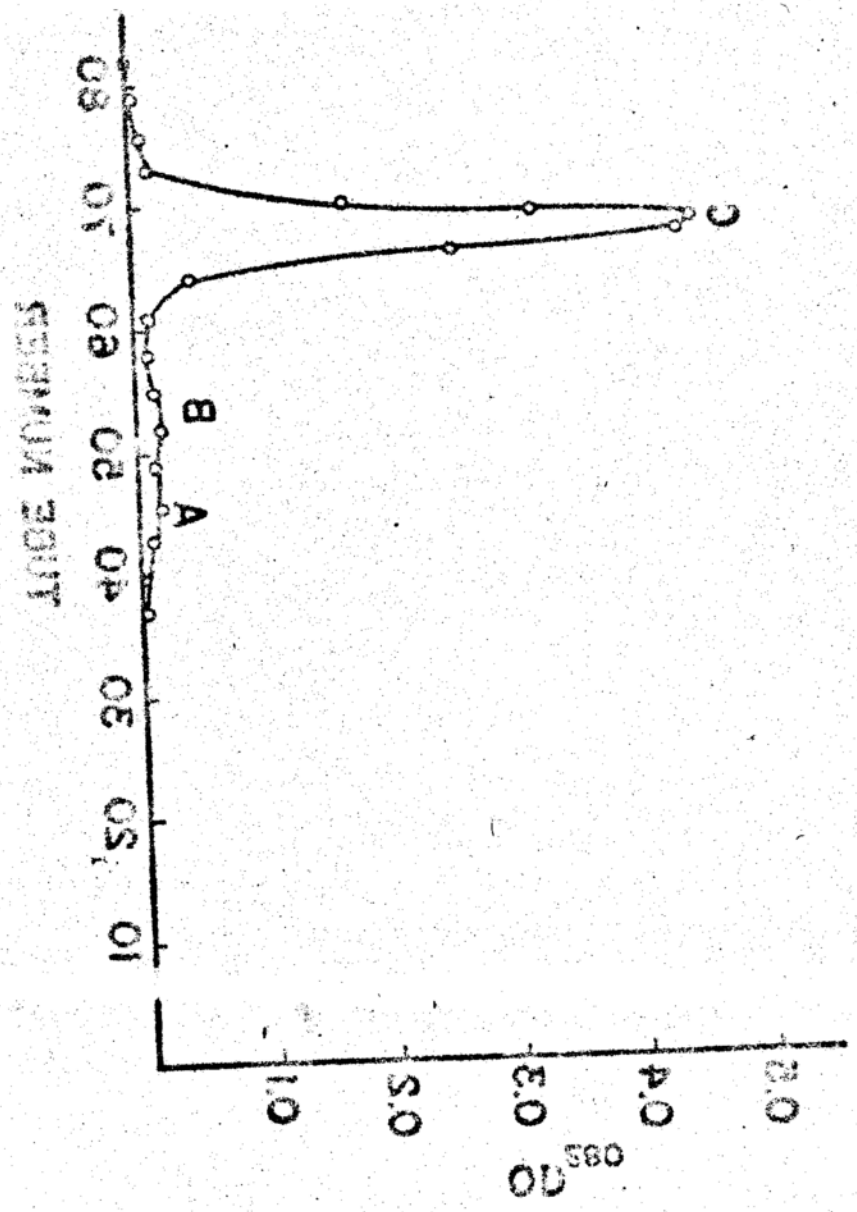


FIGURE 29. Chromatography of fraction W2 on Bio-Gel A 5m. The plot represents mg ml^{-1} as measured by Bio-Rad protein assay with bovine serum albumin as standard. The column was 1.5 x 60 cm and eluted at 4°C at a flow rate of 9.4 ml h^{-1}. Fraction volumes were 1.6 ml.

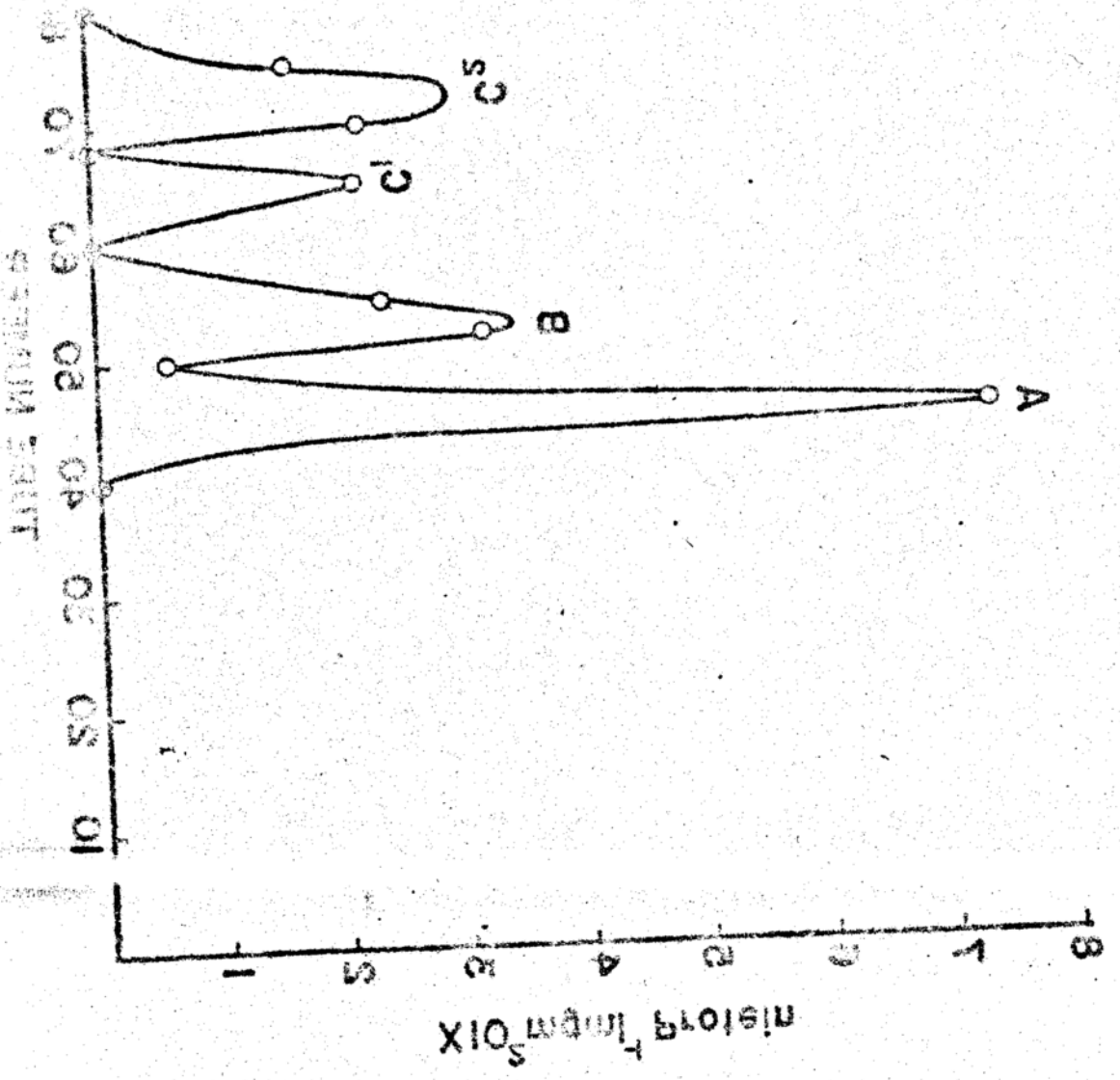


TABLE X. Elution volume of each peak and apparent molecular weight of the species contained therein following chromatography of fraction W2 on Bio-Gel A 5m.

Peak	V_e[a] (ml)	M.W.[b]
A	72	2.4×10^4
B	84	5.5×10^3
C_1	104	4.65×10^2
C_2	115.2	1.2×10^2

[a] Elution volume.

[b] Obtained from a calibration plot shown in Figure 45.

FIGURE 30. U.V. spectrum of contents in tube #67. See legend for Figure 28 for details.

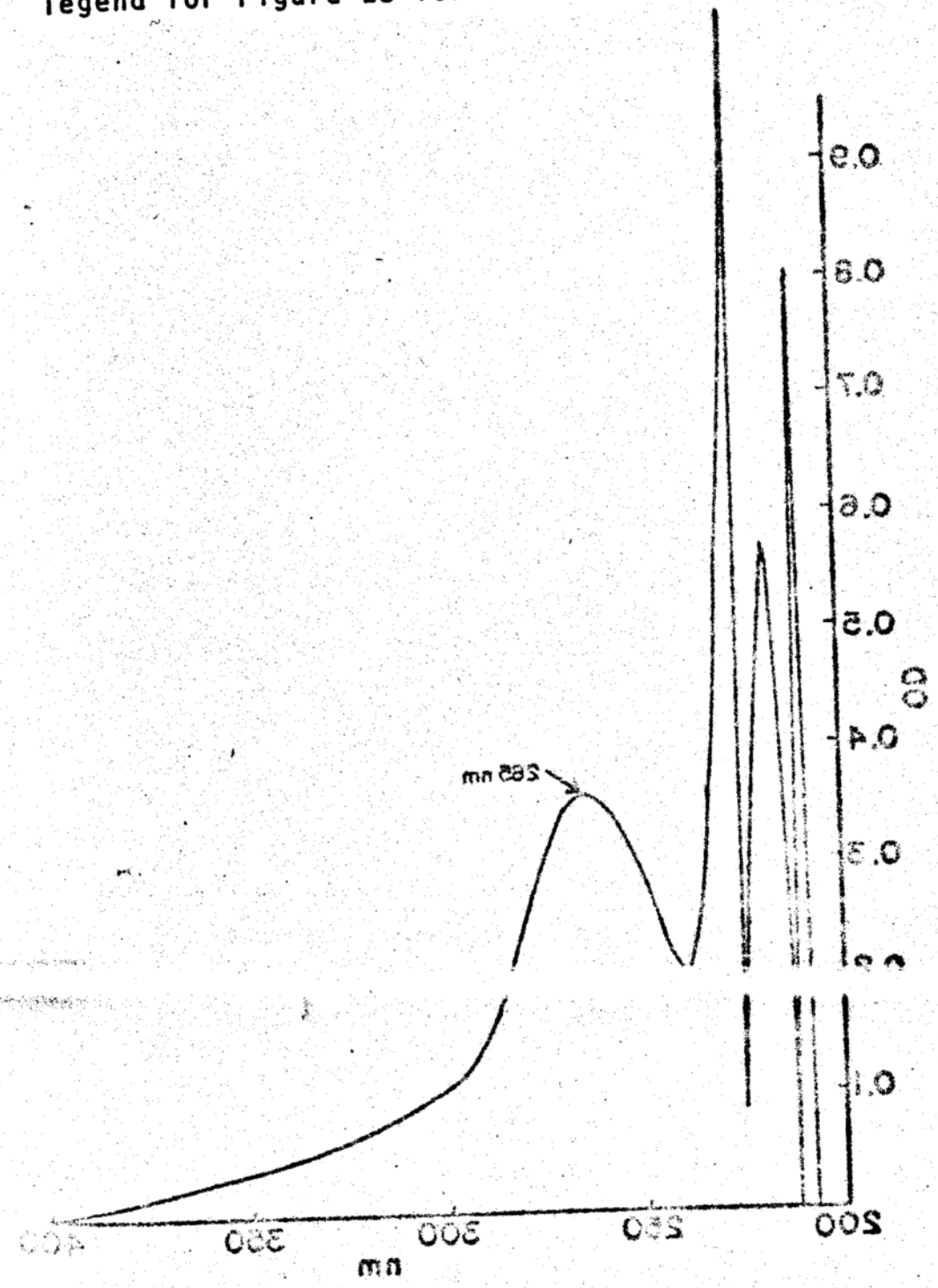


FIGURE 31. Chromatography of DEAE Bio-Gel A gradient fraction G2 on Bio-Gel A 5m. The plot represents OD_{280}. The column was 1.5 x 60 cm and eluted with a flow rate of 8.8 ml h^{-1}. Fraction volumes were 1.5 ml.

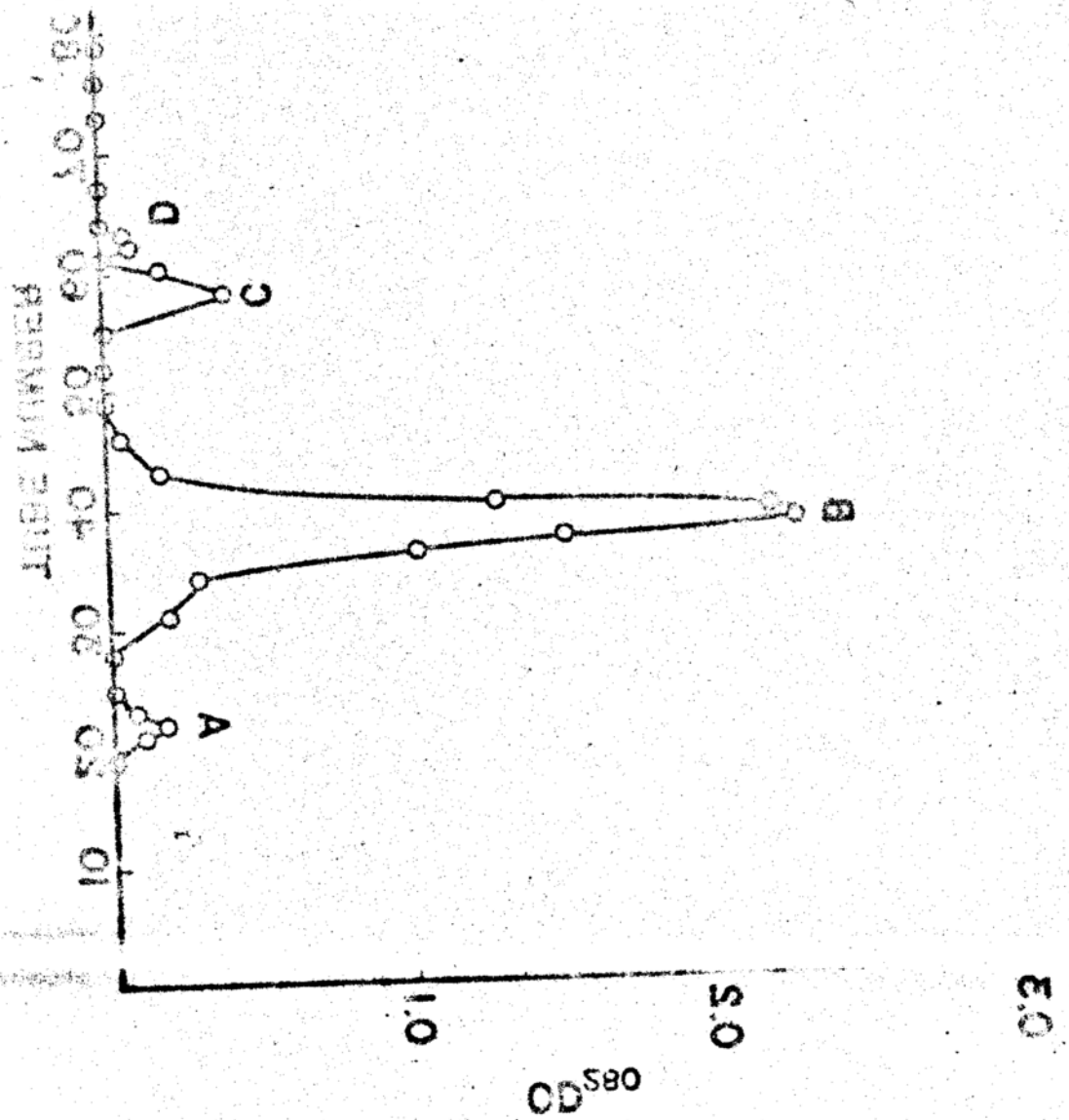


FIGURE 32. Chromatography of DEAE gradient fraction G2 on Bio-Gel A 5m. The plot represents mg ml^{-1} of protein as measured by the Bio-Rad protein assay with bovine serum albumin as standard. The column was 1.5 x 60 cm and eluted with a flow rate of 8.8 ml h^{-1}. Fraction volumes were 1.5 ml.

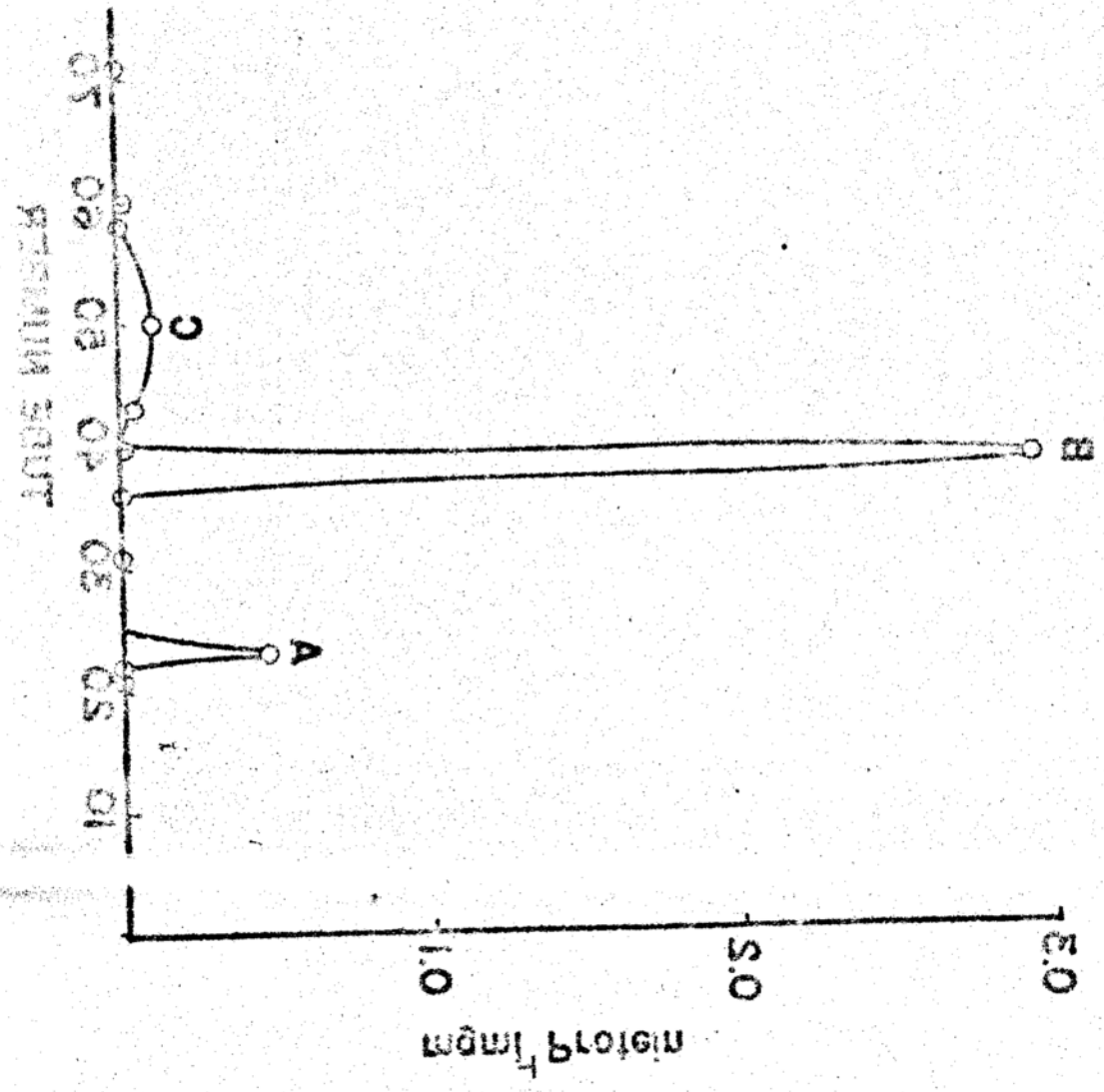


TABLE XI. Elution volume of each peak and apparent molecular weight of the species contained therein following chromatography of fraction G2 on Bio-Gel A 5m.

Peak	V_e[a] (ml)	M.W.[b]
A	33	3.0×10^6
B	57	1.7×10^5
C	87	4.9×10^3

[a] Elution volume.

[b] Obtained from a calibration plot shown in Figure 45.

0.4
0.3
0.2
0.1
µg NANA
10
20
30
40
50
60
TUBE NUMBER

TUBE NUMBER
10
20
30
40
50
60
% NANA
6
5
4
3
2
1

FIGURE 35. PAGE of fraction G2 and its components resolved on a Bio-Gel A 5m column. 4% Bio-Phore gels. The samples were; gel A -- DEAE G2; gel B -- DEAE/Bio-Gel A 5m, G2, tube #22; and gel C -- DEAE/Bio-Gel A 5m, G2, tube #38. DEAE/Bio-Gel A 5m represents chromatography of conjunctival crude extract on DEAE followed by chromatography of fraction G2 thus obtained on Bio-Gel A 5m. See text for additional details.

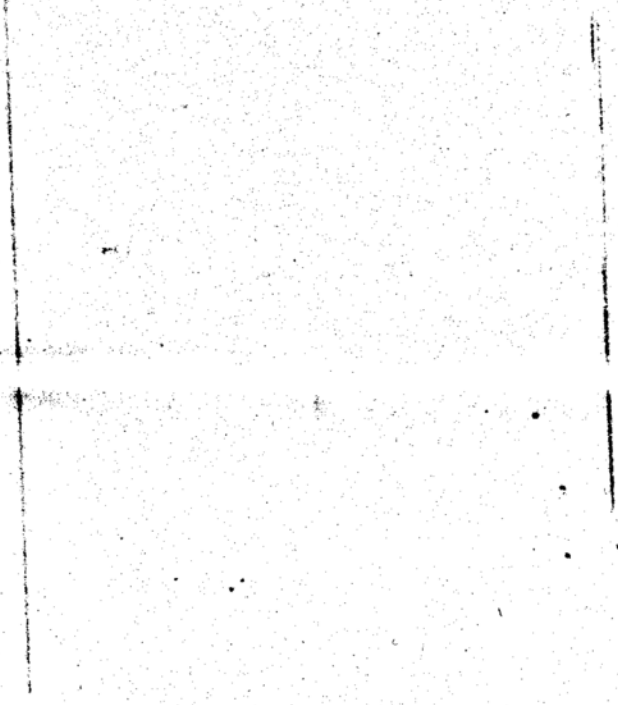


FIGURE 36. PAGE of fraction G2 and its components resolved on Bio-Gel A 5m chromatography. 7.5% Bio-Phore gels. For key and details see legend for Figure 35.

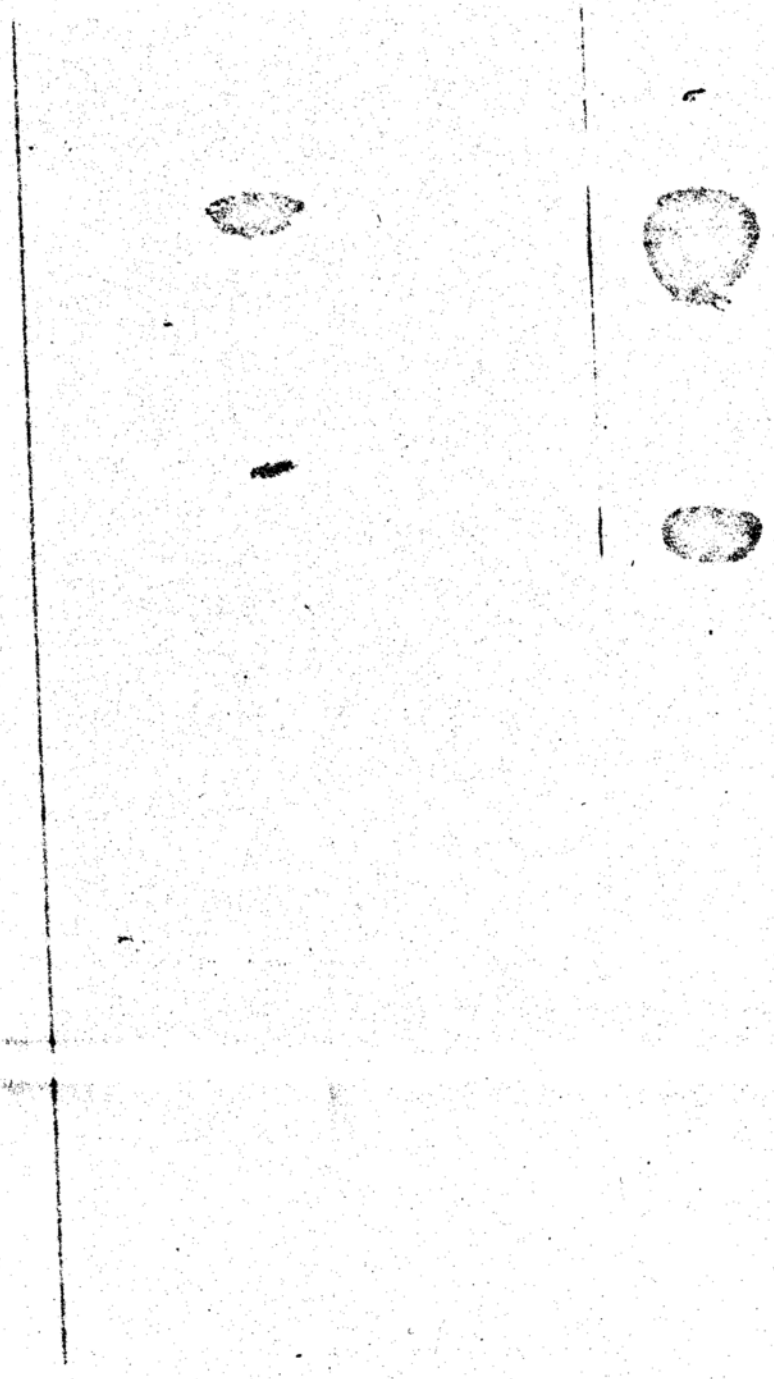


FIGURE 37. PAGE of fraction G2 and its components resolved on Bio-Gel A 5m chromatography. 12% Bio-Phore gels. For key and details see legend for Figure 35.

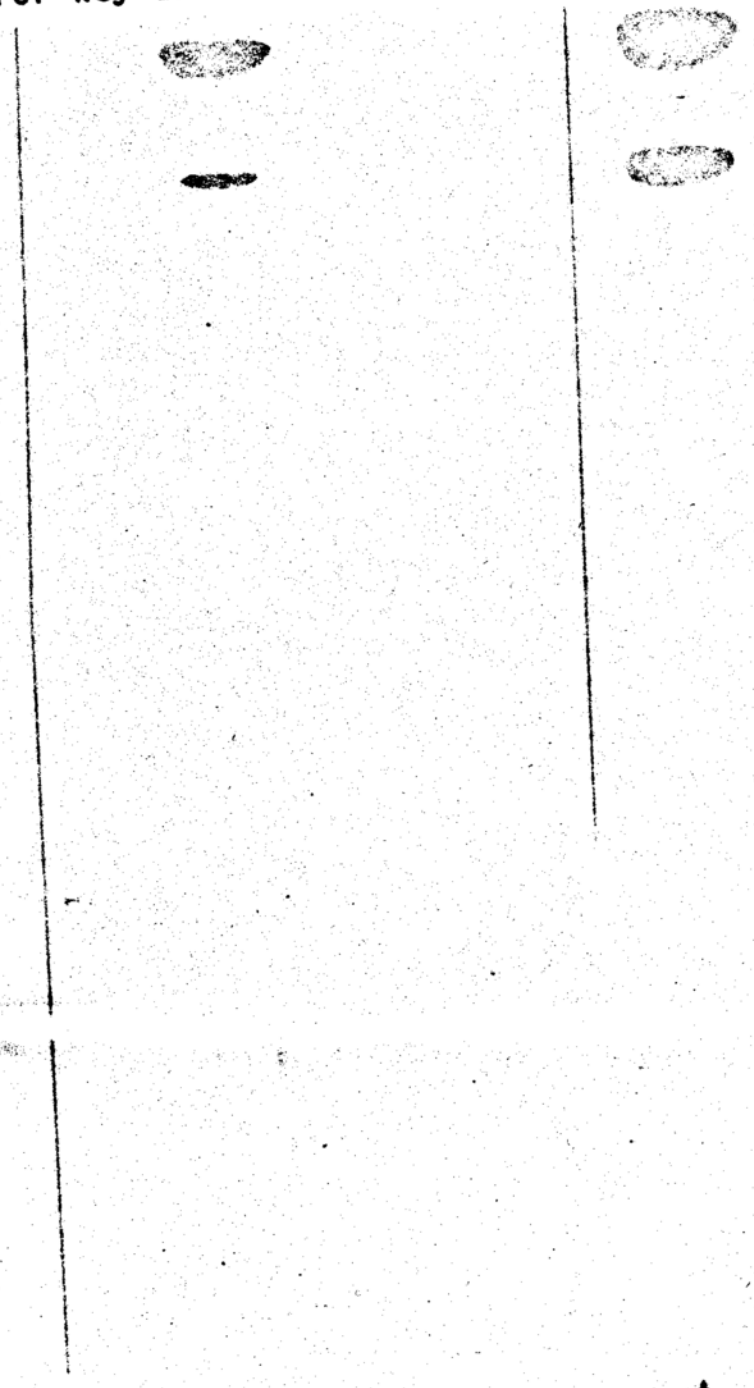
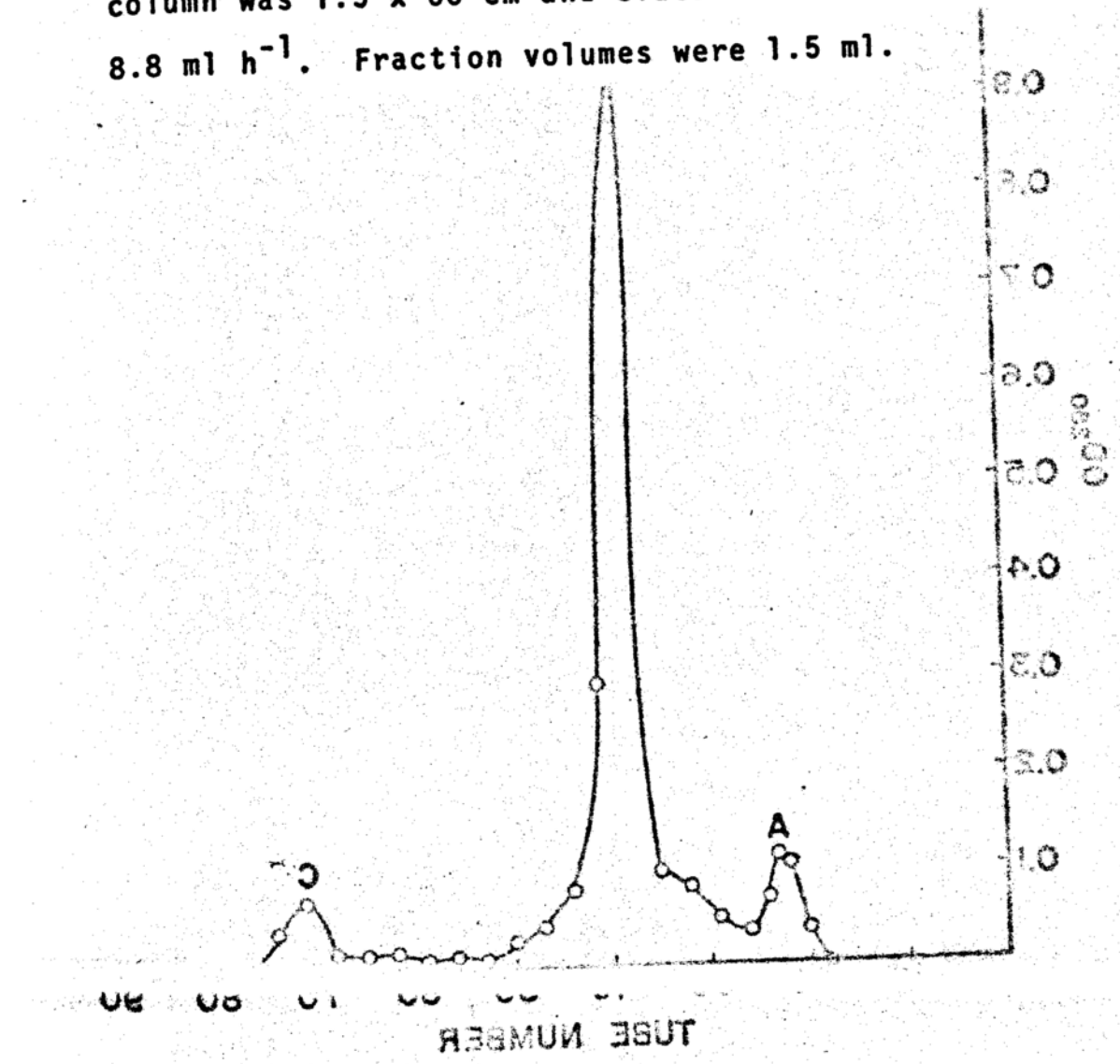


FIGURE 38. Chromatography of DEAE gradient fraction 3 (G3) on Bio-Gel A 5m. The plot represents OD_{280}. The column was 1.5 x 60 cm and eluted with a flow rate of 8.8 ml h^{-1}. Fraction volumes were 1.5 ml.



protein assay (see Figure 39). Sialic acid was present in both peaks A and B, as well as peak C that did not contain protein (see Figures 40 and 41). Indeed, a high level of sialic acid was demonstrated in the effluent eluted with total bed volume. Upon lyophilizing the contents of tubes 61-80, which encompassed peak C, a white, fluffy powder was obtained. It apparently possessed a high charge density as it was eluted off the DEAE Bio-Gel column at a relatively high NaCl concentration (0.118 M).

Table XII lists the elution volume corresponding to each peak as well as the apparent molecular weight of the species contained therein. A comparison with Table XI reveals that the species contained within peaks A and B yielded by fractions G2 and G3 are practically the same. The same is true with ratios of the areas of peak B to A given by these two fractions: 16 in the case of G2 and 14 in the case of G3. Recall that G2 and G3 produced different electrophoretic patterns. The significance of this observation will be elaborated upon in the Discussion section.

As for G2, PAGE was performed on the contents of tubes #23 and #38. Figures 42-44 present the electrophoretic patterns. Due to a 10-fold increase in the amount of proteins used, unlike G2, a band corresponding to a high molecular protein was evident in the gel (gel B) layered with the content of tube #23. From gel C it is clear that tube #38

FIGURE 40. µg of sialic acid in effluent off Bio-Gel A 5m column (1.5 x 60 cm). The sample was DEAE gradient fraction 3 (G3).

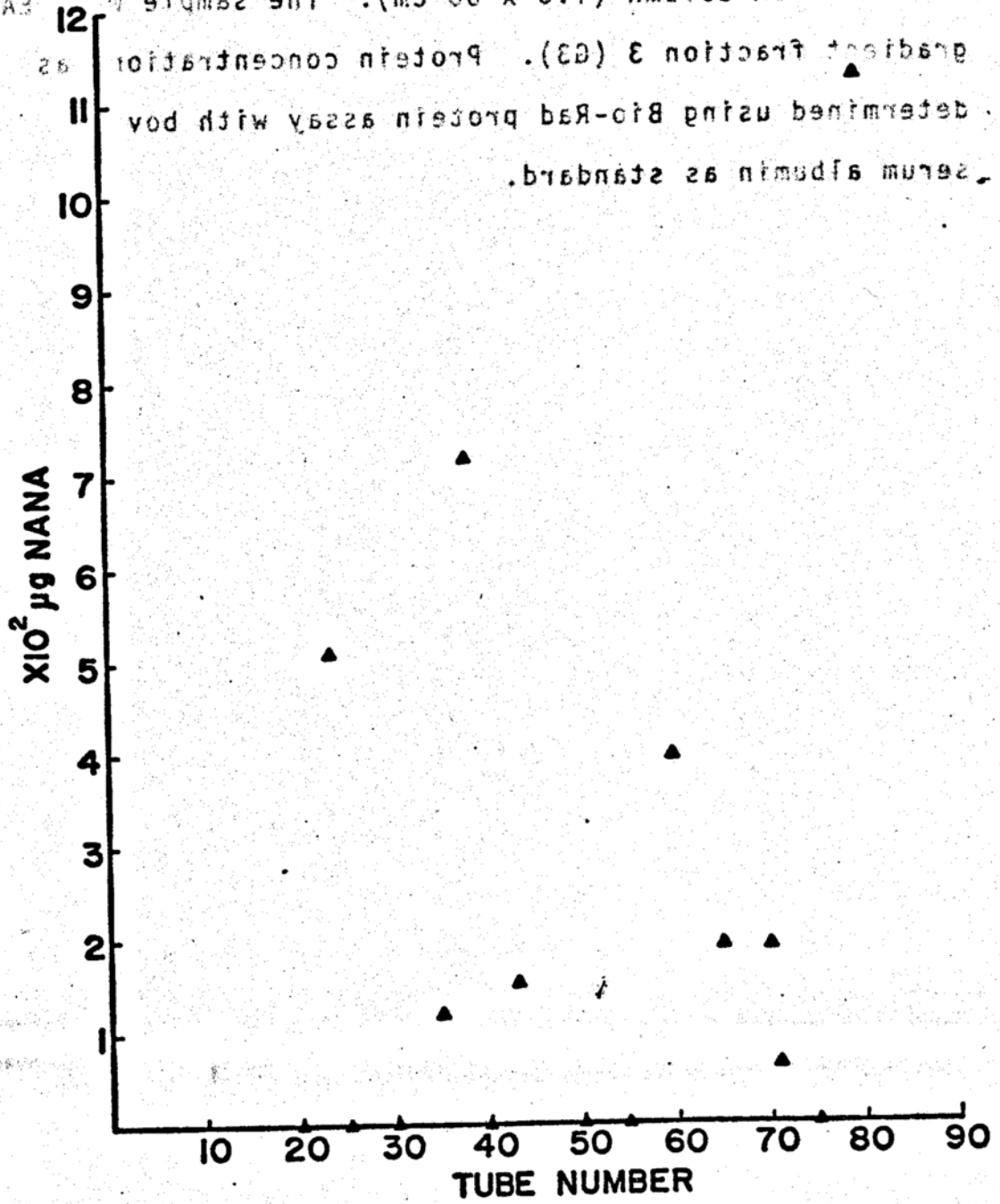

12
11
10
9
8
7
6
5
4
3
2
1
X10² µg NANA
10
20
30
40
50
60
70
80
90
TUBE NUMBER

TABLE XII. Elution volume of each peak and apparent molecular weight of the species contained therein following chromatography of fraction 63 on Bio-Gel A 5

Peak	V_e[a] (ml)	M.W.[b]
A	34.5	2.5×10^6
B	57	1.7×10^5
C	106.5	480

[a]Elution volume.

[b]Obtained from a calibration plot shown in Figure 45.

FIGURE 42. PAGE of fraction G3 and its components resolved on a Bio-Gel A 5m column. 4% Bio-Phore gels. The samples were: gel A -- DEAE G3; gel B -- DEAE/Bio-Gel A 5m, G3, tube #23; and gel C -- DEAE/Bio-Gel A 5m, G3, tube #38. DEAE/Bio-Gel A 5m represents chromatography of conjunctival crude extract on DEAE followed by chromatography of fraction G3 thus obtained on Bio-Gel A 5m. See text for additional details.

FIGURE 43. PAGE of fraction G3 and its components resolved on Bio-Gel A 5m column. 7.5% Bio-Phore gels. For key and details see legend of Figure 42.

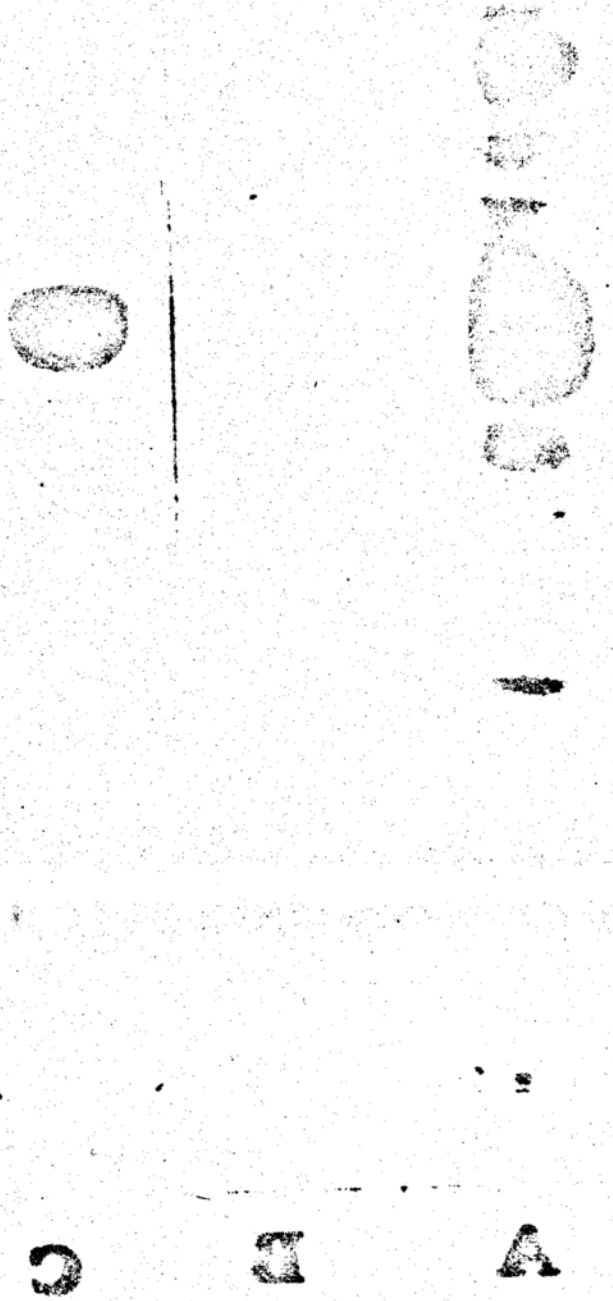


FIGURE 44. PAGE of fraction G3 and its components resolved on a Bio-Gel A 5m column. 12% Bio-Phore gels. For key and details see legend of Figure 42.

contained electrophoretically pure material possessing an apparent molecular weight of 1.7×10^5. As it migrated faster than albumin (M.W. = 6.9×10^4) in an electric field within a polyacrylamide gel matrix, it must possess an abundance of charge at pH 8.9 in order to achieve a high charge density. A comparison of the patterns shown in Figure 37 (G2 #38) and Figure 16 (mixture of crude extract, albumin and bovine submaxillary mucin) suggests the heavily stained band on gel C (Figure 37) corresponds to a protein with a molecular weight in the neighborhood of 4.9×10^3 (Table XI). The reason is that it moves faster than albumin in the electric field.

C. Chromatography of Conjunctival Crude Extract on Bio-Gel A 5m

1. Calibration of Column with Proteins of Known Molecular Weights

The determination of molecular weights, particularly of proteins, is one of the most important applications of gel chromatography (129). In a long series of careful measurements Andrews and his group (130-133) demonstrated that for most proteins there is a close correlation between molecular weight and elution behavior.

A column (1.5 x 60 cm) of Bio-Gel A 5m was calibrated using the following proteins (or protein mixture): bovine

TABLE XIII. Elution behavior of various proteins and Blue Dextran 2000 used to calibrate a Bio-Gel A 5m column (1.5 x 60 cm).

Protein	M.W.	V_e (ml)	K_{av}
Albumin	6.8×10^4	63.6	0.383
γ-Globulin	1.5×10^5	60.7	0.348
Fumarase	2.0×10^5	54.8	0.278
Blue Dextran 2000	2.0×10^6	35.5	0.052
Bovine submaxillary mucin	4.0×10^6	31.1	0

FIGURE 45. Plot of K_{av} against log(M.W.) for proteins on a Bio-Gel A 5m column (1.5 x 60 cm). Experimental details are given in the text.

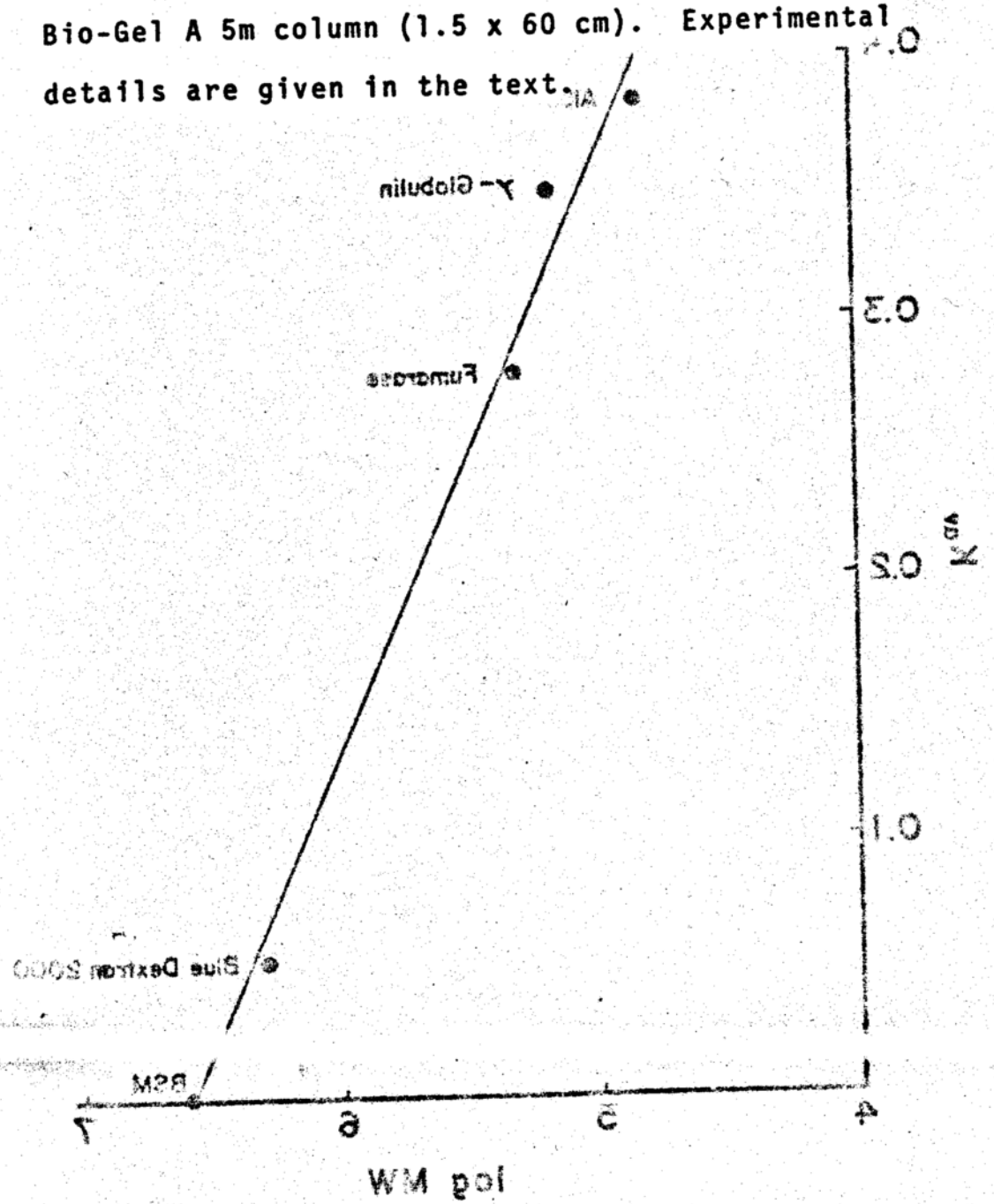
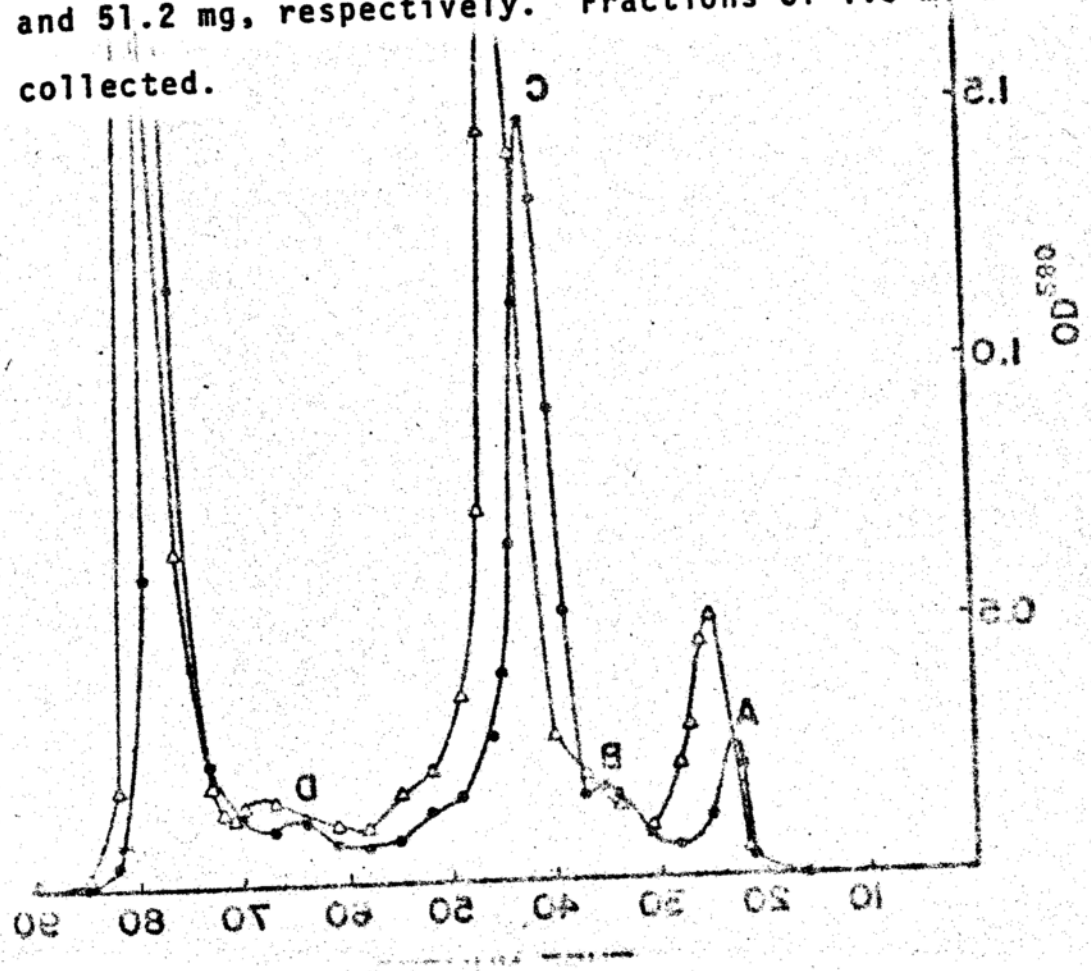
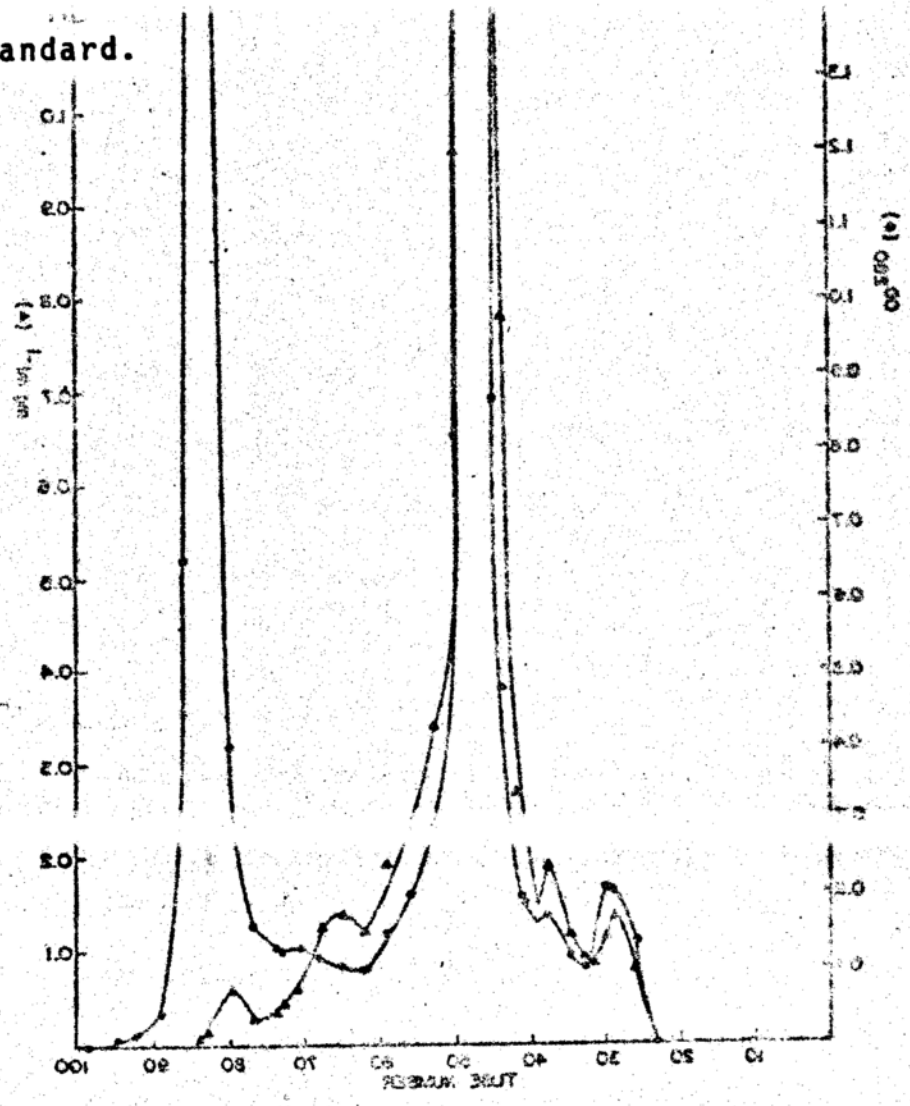


FIGURE 46. Chromatography of conjunctival crude extract on Bio-Gel A 5m column (1.5 x 60 cm). The plots represent elution of the column at a flow rate of 11.25 ml h^{-1} (●) and 8.8 ml h^{-1} (Δ). Sample applied was 38.7 mg and 51.2 mg, respectively. Fractions of 1.5 ml were collected.



159

FIGURE 47. Chromatography of conjunctival crude extract on Bio-Gel A 5m column (1.5 x 60 cm). The plots represent OD_{280} (●) and mg ml^{-1} protein (▲). The column was eluted at 8.8 ml h^{-1}. Fractions of 1.5 ml were collected. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.



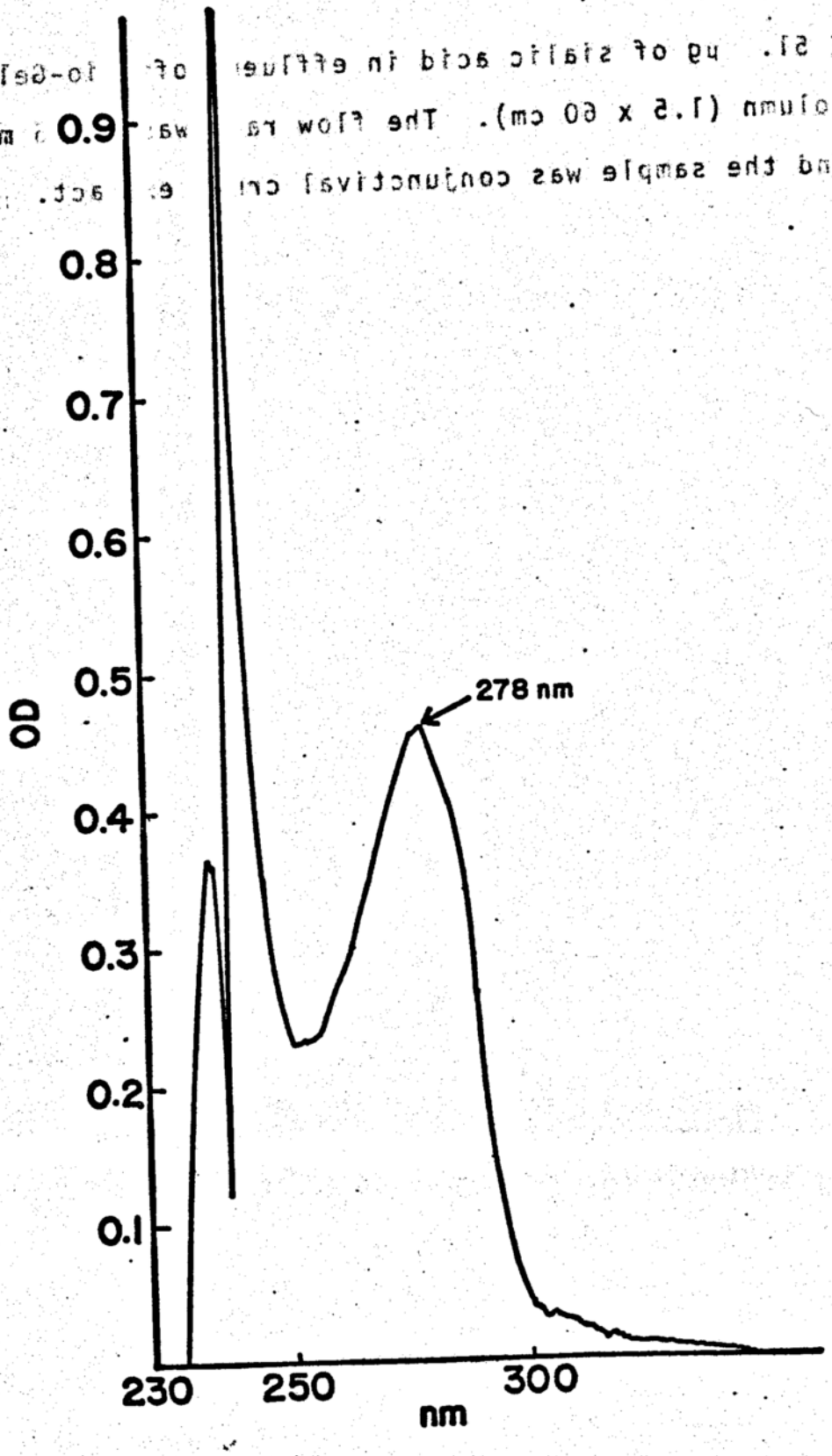
contain materials that absorbed strongly at 280 nm, however. The U.V. spectrum (Figure 48) given by the content of tube #88 shows λ_{max}'s at 270 nm and 220 nm, corresponding to the λ_{max} of the amino acid phenylalanine and a peptide bond, respectively (135). This spectrum is to be contrasted with that given by the crude extract (Figure 49) and the content of tube #44 (Figure 50), both yielding absorption maxima at roughly 235 and 280 nm. It must be mentioned that the same species may constitute peak E identified here and peak C identified in the elution profile of the chromatography of DEAE wash fraction 2 (W2) on Bio-Gel A 5m (see Figure 28). This highly U.V. absorbing peak was also present upon chromatographing tear mucoid clots on Sephadex G-150 (17). It was due to a small peptide of 9 amino acids.

Figures 51 and 52 present the sialic acid content of the effluent in terms of microgram and percent (w/w). Peak A shown in Figure 46 apparently possessed the highest sialic acid content, reaching a maximum of 16.5%. The species eluted with total bed volume also possessed a high sialic acid content and this pattern is similar to that observed with the chromatography of DEAE gradient fraction 3 (G3) on Bio-Gel A 5m (see Figure 40).

Table XIV lists the elution volume corresponding to each peak as well as the apparent molecular weight of the species contained therein. Like G2 and G3 (see Tables XI

FIGURE 49. U.V. spectrum for conjunctival crude extract.

FIGURE 50. U.V. spectrum for content of tube #44 in the elution profile (Δ) shown in Figure 46.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OD
278 nm
230
250
300
nm

0.4
0.3
0.2
0.1
µg SIALIC ACID (in 10 µl)
10
20
30
40
50
60
70
80
90
TUBE NUMBER

% SIALIC ACID
20
15
10
5
10
20
30
40
50
60
70
80
90
TUBE NUMBER

and XII), species with M.W. in the order of 10^3, 10^5 and 10^6 were present. Unlike G2 and G3, the fraction containing species with M.W. $\sim 10^5$ contributed even less to the elution profile than that containing species with M.W. $\sim 10^6$. A fraction with M.W. $\sim 10^4$ not seen with G2 and G3 now emerged.

SDS PAGE on the contents of tubes corresponding to the maximum of each peak (tubes #25, 37, 45, 64, and 79) produced the electrophoretic patterns shown in Figures 53 through 57. A single thick band was seen at the origin of the gels layered with the content of tube #25, a result that was consistent with the molecular weight of $\sim 10^6$ deduced from the elution pattern on Bio-Gel A 5m.

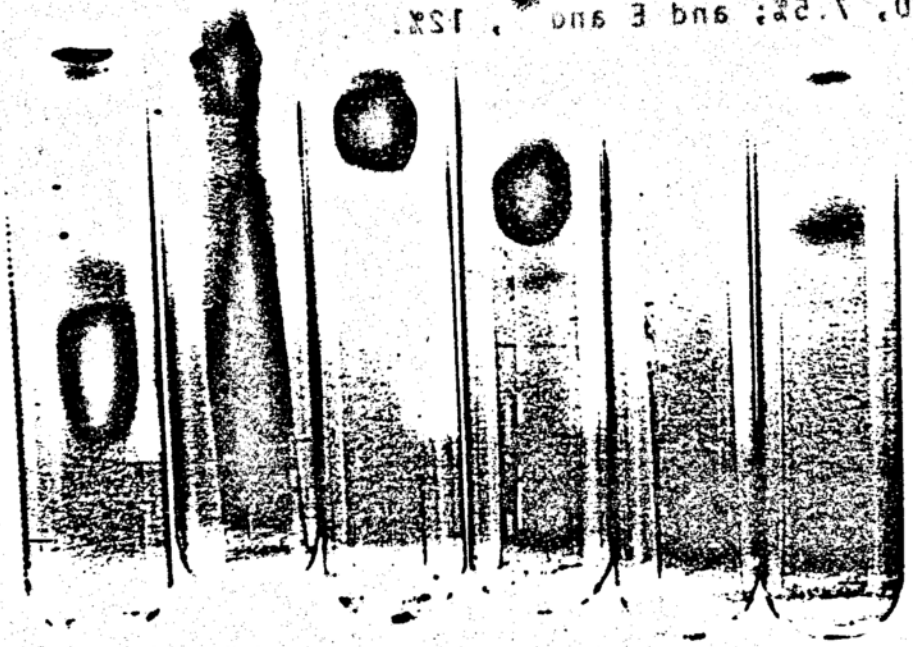
Three bands were seen on the 12% gel layered with the content of tube #37 (gel E in Figure 54). On the basis of staining intensity, the protein with M.W. $\sim 10^5$ (approximately 4 cm into the gel) appeared to be a minor component. The other two components possessed a lesser electrophoretic mobility, hence lower charge density.

The content of tube #45 (peak C) (gel E in Figure 55) also produced three (perhaps, four) bands. Based on the observation with G3 #38, the band with the greatest staining intensity probably corresponded to a protein with M.W. $\sim 1.7 \times 10^5$, and this was the major protein contained in peak C. The band next in intensity probably corresponded to a protein with M.W. $\sim 4.9 \times 10^3$. But these molecular weights

A B C D E F

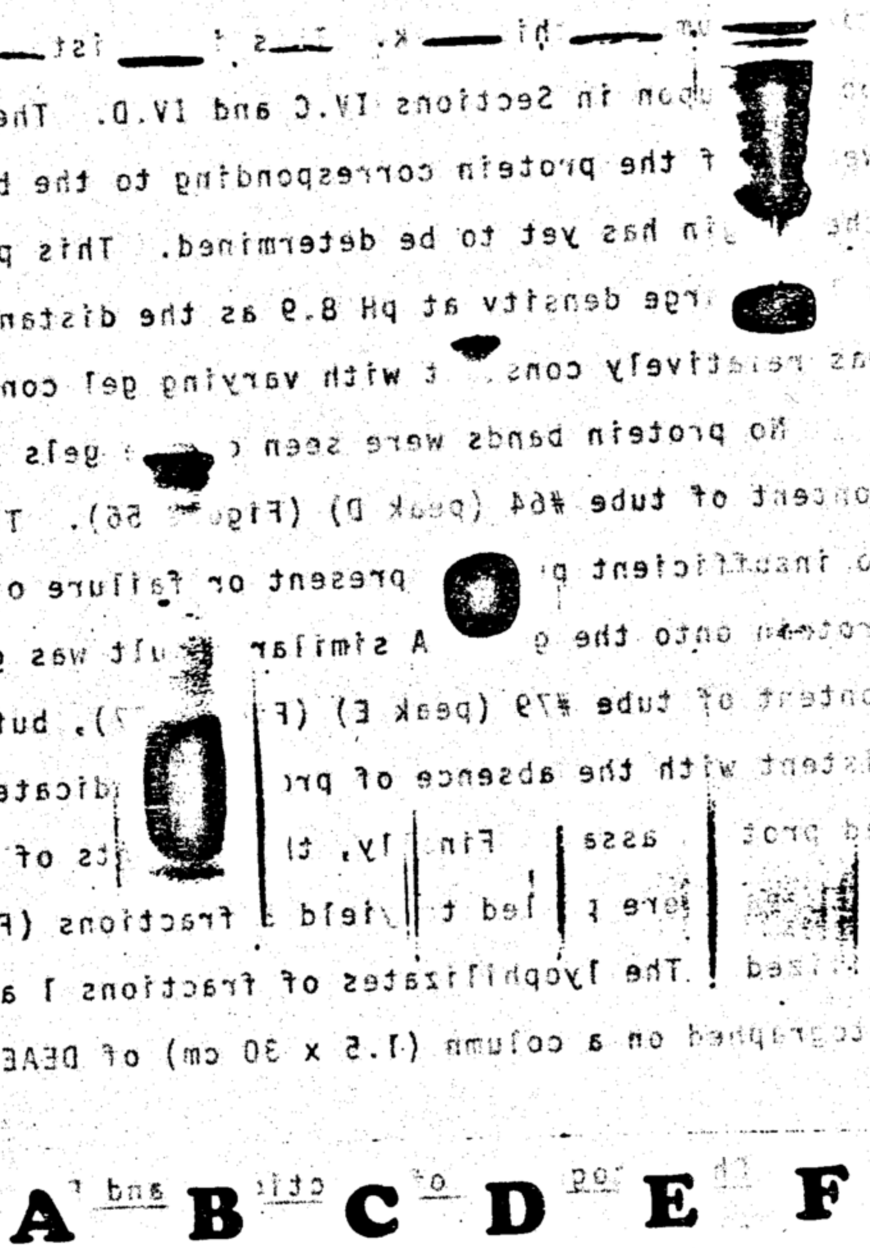
55. PAGE on peak C produced by chromatography
conjunct[illegible]l crude extract on a Bio-Gel A .5m column.
he [illegible] the [illegible] ent of tube [illegible] (see [illegible] gure 46.
To [illegible] 3 ml h^{-1}.). Gels A, C and E were from
ub[illegible] Gels B, D and F were from the conjunctival
rude extract. Gel concentration: gels A and B, 4%;
a [illegible] 7.5%+ and [illegible]nd F, 12%.

A B C D E F



A B C D E F

A B C D E F


A
B
C
D
E
F

62.4 ml h^{-1}. Fractions of 1.6 ml were collected and the OD_{280} measured for every third tube. As shown in Figure 58, a single peak emerged with total bed volume. Although its identity was not studied, the species was probably one with low charge density.

The column was next eluted at the same flow rate with a gradient composed of 200 ml each of 0.1 M and 1 M NaCl contained in a 0.01 M NH_4Ac buffer at pH 7.2. Again, 1.6 ml fractions were collected and the OD_{280} measured for every third tube. The result was that two fractions were eluted, one at 0.105 M NaCl and 65.5 ml and the other at 0.135 M NaCl and 76.5 ml (see Figure 59). This result, coupled with the observation that bovine submaxillary mucin was eluted at 0.146 M NaCl, indicates that fraction 1 contains a species that is rather similar to BSM in its charge. Moreover, they are likely to be very similar in molecular weight, differing only, in a subtle manner, their charge distribution. The sedimentation pattern shown on the view screen of a Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California) consisted of a single, sharp peak, indicating molecular weight homogeneity. This single sedimentation velocity run was conducted with a sample of fraction 1 dissolved in a 0.01 M NH_4Ac buffer (pH 7.2) containing 2 M NaCl; rotor speed was 59,780 rpm (259,700 x g).

0.03
0.02
0.01
OD_{280}
10
20
30
40
50
60
70
80
90
TUBE NUMBER

M NaCl
0.20
0.15
0.10
0.05
90
80
70
60
50
40
30
20
10
TUBE NUMBER
0.07
0.06
0.05
0.04
0.03
0.02
0.01
OD280

FIGURE 60. Chromatography of fraction 3 from Bio-Gel A 5m on DEAE Bio-Gel A. The column was 1.5 x 30 cm and eluted with a 0.01 M NH_4Ac wash (100 ml), followed by a gradient composed of 200 ml each of 0 M and 1 M NaCl in 0.01 M NH_4Ac buffer at pH 7.2. Fractions of 1.4 ml were collected. Flow rate was 56.0 ml h^{-1}.

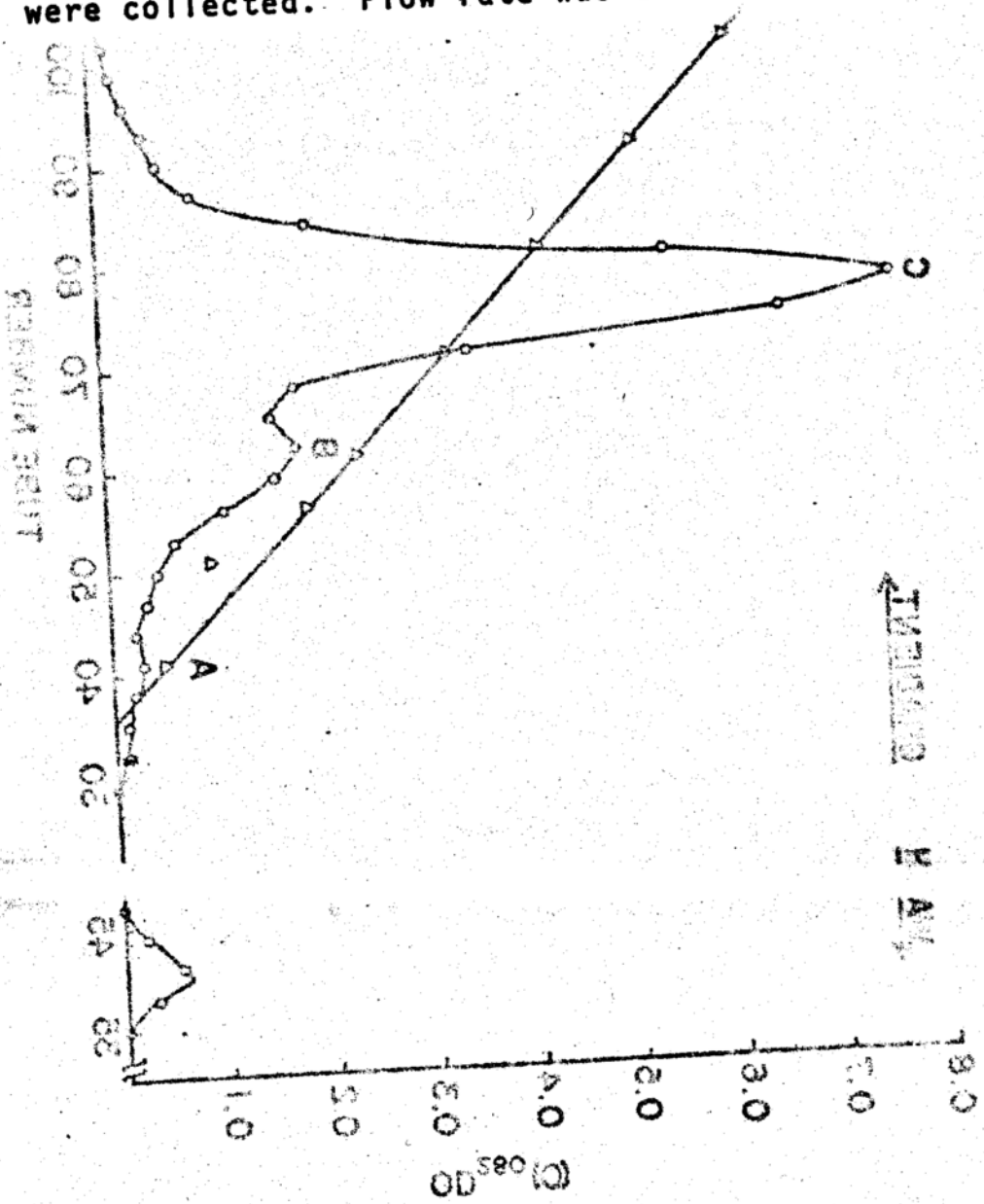
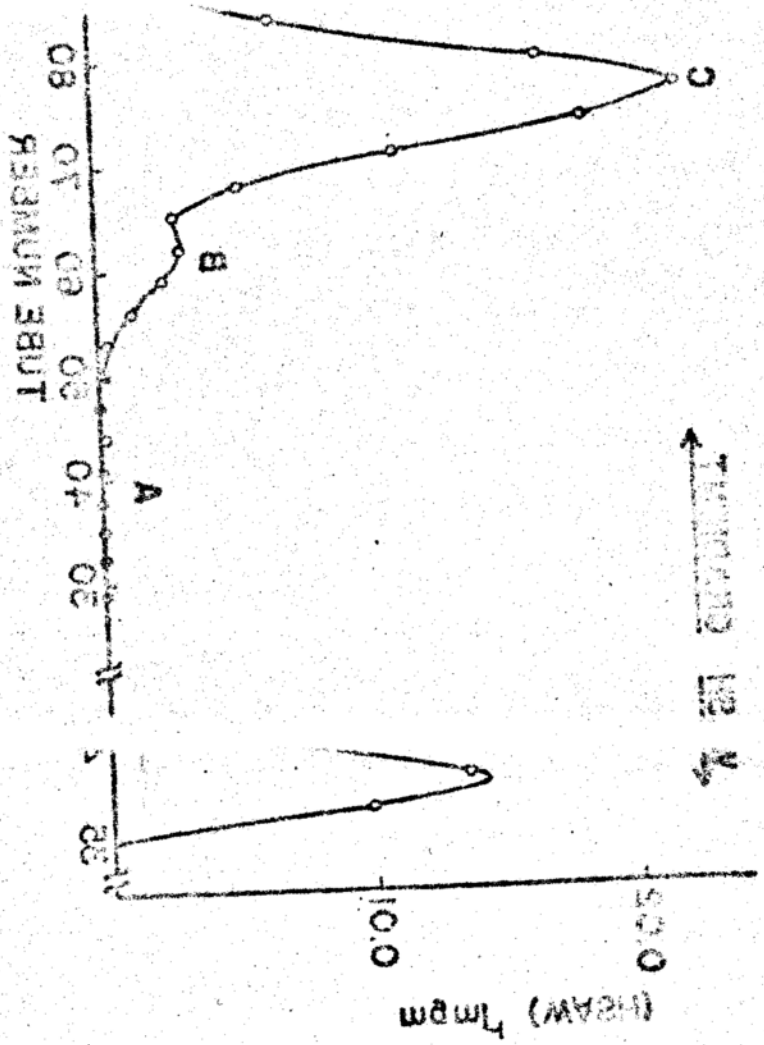


FIGURE 61. Chromatography of fraction 3 from Bio-Gel A 5m on DEAE Bio-Gel A. The column was 1.5 x 30 cm and eluted with a 0.01 M NH_4Ac wash (100 ml), followed by a gradient composed of 200 ml each of 0 M and 1 M NaCl in 0.01 M NH_4Ac buffer at pH 7.2. Fractions of 1.4 ml were collected. Flow rate was 56.0 ml h^{-1}. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard.



IV. DISCUSSION

As a result of this investigation it is apparent that conjunctival mucin exists in multiple fractions, each differing in molecular weight and charge density. This is in accord with the isolation of multiple fractions of tear mucins in the precorneal area by Iwata and Kabasawa (17). There is circumstantial evidence that the high molecular weight conjunctival mucins are aggregates of some monomer unit. The stage is now set for characterization of the various conjunctival mucins in regard to their amino acid and carbohydrate composition, structure, shape, degree of hydration and surface activity. The remainder of this report will be devoted to an evaluation of: (1) the fractionation schemes employed in this work, (2) the phenomenon of self association as it applies to conjunctival mucins, (3) proposed models for mucin structure, (4) the interrelationship between conjunctival and tear mucins, and (5) the composition of tear and selected epithelial mucins.

A. Extraction of Mucin from Rabbit Conjunctival Goblet Cells

An objective of this research was to isolate mucin from conjunctival goblet cells free from tear mucins. Therefore the manner in which the samples were handled was of primary

almost indefinitely if the temperature is low enough (140), all operations from tissue homogenization to chromatographic elution were conducted at 4°C in a cold room. Similarly, to optimize stability of the glycoproteins on storage, immediately after its removal from the rabbit, the conjunctiva was frozen at the temperature of a dry ice-acetone mixture. A consequence of this procedure is that ongoing glycoprotein synthesis would be arrested and, depending on their quantities, 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 (141) was isolated. The isolation of membrane glycoproteins often requires treatment of the cells with enzymes such as trypsin (142,143), or chemicals such as lithium di-iodosalicylate, phenol, aqueous pyridine and sodium dodecyl sulfate (144, 145). Since none of these agents was employed in the present study, the above assumption was a justifiable one to make.

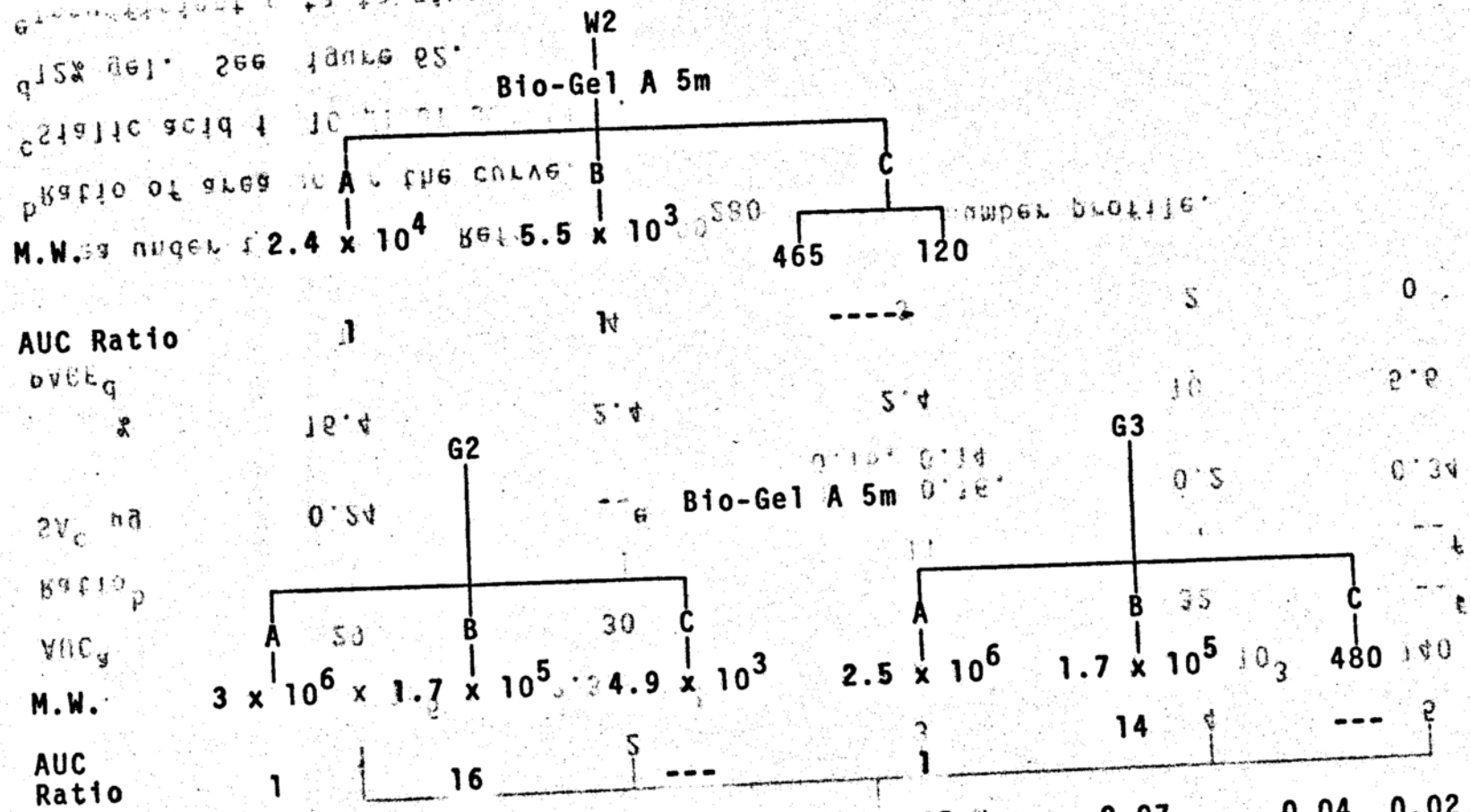
C. Summary of Chromatographic Results

At this point it is worthwhile to summarize the results obtained by chromatographing the crude protein extract according to two different schemes. Scheme III is for chromatography of the extract on DEAE Bio-Gel A followed by Bio-Gel A 5m, while Scheme IV is for chromatography of the extract in the reverse order.

Both schemes yield multiple glycoprotein fractions. This is in keeping with the observation that mucins, unlike proteins, are polydisperse and microheterogeneous macromolecules (149) and tend to yield several fractions during gel filtration or ion exchange chromatography. Table XV presents examples of mucins known to yield several fractions during isolation from their respective sources. In general, the number of fractions for a given mucin ranges from two to five and they differ primarily in their threonine, serine, sialic acid and carbohydrate contents, as well as molecular weights.

Insofar as the conjunctival crude extract is concerned, anion exchange chromatography (DEAE Bio-Gel A) yields seven fractions, in close agreement with the seven bands observed on electrophoresing the extract on a 12% polyacrylamide gel (see Figure 27). These fractions appeared in the chromatogram in the order of increasing number of negative charges. Notice that the order of elution did not strictly follow the

SCHEME III (continued)



SCHEME IV (continued)

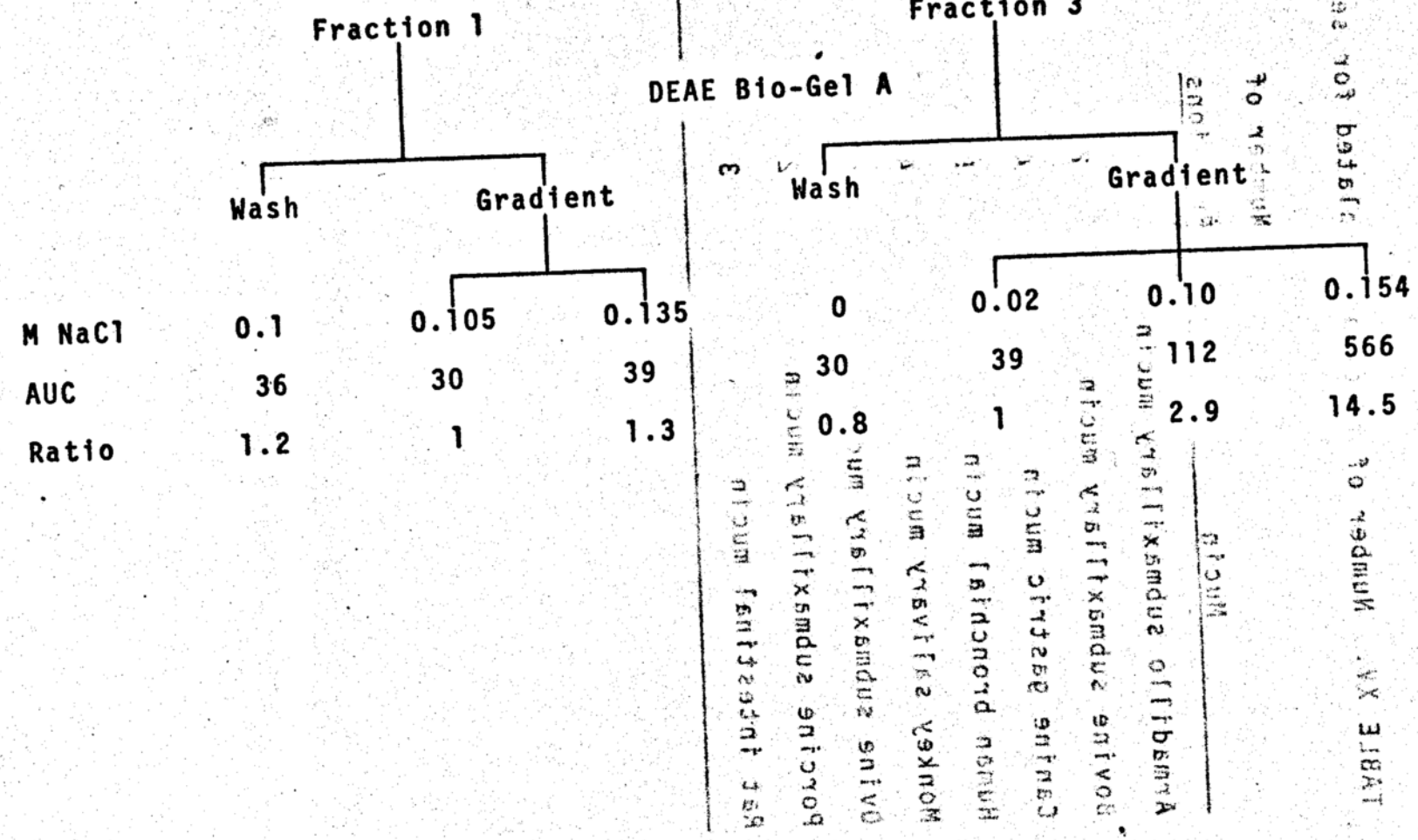
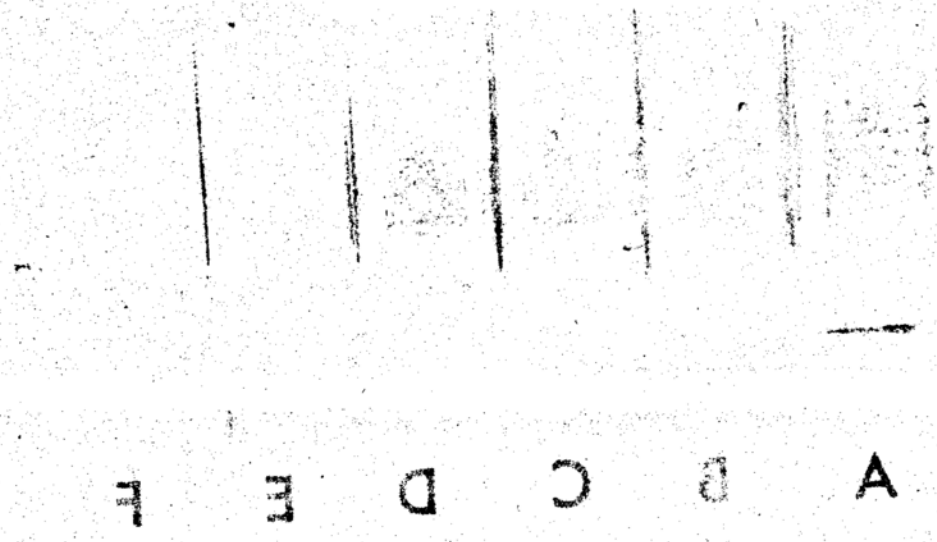


FIGURE 62. PAGE of fractions obtained by chromatographing conjunctival crude extract on a Bio-Gel A 5m column (1.5 x 60 cm). Flow rate = 8.8 ml h^{-1}. 12% Bio-Phore gels were used.

Key:

Gel	Peak	Tube # (Refer to Fig. 46)
A	A	25
B	B	37
C	C	45
D	D	64
E	E	79
F	Conjunctival crude extract	



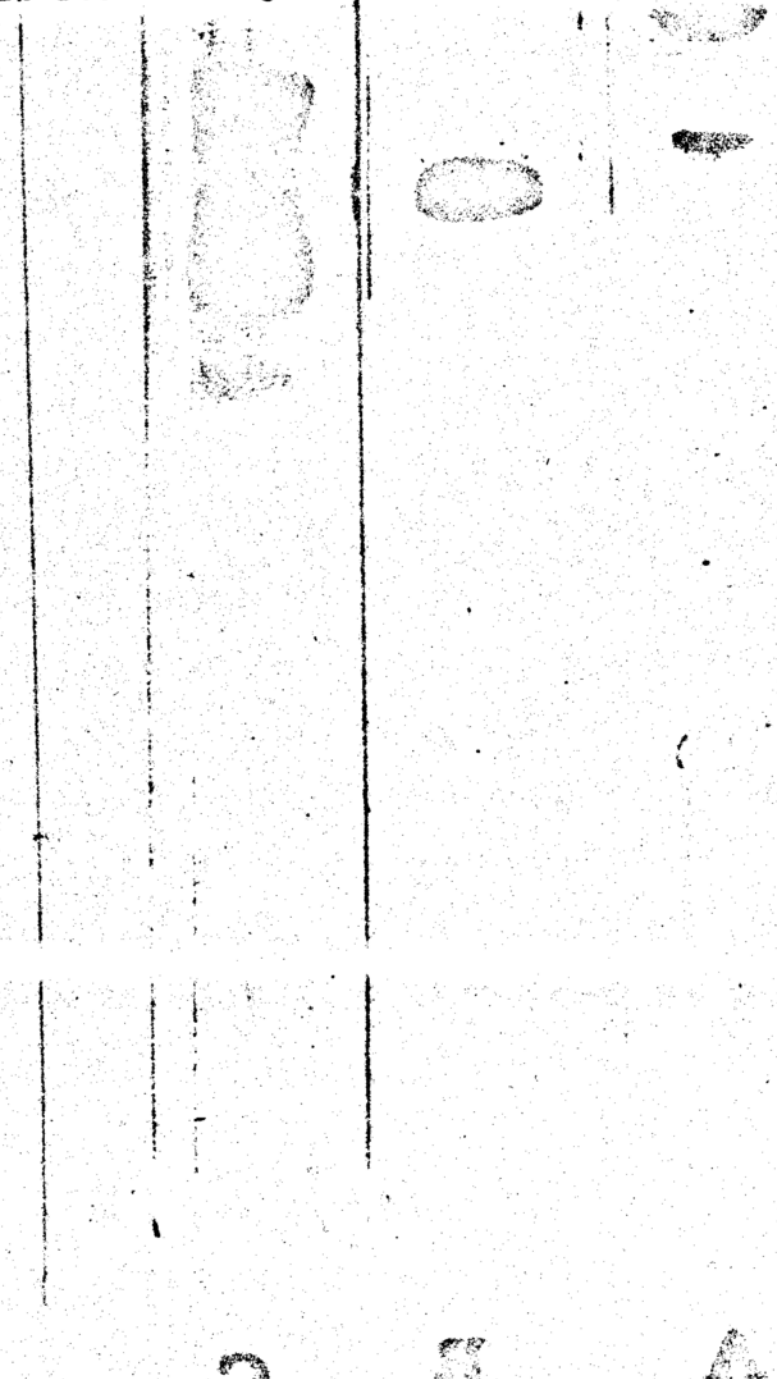
content of sialic acid, which, at pH 7.2, was practically 100% ionized.[11] This suggests that the distribution of sialic acid in the molecule, the acidic amino acids glutamic and aspartic acids, and the glycoprotein's tertiary and quaternary structures are more important determinants of the molecule's overall charge density than total sialic acid content. It must be mentioned that the order of elution of canine gastric mucin fractions from DEAE cellulose was inversely related to sialic acid content also (153).

Out of the seven DEAE Bio-Gel A fractions only three (W2, G2 and G3) were chromatographed further on the gel filtration medium Bio-Gel A 5m.[3] W1 and G1 were not examined because of limitations imposed by their minute quantities. W3 was not examined because it did not contain proteins according to the Bio-Rad protein assay.

An examination of the electrophoretic patterns due to G2 and G3 (gels A and B in Figure 27) reveals that together they practically accounted for all the bands seen in the conjunctival crude extract (gel C in Figure 27). Moreover, at least three bands (at 0, 2 and 3 cm from the origin) overlapped, reflecting, in part, the slight overlap between G2 and G3 as they were eluted from the DEAE Bio-Gel A column. Another noteworthy feature is that two bands (at 0.5 and 0.8 cm) were present in G2 but not in G3, while four bands (at 1.3, 4, 5.5, and 7.4 cm) were present in G3 but not in G2.

FIGURE 63. A comparison of electrophoretic patterns of DEAE/Bio-Gel A 5m, G2 #22 (gel A), DEAE/Bio-Gel A 5m, G3 #23 (gel B), and Bio-Gel A 5m, Fr.1 #25 (gel C) on 12% polyacrylamide gels. For key see legend of Figure 35.

FIGURE 64. A comparison of electrophoretic patterns of DEAE/Bio-Gel A 5m, G2 #38 (gel A), DEAE/Bio-Gel A 5m, G3 #38 (gel B), and Bio-Gel A 5m, Fr.3 #45 (gel C) on 12% Bio-Phore gel. See text for details.



approximately 15 times that of the one containing species with molecular weight in the order of 10^6. The significance of these observations is three-fold. First, 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 NaCl concentration. Second, each species of a given molecular weight existed in two different charge densities, since it was eluted at NaCl concentrations (0.032 M and 0.118 M) differing by a factor of approximately four. This means that four different molecular species, in suitable combinations of molecular weight and charge density, were present in G2 and G3. It should be noted that the species with molecular weight in the order of 10^6 has been shown to elute from DEAE Bio-Gel A at three NaCl concentrations: 0.1 M, 0.105 M and 0.135 M. Third, a rapid equilibrium may exist between the two species. Although aggregates have been reported for glycoproteins (see section IV.D), the 10-fold difference suggests a 10-mer with the molecular weight of the monomer being in the order of 10^5 and a 10-mer with monomer of this size is difficult to visualize geometrically.

Three bands emerged upon performing PAGE on the content of the tube corresponding to the maximum of peak B given by G2 on Bio-Gel A 5m, suggesting charge inhomogeneity (see Figure 64). In contrast, only one band was given by the

dissociation when it was subsequently exposed to a lesser charged medium, i.e., the surface of a Bio-Gel A 5m bead. This suggests hydrophobic interaction and other short-range interactions as the stabilizing factor, which is the case with protein-protein association (158). The validity of this proposed mechanism has yet to be proven since the M.W. of the species resulting from chromatographing fraction 3 (M.W. = 5.7×10^4) of Bio-Gel A 5m on DEAE Bio-Gel A had not been rechromatographed on a gel filtration medium. Interestingly, chromatographing the Bio-Gel A 5m fraction with M.W. = 5.7×10^4 on DEAE Bio-Gel A yielded a fraction that eluted at about the same NaCl concentration (0.154 M) as DEAE fraction G3 -- the fraction containing the 1.7×10^5 species. It should be instructive to study how the nature of the charge borne by the ion exchange gel influences trimer formation. Scheme V summarizes the behavior of the glycoproteins concerned when chromatographed under the specified conditions.

Finally, a species that was recovered in Scheme III but not Scheme IV was the one with M.W. = 2.4×10^4 (fraction W2 in Scheme III). This was not highly charged since it emerged prior to commencement of the NaCl gradient. Suffice it to say at this point that such a fraction also has been isolated from tear mucin clots by Iwata and Kabasawa (17). Its identity has yet to be revealed; it may be a basic

monomer unit of conjunctival mucins. Scheme VI presents the overall behavior of glycoprotein fractions when chromatographed under the conditions specified.

D. Preliminary Evidence of Self Association of Conjunctival Mucins

It has been mentioned in the preceding discussion that conjunctival mucin may self-associate to yield several species including the one with a molecular weight of $\sim 10^6$. The phenomenon of self association has been observed in over 300 proteins (especially those with high molecular weights) (159,160). According to Reithel (159), single polypeptide chains with molecular weight exceeding 6.6×10^4 do not exist, and proteins with molecular weights exceeding that should be considered as aggregates of monomer units. Dimers and tetramers are common. Multimers with odd number of monomers are rare; only 10 out of the 300 proteins listed by Klotz et al. (160) have an odd number of monomers. It must be noted that a typical monomer has a molecular weight in the order of 1×10^5. With the exception of large enzyme complexes and viruses, few proteins have more than 12 monomers.

Several factors influence self association. They are protein concentration, the presence of cofactors and of cysteine and other specific residues in a protein molecule,

and the pH of the medium. Only a few examples will be presented to illustrate each factor.

The familiar example of blood clot formation essentially is aggregation of fibrin monomers, at the proper concentration, through hydrogen bonding between tyrosine donor and histidine acceptor groups (161). Flagellin from the flagella of Proteus vulgaris at high salt concentration aggregates to molecules with M.W. = 1-2 million (162). Insulin at high salt concentration also aggregates (163). The binding of AMP to phosphorylase a, a dimer itself, has been shown (164) to trigger its transformation to phosphorylase b, a tetramer.

Several glycoproteins exist in an associated form. They include canine tracheal mucin, bovine, canine, ovine and porcine submaxillary mucins (165,166,213). These mucins are characterized by their resistance to deaggregation by urea and guanidine HCl as well as sodium dodecyl sulfate, agents known to disrupt hydrogen and hydrophobic bonds (156, 165-167). The erythrocyte membrane glycoprotein, with a M.W. $\sim 10^6$ (monomer M.W. $\sim 10^5$), behaves similarly towards 0.5% sodium dodecyl sulfate (168,169). This is to be contrasted with the 4-, 8-, 16-, and 32-mers of urinary glycoprotein (highest M.W. $\sim 7 \times 10^6$, monomer M.W. $\sim 2 \times 10^5$); they deaggregate in a 6 N urea solution (170).

One final example is on sucrase-isomaltase, a

positively charged groups, has been shown to separate the monomers of insulin and albumin from their dimers (176,177). Similarly, bovine pancreatic ribonuclease A has its aggregates, that are stable upon subsequent chromatography on Sephadex G-25, separated from the monomer by sulfoethyl-Sephadex, a cationic exchange gel (178). Horse heart cytochrome c gave similar results when chromatographed on Amberlite IRC-50, a weak cationic exchange resin (179). It is not clear if the monomer-multimer equilibrium exists in the bulk. Nevertheless, by preferentially adsorbing the multimers, the ion exchange gel appears to shift the equilibrium in favor of multimers, thus promoting aggregation.

The mechanism by which aggregation is promoted in the microenvironment of the gel surface has yet to be elucidated. It cannot be a pH effect because the microenvironment pH is higher than the bulk pH due to repulsion of H^+ ions from the positively charged surface. The higher pH favors ionization of sialic, glutamic and aspartic acids, and the attendant electrostatic repulsion among the molecules concerned is unfavorable towards 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 conceivably can

by oligosaccharide chain entanglement. Hill et al. (183) has studied in detail the aggregation of ovine submaxillary mucin and its desialylated (asialomucin) and deglycosylated (apomucin) variants. They found that the apomucin did not aggregate whereas the parent ovine submaxillary mucin and asialomucin did, indicating that the carbohydrate portion of the molecule, with a propensity of hydrogen bonding sites, was necessary for aggregation.

In section IV.F, the physiological significance of trimer formation will be explored.

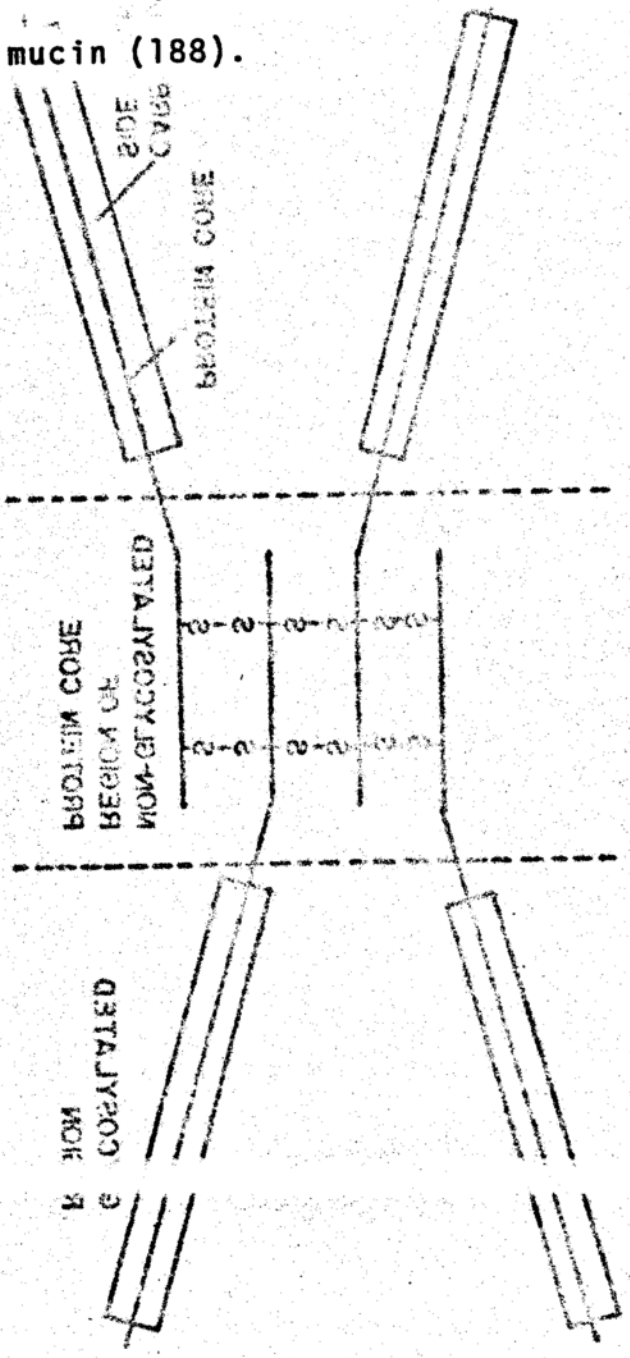
E. Proposed Models of Mucin Structure

Closely related to self aggregation of glycoproteins is their structure. The definitive structures of mucin are, at present, unknown, but several proposed structures are available. The proposed structures to be discussed here concern bovine cervical mucin and porcine gastric mucin. In addition to hydrophobic and hydrogen bonding that also plays an important role in self aggregation, the covalent disulfide bonds are an important element in the proposed structures for these mucins.

Figure 65 depicts a model (Model A) for the structure of bovine cervical mucin as proposed by Bhushano Rao and Masson (184) and Roberts (185). It is postulated that bovine cervical mucin consists of an array of subunits (with

N
SS
S
S
C
① Glycosylated Region
② Nonglycosylated Region
③ Disulfide Bridge
④ Hydrophobic Bonding

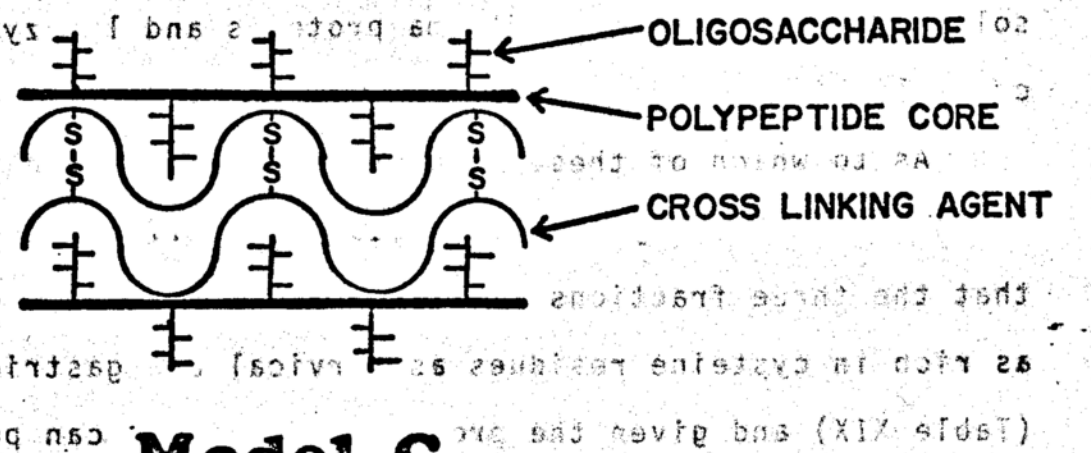
FIGURE 66. Tentative model (Model B) for the structure of porcine gastric mucin (188).



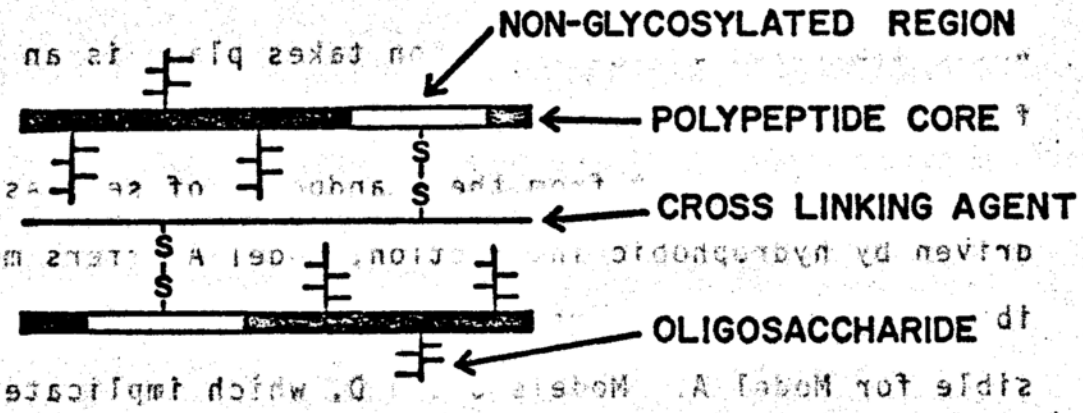
stable in 6 M guanidine HCl, 2% sodium dodecyl sulfate and 3 M KCl, suggesting that neither hydrogen, hydrophobic nor ionic bonding was the dominant factor in subunit linking. It was, however, unstable in 0.2 M mercaptoethanol, with a concomitant reduction in its molecular weight as was evident in ultracentrifugation equilibrium. This indicates disulfide bridge formation as an important link between subunits (189,190).

Figure 67 presents two additional models (Models C and D) for bovine cervical mucin (191). Both stress the importance of a crosslinking agent that is interposed between the various mucin molecules in such a manner that the negative charges of two mucin molecules are kept as far apart as possible. The crosslinking agent is bound to the mucin molecule by hydrogen or electrostatic bonds. In Model C, the disulfide bridges are important for integrity of the crosslinking agent. This model is compatible with the observation that such mucins as bovine and ovine submaxillary mucins are practically devoid of cysteine residues (151).

In Model D, the mucin molecules are interwoven and held together by disulfide bridges located in the non-glycosylated portion of the mucin molecule. The existence of a crosslinking agent for bovine cervical mucin was first suggested by Gibbons (192). Schumacher and Pearl (193) and Elstein (194) have speculated on the possible involvement of



Model C



Model D

detects only free lysozyme. Needless to say, the choice of a model must, among others, be consistent with the physiological roles performed by conjunctival mucin. An attempt will be made here to delineate some of the constraints imposed on conjunctival mucin structure insofar as present knowledge in its physiological roles allows.

As indicated in the Introduction, mucin, despite its high negative charge density, is the most surface active component in the tear film. An important role assigned to it is its transformation of a low energy surface presented by the corneal epithelium to a higher one upon its adsorption to the surface. For the moment let us ignore the glycocalyx on the corneal epithelial surface. The corneal epithelium, with its high lipid content, is a region of low dielectric constant. Therefore, considerable work has to be expended to bring an unshielded ionized group on a glycoprotein molecule against the potential, due to the single image charge within the surface, to a given distance from the surface (197). Unless there is a hydrophobic region in the mucin molecule to at least partially offset the electrostatic charges, the work required would be so enormous that adsorption onto the corneal epithelium is hindered. While this picture is probably realistic for the adsorption of mucin to the monolayer formed by meibomian lipids at the air/tear interface, it is unlikely to be so at

acids) portions, as proposed in Model A, appears to be the one most favorable towards adsorption. Such a structure would readily allow interaction with both interfaces to occur. As a corollary, multimers formed as a result of hydrophobic bonding among the monomers will now experience difficulties, from the thermodynamic and kinetic points of view, in adsorbing onto both interfaces. This is because the hydrophobic regions would be shielded by the oligosaccharide side chains and deaggregation is necessary prior to adsorption. Nevertheless, as the oligosaccharide side chains gain prominence in the multimer, the solubility of mucin in tears and, as a consequence, tear film stability should be improved. Therefore, according to this picture, tear film stability depends on a delicate balance in the relative distribution of mucin monomers and multimers.

A physiological function frequently attributed to mucins is their lubricating properties. This is related to their ability to form gels and has been studied most thoroughly in gastric and intestinal mucins (156,157,201). As one might expect gel formation is closely related to self association. Human small intestinal mucin as well as porcine gastric mucin undergoes a rather sharp sol-gel transition within a narrow concentration range (157). Aggregation beyond a certain concentration is mediated by non-covalent forces (202), although covalent bonds, notably the disulfide

of mucin molecules.[9] Work must be expended to overcome the repulsive potential energy barrier due to the ionized sialic acid and geometry of the side chains.[10] Since, during the process, some of the hydrated water molecules are excluded from the interaction zone, the decrease in segmental entropy in the interaction zone conceivably can be overriden by the increase in configurational entropy of the excluded water molecules.[2] The net result is a decrease in the overall free energy of the system, thus favoring multilayer formation. Here again, different mucin fractions conceivably show different tendencies toward phase separation and the success of multilayer formation may depend on a delicate balance in distribution among them.

F. Mucin Fractions in Conjunctival Crude Extract and Tear Mucoid Clots

With the isolation of various glycoprotein fractions from the conjunctiva and its goblet cells, it is now possible to explore the interrelationships among conjunctival mucins and those in the precorneal area.

Table XVI lists the number of glycoprotein fractions isolated from the conjunctiva in the present study and tear mucoid clots in the study by Iwata and Kabasawa (17). Quite unexpectedly the number of glycoprotein fractions from the conjunctiva was not much larger than that from tear mucoid

clots. It is clear that the conjunctiva contained a high molecular weight species (M.W. = 1.9×10^6) that apparently was absent from tear mucoids. But this was an artifact since the Sephadex G-150 employed by Iwata and Kabasawa (17) had an exclusion limit of 4×10^5 which was lower than that of Bio-Gel A 5m (5×10^6) employed in the present study. Hence the species with M.W. = 1.9×10^6 might be present in tear mucoid also. While the fraction with M.W. = 4×10^5 constituted the major fraction in tear mucoid, this was not the case for the conjunctiva fraction with M.W. = 2.3×10^5. Instead the fraction with a M.W. = 5.7×10^4 was the major conjunctival mucin fraction. This fraction had a sialic acid content of 2.4% (compared with the corresponding tear mucoid fraction with a sialic acid content of 0.5%[7]), which was about seven times less than the fraction with a M.W. = 1.9×10^6. Supposing that the species with M.W. = 5.7×10^4 is a monomer for the one with M.W. = 1.9×10^6, association must have occurred in such a way to bury the sialic acid in the resulting aggregate, since the sialic acid content did not undergo a corresponding 33-fold increase. If the sialic

[7] The discrepancy in sialic acid is due to different sialic acid assays. The thiobarbituric acid assay was employed in the study by Iwata and Kabasawa (17) and, as indicated in the Experimental section, it underestimated sialic acid content due to degradation of the sialic acid liberated from the glycoproteins.

TABLE XVII. Molecular weight of fractions obtained by chromatographing (a) the crude extract directly on Bio-Gel A 5m and (b) the effluent from chromatographing the conjunctival crude extract on DEAE Bio-Gel A on Bio-Gel A 5m.

Fraction	Molecular Weight
A1[a,d]	1.9×10^6
G2[b]	2.5×10^6
G3	3×10^6
A2	2.3×10^5
G2	1.7×10^5
G3	1.7×10^5
A3	5.7×10^4
W2[c]	2.4×10^4
A4	1.2×10^3
W2	5.5×10^3
G3	4.9×10^3

[a] A denotes fractions obtained by chromatographing the conjunctival crude extract directly on Bio-Gel A 5m.

[b] G denotes fractions obtained by chromatographing the gradient effluent (from chromatographing the conjunctival crude extract on DEAE Bio-Gel A) on Bio-Gel A 5m.

[c] W denotes fractions obtained by chromatographing the wash effluent (from chromatographing the conjunctival crude extract on DEAE Bio-Gel A) on Bio-Gel A 5m.

[d] The number represents fraction number.

cationic exchange properties. Nevertheless, the possibility that an anionic exchange matrix exists between the cell membrane proper and the glycocalyx cannot be ruled out. Thus, loss of integrity of the matrix, coupled with disturbances in electrolyte balance and fluid flow, will cause a shift in the chemical nature of mucin fractions in the precorneal area. Its consequence is tear film instability.

G. A Comparison of Tear Mucin Composition with Selected Mucins (Glycoproteins)

Although the characterization of various glycoprotein fractions isolated from the conjunctiva is outside the scope of this work, there is circumstantial evidence that conjunctival and tear mucins are similar. It is of interest to compare tear mucins in regard to their carbohydrate and amino acid composition, as well as their molecular weights, with other mucins (glycoproteins). These parameters are presented in Tables XVIII-XX.

The molecular weights of conjunctival and tear mucin fractions span the spectrum of molecular weights encountered with epithelial mucins such as bovine submaxillary mucin and plasma glycoprotein such as α_1-acid glycoprotein. In Table XVIII it can be seen that tear mucins contain 4 to 10 times less hexosamines than the other glycoproteins listed. Like α_1-acid glycoprotein there is a higher protein than

TABLE XIX. Amino acid composition (moles/100 moles) of tear mucins and selected mucins (glycoproteins).

									Tear		
Amino Acid	BSM	OSM	MSM	RSIM	HBM	BCM	HESG	AAG	I	II	III
Lys	0.1	0.3	1.4	3.3	2.9	2.9	3.5	7.8	5.9	8.0	6.8
His	--	0.7	1.1	1.6	1.7	0.9	3.8	1.8	2.2	3.0	1.1
Arg	4.2	3.6	1.7	1.4	1.9	3.3	4.1	5.0	4.3	3.3	3.5
Asx	1.7	1.7	2.0	5.6	6.5	4.5	--	11.0	9.4	8.4	8.2
Thr	14.3	14.8	20.0	23.9	18.0	23.3	13.8	8.6	7.5	8.5	5.1
Ser	20.0	17.5	15.9	12.2	10.6	13.5	13.6	3.5	7.0	5.7	4.5
Glx	6.3	5.3	5.0	6.8	9.0	7.4	10.0	18.3	11.3	10.0	14.3
Pro	11.2	9.6	5.5	12.2	17.4	9.8	6.5	4.0	6.4	7.8	4.6
Gly	18.8	19.8	23.8	9.3	7.5	6.4	6.8	3.5	7.3	4.1	3.8
Ala	12.8	13.6	10.2	5.7	7.5	8.5	6.8	4.4	7.4	11.6	12.1
Cys	--	0.8	0.6	2.0	--	2.6	tr	1.4	2.7	4.3	3.2
Val	6.7	6.0	6.4	4.7	5.5	7.6	7.7	4.7	7.5	5.9	8.8
Met	--	0.4	0.1	1.1	--	1.1	tr	0.6	1.2	0.3	0.6
Ile	1.4	1.3	2.5	3.3	4.3	1.5	4.5	4.8	4.1	2.7	3.8

TABLE XX. Distribution of amino acids (moles/100 moles) in selected mucins (glycoproteins).

Amino Acids	BSM[a]	OSM	MSM	RSIM	HBM	BCM
Ser, Thr, Pro	45.5	41.9	41.4	45.5	46.0	46.6
Acidic	8.0	7.0	7.0	15	15.5	11.9
Basic	4.3	3.9	4.2	6.9	6.5	7.1
Aromatic	--	--	1.9	4.4	2.9	3.6
Hydrophobic[b]	35.9	38.3	28.3	32.8	41.3	33.9
Thr/Ser	0.72	0.85	1.26	1.96	1.70	1.73

			Tear		
Amino Acids	HESG	AAG	I	II	III
Ser, Thr, Pro	33.9	16.1	20.9	22.0	14.2
Acidic	10.0	29.3	20.7	18.4	22.5
Basic	11.4	14.6	12.4	14.3	14.1
Aromatic	10.9	12.7	9.6	10.4	10.3
Hydrophobic	29.0	31.3	39.5	41.7	45.8
Thr/Ser	1.01	2.46	1.07	1.49	1.13

[a] For abbreviations see footnotes of Table XVIII.

[b] Hydrophobic amino acids include Ala, Ile, Leu, Met, Phe, Pro and Val.

oligosaccharide side chains, or to the degree of saturation of the peptide core; in short, mucin structure. The proline content is also low, meaning a more rigid polypeptide backbone. There is, however, a high content of lysine, aspartic acid, glutamic acid, tyrosine and phenylalanine. As can be seen in Table XIX, tear mucins possess the highest cysteine content and this may be significant in either intra- or interchain disulfide bond formation, an important element in mucin structure. Notice in Table XX the relatively high content of hydrophobic amino acids found in tear mucins. This can influence not only their tendency towards self association but also their structure.

This brief survey of amino acid and carbohydrate composition indicates that tear (and probably conjunctival) mucins are not typical epithelial mucins. In some aspects they resemble α_1-acid glycoprotein. But, on balance, tear mucins appear to be glycoproteins in their own class. The high mannose content, the low sialic acid content, the absence of galactosamine, and the relatively high cysteine content may be some of the factors responsible for conferring unique structural features to tear mucins. Given the uniqueness of the eye as an organ, it should not be surprising to find tear mucins as unique epithelial glycoproteins. Finally, since the structure of a molecule is influenced by its composition and the function it serves depends

V. CONCLUSIONS

The work reported in this thesis represents the first of a series of studies designed to ultimately understand the structure of conjunctival mucin, the mechanisms by which it maintains a stable tear film, and its role in drug absorption.

Several glycoprotein fractions have been isolated from the conjunctiva. The preponderance of each fraction varies depending on whether gel filtration or anion exchange chromatography is the first step in the fractionation scheme. It is found that the conjunctival glycoprotein fractions bear a closer resemblance to tear mucin fractions in their molecular weights when anion exchange chromatography is the first step in the scheme. As discussed earlier, the discrepancy centers on the anion exchange fraction with a M.W. = 1.7×10^5 and the gel filtration fraction with a M.W. = 5.7×10^4. It is postulated that self association (trimer formation) induced by the microenvironment of the ion exchange gel occurs. The trimer appears to be more charged than the monomer, and its preferential removal by the ion exchange gel shifts the monomer-trimer equilibrium in its favor. It is further postulated that the same sequence of events exists during the transit of conjunctival mucin from the goblet cell to the precorneal area of the eye.

The observation that different isolation schemes lead to different conjunctival mucin fractions calls for a continuing effort to search for a scheme that yields the same conjunctival mucin fractions as they are synthesized in the goblet cells. The dependency of conjunctival mucin identity on the isolation scheme employed is intriguing because it yields information on how mucin structure and composition can be perturbed by a charged surface.

To separate conjunctival mucin free from protein contaminants one can employ affinity chromatography. This involves coupling onto a matrix lectins that have been shown to exhibit a specificity for the sialic acid found in mucins (210,211). To ascertain that the conjunctival mucin isolated is indeed derived from conjunctival goblet cells one can employ an immunofluorescence technique (156) or by injecting the animal with radiolabeled glucosamine followed by monitoring its appearance in the conjunctival goblet cells.

The importance of conducting a complete characterization of conjunctival mucin must be emphasized. Such an analysis should include elucidation of its chemical composition, as well as its primary, secondary, tertiary and quaternary structures. Attention needs to be focused on the heterogeneity in the carbohydrate portion of the molecule and this requires a specific separation technique such as

investigate tear and conjunctival mucins for their ability to adhere to cell surfaces including those of the corneal epithelium. Conjunctival mucin can play an important role in drug penetration into the eye, too. To date, virtually nothing is known about this aspect of drug disposition. About a decade ago Kakemi and his coworkers (213-216) observed that the amount of drug absorbed from the G.I. tract correlated well with its extent of binding to mucin lining the mucosa. They therefore postulated that drug binding to mucin constituted the first step in the absorption process. Their finding, however, was at variance with an earlier finding of Schanker et al. (217). These investigators observed negligible drug binding to mucin and the same extent of drug absorption regardless of the presence of mucin. Much of this contradiction necessarily arises from differences in drug selection and experimental design. Therefore, further studies are warranted to clarify the role of mucin as another barrier in drug absorption. Meanwhile, attention should be directed toward drug binding to mucin within the conjunctival goblet cells as a potential drug reservoir in the precorneal area.

Finally, although the concern of this thesis has been on an evaluation of conjunctival mucin in regard to its role in tear film stability, there is a need to explore that of

TABLE XXI. Current state of knowledge about tear film components and their properties.

Known Elements

1. Gross structure of tear film
2. Goblet cells as source of mucin
3. Presence of multiple mucin fractions in the precorneal area of the eye
4. Gross chemical composition of mucin

Unknown Elements

1. Microstructure of tear film
2. Mechanism by which mucin stabilizes tear film
3. Relative contributions of charges, wetting properties of mucin and surface characteristics of microvilli in tear film stability
4. Structure of mucin monomer and aggregates
5. Factors influencing relative proportion of mucin fractions
6. Manner and rate of turnover of mucin in the precorneal area
7. Nature and strength of forces of interaction between mucin and corneal epithelial surface, including the glycocalyx
8. Physiological functions of conjunctival mucin
9. Role of meibomian lipids in tear film stability
10. Role of mucin in ocular drug absorption

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VII. APPENDICES

Corneal Metabolism of Pilocarpine in Pigmented Rabbits

by

Vincent Hon-Leung Lee, Ho-Wah Hui and Joseph R. Robinson

ABSTRACT

Cornea, aqueous humor and iris-ciliary body levels of pilocarpine and its metabolite pilocarpic acid were determined in mixed breed rabbits following topical dosing with 25 µl of 1×10^{-2} M pilocarpine. From the time-drug concentration profile it is clear that extensive metabolism of pilocarpine occurs in the cornea of pigmented rabbits. This binding contrasts sharply with similar studies in albino rabbits where relatively low levels of pilocarpic acid were observed. It is found that the first-order metabolism rate constant in albino rabbits is approximately two orders of magnitude smaller than pigmented animals. A significant observation from this finding is the possibility that the reported greater dose requirement for heavily pigmented individuals may not be due to drug-pigment binding alone but also to extensive corneal drug metabolism.

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chemicals used were either reagent or analytical grade and were used as received.

Methods

- Preparation of 0.01 M Pilocarpine Solution - A 0.01 M pilocarpine solution, isoosmotic with tears, was prepared using pH 6.24 Sorensens phosphate buffer. Either 0.25 or 0.50 mCi, depending on the counting efficiency required, of tritiated pilocarpine was added to the 0.01 M pilocarpine solution immediately prior to each experiment. Addition of the radiolabelled pilocarpine to the drug solution did not appreciably influence the solution concentration.

- Drug Disposition Studies - Twenty-five microliters of drug solution were instilled onto the cornea of fully awake rabbits. During instillation the lids were withdrawn from the globe of the eye but were immediately returned to their normal position after instillation. Both eyes of the test animal were used but the dosing time was staggered so that one animal might, for example, be used for a 10 and 30 minute time point. Animals were sacrificed at varying times, the corneal surface thoroughly rinsed with normal saline and blotted dry, and anterior segment samples obtained. Aqueous humor samples were aspirated from the anterior chamber followed by removal of the cornea and iris-ciliary body. Drug levels were then determined in each

extraction efficiency was highly reproducible and remained at the 14.8 ± 0.2% over the concentration range of interest. Extraction efficiency was determined using excised corneas and known quantities of pilocarpine and pilocarpic acid.

Iris-ciliary body samples were treated in a manner identical to that for the cornea. Pilocarpic acid extraction efficiency from iris-ciliary body was 23 ± 0.95% and was highly reproducible.

For all samples, suitable corrections were made for background counts and the data in counts/minute were converted to micrograms through the use of standards.

Results

A complete description of ocular disposition of pilocarpine in pigmented rabbits will be reported elsewhere. Of concern in this report is the question of corneal metabolism of pilocarpine. Table I presents the percent of total drug, due to pilocarpic acid, at various times post-instillation of pilocarpine in the cornea, aqueous humor and iris-ciliary body. Each entry in the table for the cornea and iris-ciliary body was determined with corrections for extraction efficiency. Such corrections were unnecessary in the case of aqueous humor.

The data in Table I were analyzed for statistical differences and the results are summarized in Tables II and III.

TABLE II. Significance testing[a] for percent of total drug due to pilocarpic acid in the cornea, aqueous humor and iris-ciliary body at selected times post-dosing.

Time (min)	Cornea vs Aqueous Humor vs Iris-Ciliary Body	p
10	30 minutes	0.5878 (N.S.)[b]
30	30 minutes	0.3529 (N.S.)
60		0.3408 (N.S.)
120	120 minutes	0.0038

[a]F-test.

[b]Not significant. The significance level chosen was 95%.

Aqueous Humor	30 vs 60 minutes	0.9561
Cornea	30 vs 60 minutes	0.9999
Iris-Ciliary Body	30 vs 60 minutes	0.9891

[a]Student's t-test.

[b]Not significant. The significance level chosen was 95%

As can be seen from Table II there is no statistical difference in the pilocarpic acid level in the cornea, aqueous humor and iris-ciliary body at the 10, 30 and 60 minute points. However, there is a significant difference at 120 minutes. Specifically, the pilocarpic acid level in the cornea, at this time, is 1.2 times higher than that in the aqueous humor and iris-ciliary body. One can also see in Table III that the level of pilocarpic acid in the cornea at 120 minutes is significantly higher than that at 30 and 60 minutes.

Discussion

Several analytical techniques were unsuccessfully attempted to resolve pilocarpine from its metabolites pilocarpic acid and isopilocarpine. The glc procedure described by Dziedzic et al. (4) for pilocarpine was unsuccessful in our hands. The assay involves derivatization of the compounds with heptafluorobutyric anhydride in the presence of trimethylamine and benzene, followed by glc separation and identification. This procedure was found to be incapable of resolving pilocarpic acid and pilocarpine, despite extensive variation in column packing and temperature conditions. HPLC appears to show promise as a technique to resolve pilocarpine from its two metabolites and, indeed, Urbanyi et al. (5), have reported an HPLC procedure for simultaneous

acid in the cornea at all time points implicates the cornea as the site of metabolism. The 120-minute point is especially noteworthy in this regard since almost all of the drug in the cornea is in the form of pilocarpic acid. One can create an alternative scenario to corneal metabolism by assuming metabolism occurs elsewhere and the metabolite is transferred to and stored in the cornea. This seems unlikely given the substantial level of pilocarpic acid at the 10-minute point and the difficulty of back diffusion to the cornea from either the aqueous humor or the iris-ciliary body.

That extensive metabolism should occur in the cornea is not surprising. Anderson et al. (6) demonstrated esterase activity in the cornea of the albino rabbit in the study of conversion of the prodrug dipivalyl epinephrine (DPE) to epinephrine. The metabolism rate constant in that study was 0.17 min^{-1}. What is surprising is that the corneal metabolism of pilocarpine should be so much greater in the pigmented than the albino rabbit. It is possible that either a specific esterase was involved in the corneal conversion of DPE to epinephrine or that a specific esterase for pilocarpine is present in pigmented but not albino rabbits. A third possibility may relate to the permeability characteristics of DPE and epinephrine as contrasted to pilocarpine and pilocarpic acid, i.e., esterases may be membrane bound

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APPENDIX B

by

Ion Exchange Resin as a Potential Topical Ocular Drug Delivery System

Vincent Hon-Leung Lee and Joseph R. Robinson

terms of achieving a therapeutically effective tissue level over a suitable length of time is the ultimate goal of ocular drug therapy. It is well known that before a drug can gain entry into the eye it must surmount several barriers imposed by the cornea as well as various physiological processes in the conjunctival sac. These physiological processes include solution drainage, tear turnover, conjunctival absorption and, in the case of pilocarpine, vasodilation (1). These processes act in concert to reduce the fraction of instilled dose available for corneal permeation. Over the years, attempts to improve ocular bioavailability have centered on delaying or eliminating solution drainage, hence the incorporation of viscosity builders (usually polymers) into a solution, and such dosage forms as suspensions, ointments, and ocular therapeutic systems including soft contact lenses and the Ocusert. With a reduction in solution drainage rate, contact time of the dosage form with the cornea increases, resulting in a modest increase in bioavailability. Some of these dosage forms have been systematically evaluated for the anti-glaucoma drug pilocarpine (2) and is the subject of a recent text (3).

The dosage form that has yet to be exploited for ocular drug delivery is the one that employs ion exchange resins. Ion exchange is an attractive method for sustaining drug delivery because, in theory, it relies only on the ionic

Materials and Methods

Materials. Tritiated pilocarpine alkaloid (New England Nuclear, Boston, MA), specific activity 4.165 Ci mmole^{-1}, was purified by vacuum evaporation immediately prior to use. Male albino rabbits (Klubertanz, Edgerton, WI), 1.5-2.4 Kg, were used throughout the study. Amberlite IRP-69 and IRP-88 were a gift from Rohm and Haas (Philadelphia, PA). Pilocarpine nitrate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were obtained commercially and were used as received.

Methods

-- Characterization of resins. The resins were conditioned according to the instructions supplied by Rohm and Haas[1], except that a batch technique was employed. The conditioned resin was characterized with respect to its volume/surface diameter (d_{vs}), solid content and exchange capacity. The results of such analyses are summarized in Table I.

-- Analytical methods

Pilocarpine nitrate was analyzed using standard isotopic techniques as well as the ferric hydroxamate method described by Brochmann-Hanssen et al. (13). The latter method was modified for assaying 1 ml of sample solution

[1]Amberlite Ion Exchange Resins. Laboratory Guide. Rohm and Haas Co., Philadelphia, PA.

using 1.75 M NaOH and 1.75 M HCl. The Beer's Law plot was linear over the concentration range of 1-4 x 10^{-4} M pilocarpine nitrate. The molar absorptivity at λ_{max} = 500 nm was 1.87 x 10^{2} M^{-1} cm^{-1}.

-- Uptake of drug by resin #69. Approximately 2.5 g of conditioned resin #69 were dispersed in 50 ml of a 0.1 M pilocarpine nitrate solution. The suspension was stirred at a preset speed for a period of 3 hours. At selected times 1 ml samples were withdrawn from the medium through a pipet appropriately fitted with glass wool at its tip. The withdrawn volume was replaced with 1 ml of double-distilled water. The sample was analyzed spectrophotometrically using the ferric hydroxamate method. When the isotopic assay was employed, the uptake experiment was modified using 500 µl of pilocarpine nitrate solution and 25 mg of resin. Vacuum purified tritiated pilocarpine alkaloid was incorporated into the suspension to yield an activity of 6 x 10^{4} cpm per microliter of solution. The suspension was shaken manually and intermittently for three hours at room temperature.

-- Release of drug from resin #69. At the end of the uptake experiment, the resin was washed with three 10-ml portions of double-distilled water and allowed to dry to

reading was obtained. This was achieved within 600 seconds. The average pupil diameter thus obtained was 5.4 ± 0.03 cm (n = 103). Subsequent to dosing, miosis measurements were recorded every 100 seconds for the first 2400 seconds and every 300 seconds for the next 4800 seconds.

Miosis measurements were made using the following preparations:

1. Isotonic Sorensen's (SØR) buffer at pH 7.38
2. 0.05% suspension of resin #69 in isotonic SØR buffer at pH 7.38
3. 0.01, 0.05, 0.1 and 0.5 M pilocarpine nitrate solutions in isotonic SØR buffer at pH 7.38
4. 0.05% suspensions of resin #69 incorporated with the pilocarpine nitrate solutions listed in (3) above, and
5. 0.05% and 0.1% suspensions of resin #88 incorporated with 0.1 M pilocarpine nitrate solution.

It must be noted that the suspensions were prepared using the fines of the resins. Their particle size was estimated to be less than five microns.

Results

Uptake of pilocarpine by resin #69. Uptake of pilocarpine by resin #69 (functional group $-SO_3^-Na^+$) was almost instantaneous and maximal levels were achieved within 30

FIGURE 1. Release of pilocarpine nitrate from Amberlite IRP #69 into 0.154 M NaCl (●) and KCl (▲) solutions. Each time point represents an average of three trials. The error bars are smaller than the symbol and therefore omitted.

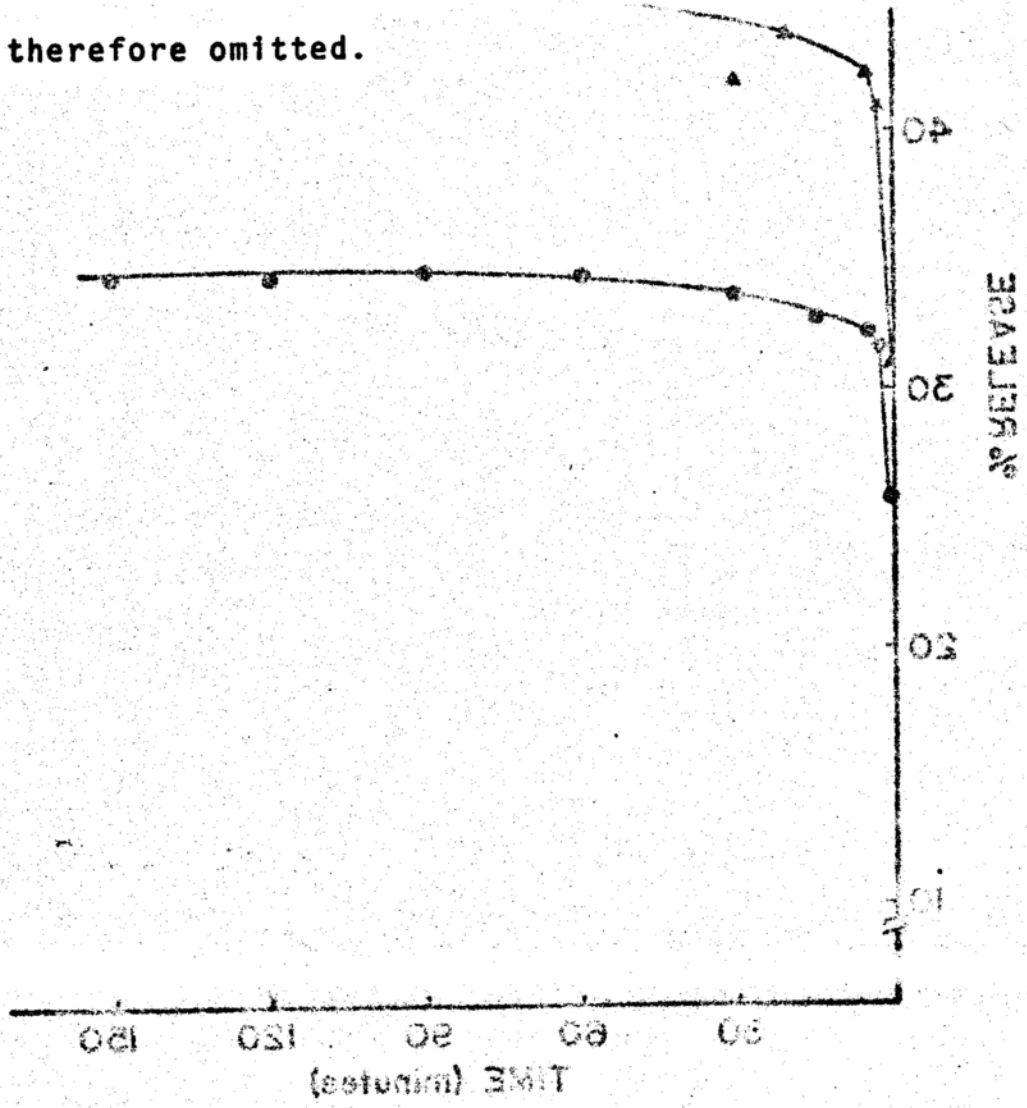


TABLE II. Uptake and release results for pilocarpine nitrate. The resin was Amberlite IRP #69.

Experiment	Isotopic Assay	Colorimetric Assay[a]
Uptake	58.5	94
Release KCl	5.0	45
Release NaCl	3.4	35

[a]Ferric hydroxamate method (13).

FIGURE 2. Miosis-time profiles for an aqueous solution (●), 0.05% suspensions of Amberlite IRP #69 (▲) and IRP #88 (■), all being 0.1 M in pilocarpine nitrate. Error bars are omitted for clarity. An average of 8 eyes were used for each profile.

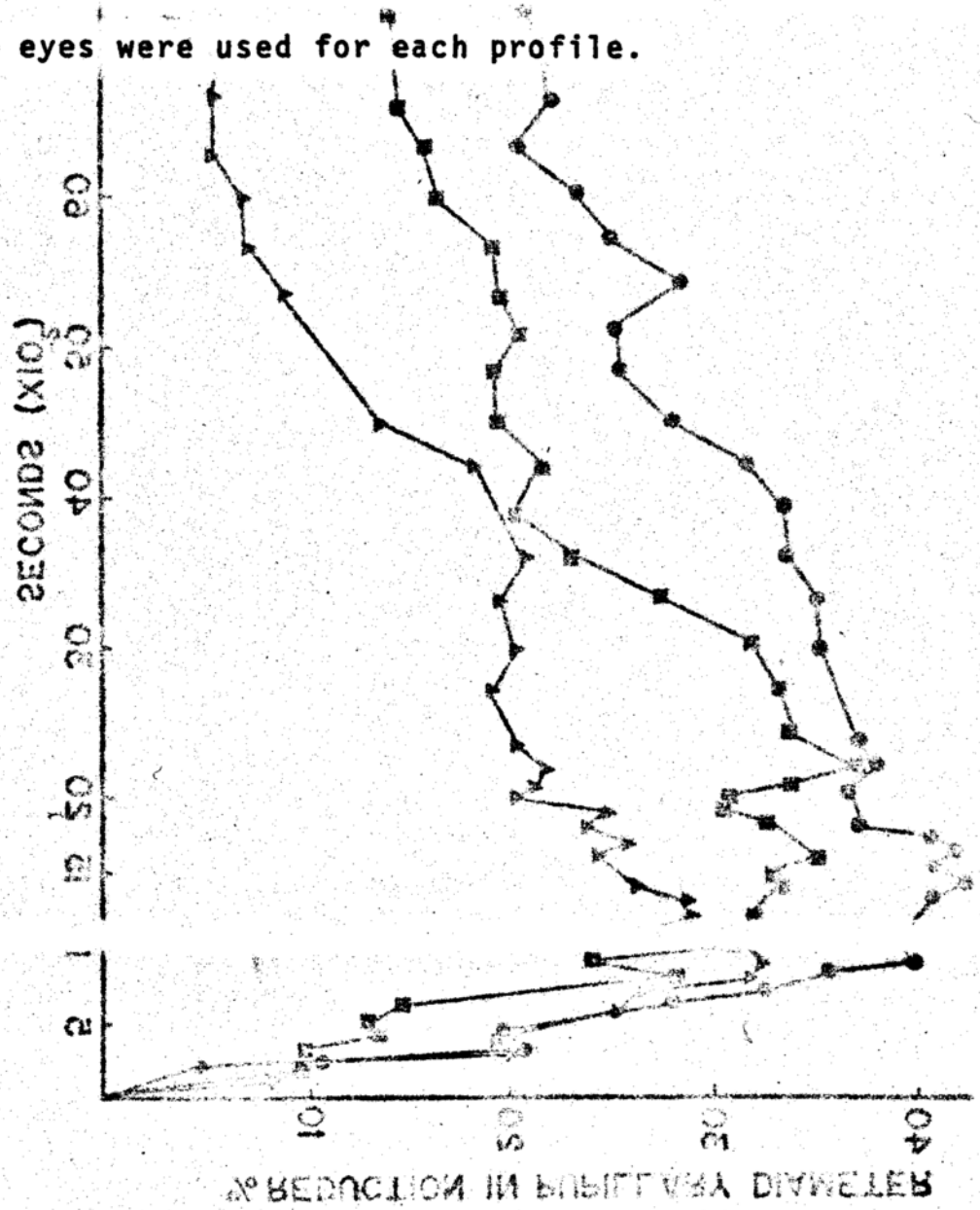


FIGURE 3. Miosis-time profiles for an aqueous solution (●) and a 0.05% suspension of Amberlite IRP #69 (▲), both containing 0.5 M pilocarpine nitrate. Error bars are omitted for clarity. An average of 8 eyes were used for each profile.

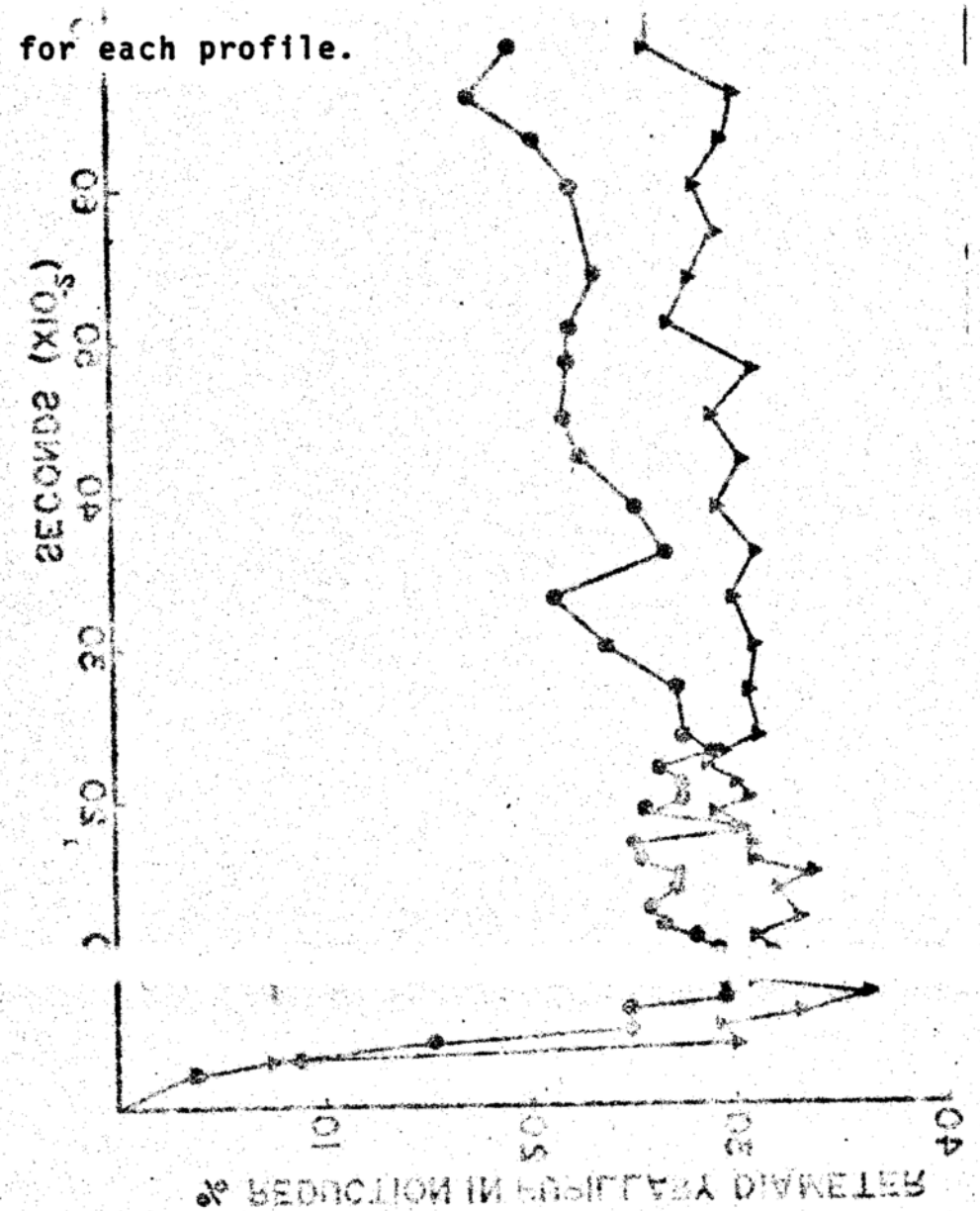
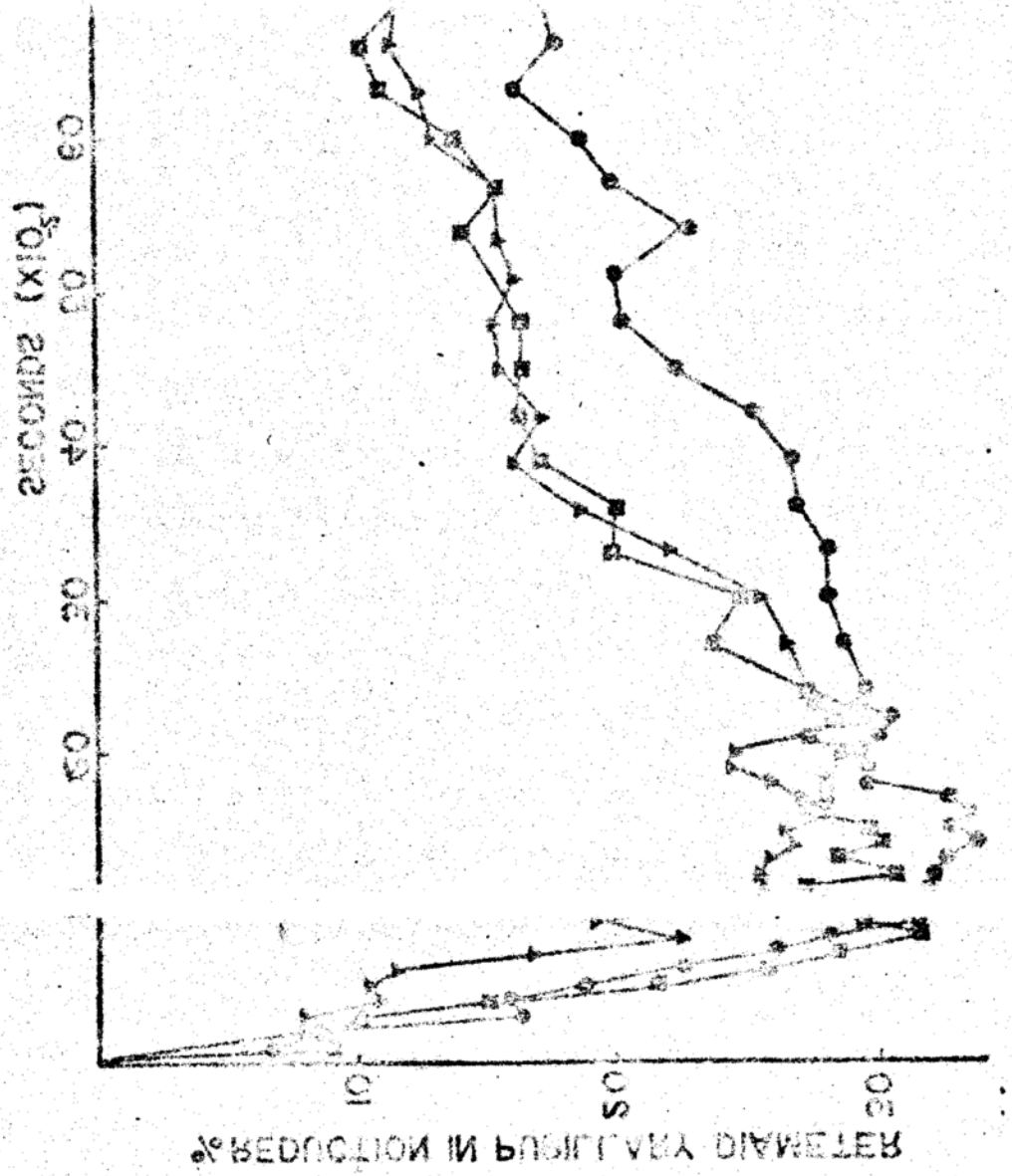


FIGURE 4. Miosis-time profiles for an aqueous solution (●) and 0.05% (▲) and 0.1% (■) suspensions of Amberlite IRP #88, all containing 0.1 M pilocarpine nitrate. Error bars are omitted for clarity. An average of 8 eyes were used for each profile.



(resin #88) concentration on miosis. At the 95% confidence level, the same degree of miosis was observed with both a 0.05% and 0.1% suspension. As in the case of resin #69, the resin suspension caused a less pronounced miosis than the corresponding solution.

Discussion For a given amount of pilocarpine administered as an ion exchange resin and a solution, an ion exchange resin is expected to produce a lesser miosis initially than a solution simply because a fraction of the dose is bound to the resin and this is unavailable for immediate absorption. As drug absorption proceeds, provided that a constant ionic composition in the environment about the resin surface is available, the bound pilocarpine will be released into tears eventually leading to a sustained miotic response. As shown in Figures 2-4, this was not observed with pilocarpine nitrate concentrations below 0.5 M. In fact, no statistically significant difference in miotic response was detected for a solution and a resin suspension, although the resin appeared to give a lesser miotic response than the solution. The roughly equivalent initial miosis observed with both dosage forms is not surprising in light of the enormous loss of drug solution to the drainage apparatus within minutes of instillation. It is an interesting

explained by loss of most of the resin to the nasolacrimal apparatus. This is likely since the particle size was less than the diameter of the smallest passage ways (0.3 mm, Ref. 14) composing the drainage apparatus. It may also be due to lack of affinity of the resin for the corneal or conjunctival surfaces. This is likely since the charge of the resin is similar to that of the corneal and conjunctival surfaces, both being negative. As indicated in the preceding paragraph, all the bound pilocarpine should eventually be released. So, there is a distinct possibility that sustained miosis was provided by the resin but not detected due to insensitivity of miosis to variation in pilocarpine concentration (15).

Another explanation for the lack of sustained response over the time course of observation is that the drug was removed more rapidly than was released, or corneal permeation, not drug release, was rate-limiting. In principle, the first alternative can be partly overcome by increasing the dose administered through dispersing the resin in a more concentrated pilocarpine nitrate solution. Indeed, with a 0.5 M pilocarpine nitrate solution, a sustained response was obtained with the resin. In this instance, the larger amount of pilocarpine initially in solution rapidly established a high initial tear drug concentration, thus saturating a sizeable fraction of the receptors responsible for

advantage of sustaining miosis at the usual therapeutic dose.[6] Whether this extends to intraocular pressure lowering, the therapeutic effect sought, is not known.[6] However, this conclusion may have to be modified if drug concentration in various ocular tissues is monitored because relative to miosis it is more sensitive to variation in drug concentration in the precorneal area.[6]

At this point it is appropriate to establish several criteria for a successful ocular sustained release system utilizing the ion exchange principle.[7] In addition to the availability of a proper ionic composition at the absorption site, an adequate tear flow rate can be important because it influences tear ionic composition.[4] This can be assessed by repeating the experiment using anesthetized rabbits. Insofar as the resin is concerned, the degree of saturation of the resin by the drug is important because both the bound drug and the native counterions will compete for the counterions in the eluting medium.[7] Probably the native counterion will be preferentially displaced since with the bound drug there is the additional hydrophobic bonding to be overcome.[7] Saturation of the resin's binding sites, i.e., absence of native counterions, may explain the sustained miosis only observed with the resin loaded with 0.5 M pilocarpine nitrate.

As a result of this study it is clear that additional

released from resins entrapped in the lower fornix.

Finally, a noteworthy finding in this study concerns the divergence in uptake and release results given by the isotopic and colorimetric assays. Even though the amount of pilocarpine available for binding to the resin was below the resin's binding capacity (see Table I), the isotopic assay indicated an uptake much below 100%. This may be attributed to tritium exchange between the label on the pilocarpine molecule and water molecules in the dispersion medium. One would expect, therefore, that the isotopic assay overestimated the extent of release. That this was not observed suggested that upon binding onto the resin, the pilocarpine label exchanged with the water of hydration about the ionogenic groups, thus unavailable for release. Based on this result binding studies utilizing ^{3}H labels should be avoided and some other analytical methods sought.

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