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The Acid Mucopolysaccharides of Cattle Retina

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1. Two polysaccharides were isolated from the interstitial matrix surrounding the photoreceptor cells of cattle retina. They were liberated from this region of the tissue in a soluble form after agitation of whole retinas in 0·9% sodium chloride. One, which comprises two-thirds of the polysaccharides present, is a hyaluronidase-sensitive 'half-sulphated' chondroitin sulphate containing uronic acid, galactosamine and sulphate in the molar proportions 1·27:1·0:0·54. The other is a hyaluronidase-resistant non-sulphated heteropolysaccharide for which the name sialoglycan is proposed. It contains galactose, glucosamine and sialic acid in the molar proportions 2·4:1·0:0·4. Both polysaccharides contain only small amounts of nitrogen in excess of the amount calculated from their amino sugar and sialic acid content. 2. A similar combination of mucopolysaccharides is associated with the pigment epithelial-cell layer but in quantities only one-fifth of those present in the adjacent matrix area. 3. The ease with which they are released into aqueous media is consistent with the assumption that they are present in the extracellular spaces in both of these tissue layers. 4. The retinal residue left after removal of the two soluble polysaccharides is rich in amino sugar- and sialic acid-containing polymers, which appear to be firmly bound to the tissue fragments. 5. About one-third of the sialic acid and one-tenth of the amino sugar could be extracted with chloroform–methanol. The components in this fraction were tentatively identified as gangliosides. 6. Digestion of the chloroform–methanol-insoluble residue with Pronase yielded as the principal product a heteropolysaccharide containing 16·5% of glucosamine, 24·3% of neutral sugar (galactose plus fucose) and 18·1% of sialic acid. This substance has been classified as a sialoglycan of composition similar to (but not identical with) that of the soluble one isolated from the matrix area of the tissue.

The acid mucopolysaccharides detected histochemically in the retina (Sidman, 1958; Zimmerman & Eastham, 1959; Fine & Zimmerman, 1963) appear to be localized mainly in the interstitial matrix in which the photoreceptor cells are embedded. This area comprises an intercellular space, extending from the outer limiting membrane to the pigment epithelial-cell layer, and the mucopolysaccharides in it were at first believed to be resistant to both testicular and streptococcal hyaluronidase (Zimmerman & Eastham, 1959; Fine & Zimmerman, 1963). However, more recent work has shown that they are in fact partially degraded by this enzyme (Berman, 1964; Hall, Ocumpaugh & Young, 1965; Ocumpaugh & Young, 1966).

A definitive chemical characterization of the retinal mucopolysaccharides has been hindered by several factors, one of which is the limited amount of material in the tissue. Another difficulty encountered is that the large amounts of interfering substances (principally nucleic acids and lipids) present in the retina make colorimetric analyses extremely unreliable. Although mucopolysaccharides with electrophoretic mobilities similar to those of chondroitin sulphate and keratan sulphate have been isolated from papain extracts of acetone-dried cattle retina (Wortman, 1959), further investigations revealed that other components, namely non-sulphated mucopolysaccharides and 'neutral heteropolysaccharides', may also be present (Freeman & Wortman, 1966). The difficulty in separating and identifying these substances is further evidenced by the findings that both glucosamine- and galactosamine-containing mucopolysaccharides were present in fractions that were eluted as single peaks in ECTEOLA-cellulose (Freeman & Wortman, 1966) as well as DEAE-Sephadex (Berman, 1965; Hall *et al.* 1965) chromatography. Analyses of a purified fraction isolated from cattle retina suggested that the galactosamine-containing component was an undersulphated chondroitin sulphate (Berman, 1965). The glucosamine-containing mucopolysaccharide could not be identified. Neutral sugar (mainly galactose) was

present in amounts far higher than the stoicheiometric ratio expected for keratan sulphate; the low protein content of the preparation excluded the possibility of contamination with glycoprotein in significant amounts.

A critical evaluation of the experimental data presently available suggested the need for a systematic examination of all the amino sugar-containing macromolecules of the retina. The present paper, which is an extension of earlier investigations (Berman, 1964, 1965), describes the isolation, approximate anatomical distribution and chemical composition of the principal mucopolysaccharide components of cattle retina.

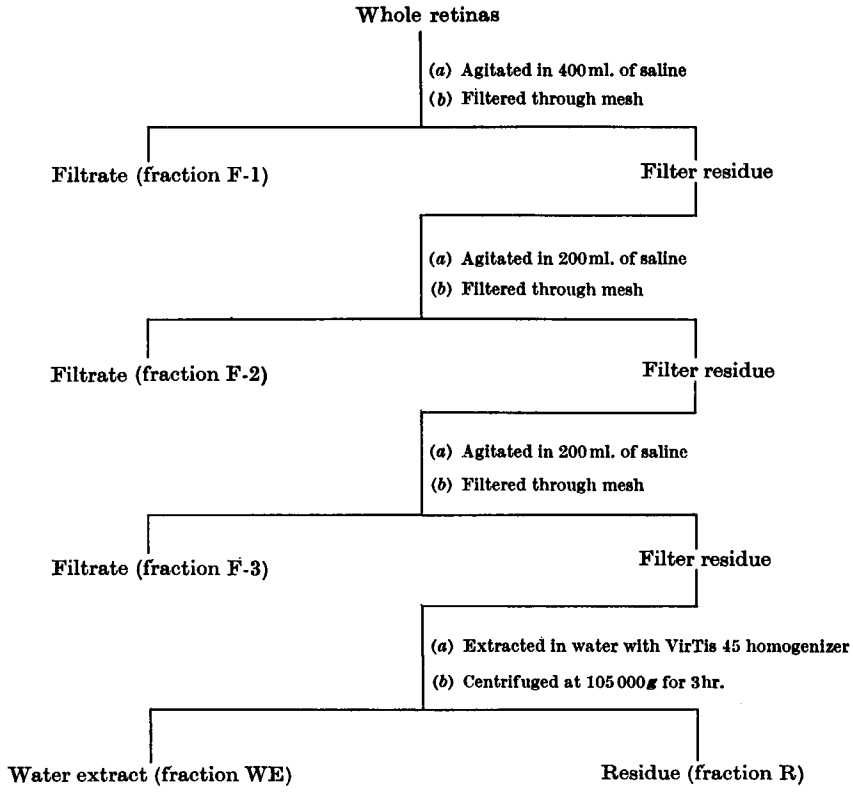
EXPERIMENTAL

Preparation of retinas and pigment epithelial cells. Cattle eyes, received from the slaughterhouse within 2hr. after death, were dissected as quickly as possible, with great care being taken to exclude completely any traces of vitreous humour before the retina itself was removed from the optic cup. Each retina was then lifted out with fine tweezers and placed immediately in cold 0·9% NaCl. The pigment epithelial cells remaining in the optic cup were collected by gently rubbing the exposed surface with a camel-hair brush after the addition of approx. 0·5ml. of 0·9% NaCl (Glocklin & Potts, 1962). The resulting suspension of cells was then aspirated with a syringe attached to a no. 18 gauge needle, the tip of which had been cut horizontally and bevelled. Two or three rinses sufficed to remove all the cells.

A schematic diagram of the retinal cell layers is given in the Appendix.

Preparation of crude mucopolysaccharides from retina. On the assumption that most of the uronic acid-containing mucopolysaccharides of the retina *in situ* are localized in the area immediately surrounding the photoreceptor cells (see the Appendix), isolation of this cell layer should result in the simultaneous release of the mucopolysaccharide-rich matrix substances. One of the more commonly used procedures for liberating outer segments from the intact retina is to agitate the tissue gently in buffer, saline or sucrose media (Saito, 1938; Collins, Love & Morton, 1952). Previous investigations (Berman, 1965) had shown that this simple procedure is effective in releasing 80–90% of the total detectable uronic acid-containing mucopolysaccharides of cattle retina, a finding that served as a basis for all subsequent work.

In the first step, 40–50 retinas were agitated in 400ml. of 0·9% NaCl on a magnetic stirrer at 4° for 30min. (Scheme 1). The resulting suspension was poured through a stainless-steel mesh (pore size approx. 1mm. diam.), yielding a



Scheme 1. Procedure for the preparation of fractions from whole retinas.

filtrate, which was set aside for further analysis, and a filter residue, which was agitated again in 200ml. of saline. This was done a third time, and the filter residue was then extracted with 100ml. of water in a VirTis 45 homogenizer for 30min. This procedure yielded five fractions designated as F-1, F-2, F-3, WE (water extract) and R (residue).

Fractions F-1, F-2 and F-3 were centrifuged at 37000*g* for 2hr. at 4°, dialysed and concentrated by freeze-drying. The water extract (fraction WE) was dialysed, concentrated and centrifuged at 105000*g* for 3hr. at 4°. The residue (fraction R) was suspended in water and dialysed before analysis.

Preparation of crude mucopolysaccharides from the pigment epithelial-cell layer. The cell suspension was agitated in 0·9% NaCl on a magnetic stirrer for 30min. and centrifuged at 10000*g* for ½hr. The pellet was then suspended in 0·9% NaCl, agitated and centrifuged again. This was done a third time and the combined supernatants were dialysed and concentrated. The cell fragments remaining were suspended in water and dialysed before further chemical analysis.

Chromatographic procedures. Column chromatography with SE- or CM-Sephadex (Pharmacia, Uppsala, Sweden) was carried out in 0·02M-citrate–phosphate buffer, pH5·2 (Berman, 1966). Amino sugars, after hydrolysis in 2N-HCl at 100° for 14hr., were identified by the procedure of Gardell (1953) with Bio-Rad AG50W (X8; H^+ form; 200–400 mesh). As a further check on the amino sugar composition, the samples were also analysed on a Beckman–Spinco model 120B amino acid analyser. In all cases the values found were in good agreement, but because of the greater accuracy and sensitivity of the amino acid analyser all results given are those obtained with this instrument.

The mucopolysaccharides were fractionated on columns (1cm.×20cm.) of DEAE-Sephadex A-50 (medium grade). The details of this procedure were as described by Berman (1963, 1964). Both sample and column were equilibrated overnight with 5mM-phosphate buffer, pH7, and the mucopolysaccharides were eluted in a convex gradient of NaCl.

Separation of mucopolysaccharides with cetylpyridinium chloride. The mucopolysaccharides were dissolved in 0·04M-NaCl and 0·1ml. of 1% cetylpyridinium chloride was added/ml. of solution. After standing for ½hr. at 35°, the solution was centrifuged at room temperature for 20min. at 17000*g*. The supernatant was drawn off and the mucopolysaccharides were collected after the addition of 5vol. of ethanol. The precipitate was washed with 0·04M-NaCl, centrifuged and dissolved in 2·1M-NaCl containing methanol (10%, v/v). The mucopolysaccharides were precipitated by addition of 5vol. of ethanol.

Precipitation of mucopolysaccharides with ammonium sulphate. Solid $(NH_4)_2SO_4$ was slowly added to the mucopolysaccharide solution until 80% saturation was reached. After standing overnight at 4°, the precipitate was removed by centrifugation at 15000*g* for ½hr., dissolved in water and exhaustively dialysed. The supernatant was dialysed against 12l. of running tap water, concentrated to a small volume and then dialysed again against distilled water. The sulphate content of both preparations was checked before and after treatment with $(NH_4)_2SO_4$ to be certain that all of it had been removed.

Isolation of sialic acid-containing polymers from the retinal residue. The residue (fraction R) remaining after the water extraction was homogenized with 19vol. of chloroform–methanol (1:2, v/v)/g. of wet tissue according to Suzuki's (1965) modification of the procedure of Folch, Lees & Sloane-Stanley (1957). The residue was dried and set aside for further treatment, and the chloroform–methanol extract was partitioned into two phases by addition of 0·5vol. of 0·1M-KCl. After centrifugation, the lower (chloroform) phase was discarded and the upper (methanol–water) phase was dialysed and concentrated. The insoluble residue remaining after chloroform–methanol extraction was digested with Pronase (Calbiochem A.-G., Lucerne, Switzerland), by using 2mg. of enzyme/g. of dry tissue suspended in 30ml. of 0·1M-phosphate buffer, pH7·2. The incubation was carried out for 16hr. at 37° in the presence of added toluene.

Enzymic procedures. To standardize the procedure for testing the susceptibility of the retinal mucopolysaccharides to hyaluronidase degradation, both the release of free *N*-acetylhexosamine (Reissig, Strominger & Leloir, 1955) and the amount of non-diffusible uronic acid (or hexosamine) remaining after incubation with the enzyme were measured. The results obtained by the two methods were in good agreement, but because of its greater sensitivity the uronic acid (or hexosamine) assay system was chosen. Samples of mucopolysaccharide containing the equivalent of 15μg. of uronic acid (or hexosamine) were incubated with 10μg. of testicular hyaluronidase (20000units/mg.) (supplied by the A. B. Leo Co., Hälsingborg, Sweden), in 1·0ml. of 0·1M-citrate–acetate buffer, pH5·0. The mixture was placed in a small dialysis sac in 100ml. of buffer (containing added toluene) and incubated for 20hr., with continuous shaking, at 37°. At the end of the incubation the contents of the dialysis sac were removed and assayed.

Sialic acid was liberated with neuraminidase (Behringwerke A.-G., Marburg/Lahn, Germany) by using 3 units of enzyme/μg. of sialic acid. Incubation was carried out for ½hr. at 37°.

Identification of neutral sugars. It was found that even trace amounts of lipids interfered with the chromatographic procedure used. Therefore the samples were first extracted with chloroform–methanol and then hydrolysed for 12hr. at 100° in 1N-HCl. After removal of the HCl *in vacuo*, the residue was taken up in water and passed through a mixed-bed column (0·8cm.×10cm.) of Dowex 1 (Cl^- form) and Dowex 50 (H^+ form). The effluent, containing only neutral substances, was concentrated and chromatographed on thin-layer sheets of silica gel (Eastman Chromagram) pretreated with 0·02M-sodium acetate. The sheets were developed in the solvent ethyl acetate–propan-2-ol–0·1N-boric acid (65:63:12, by vol.) and sprayed with $AgNO_3$ solution.

Chemical analyses. Amino sugars were determined by a modification (Gatt & Berman, 1966) of the Elson & Morgan (1933) reaction, under the hydrolytic conditions described above. Though a maximum yield of hexosamine was thus obtained, it was later found that in this modification the extinction values of amino sugars were greatly enhanced in the presence of NaCl. Therefore, when samples containing NaCl (e.g. the chromatographic effluents from DEAE-Sephadex) were analysed, the amount of amino sugar was calculated against standards containing equivalent amounts of salt. Protein and sulphate were assayed as described by Berman (1965). Sialic acid was measured by the method of Warren (1959) and the extinction

Table 1. *Molar extinction coefficients of various monosaccharides in three colorimetric reactions*

Monosaccharide	$10^{-3}\epsilon$ Orcinol–H_2SO_4 (430 mμ)	Anthrone (625 mμ)	Carbazole (530 mμ)
Galactose	24·9	4·6	2·2
Glucose	26·6	6·6	2·0
Glucuronolactone	9·4	0·5	20·9
Glucosamine hydrochloride	2·6	0·1	1·3
Galactosamine hydrochloride	1·7	0·3	1·4

at 549 mμ corrected for interfering substances when necessary. In crude samples of retina, sialic acid could not be determined by this reaction because of the excessive amounts of deoxyribose present. In these cases it was measured after enzymic release with neuraminidase.

Neutral sugar was analysed both by a modification (Berman, 1965) of the orcinol–H_2SO_4 reaction (Winzler, 1955) and with the anthrone reagent (Trevelyan & Harrison, 1952). Neither of these colorimetric reactions is entirely specific for neutral sugars, since both hexuronic acids and amino sugars contribute to the total extinction (Yemm & Willis, 1954; Doganges & Schubert, 1964; Berman, 1965; Luscombe & Phelps, 1967*a*). Appropriate corrections were therefore made on all the purified preparations according to the experimentally determined molar extinction coefficients shown in Table 1. Protein may also be the cause of erroneously high values in both the orcinol–H_2SO_4 and the anthrone reactions. Although bovine albumin (Cohn fraction V) alone gave little or no absorption in either of these reactions, when it was added to standard solutions of neutral sugar an increase in absorption of approx. 1 *E* unit/μg. of protein was observed. In contrast, protein (100μg.) depressed the carbazole reaction by approx. 15%. The values reported here for hexuronic acid and amino sugars in the anthrone reaction are somewhat higher than those found by Doganges & Schubert (1964) or Luscombe & Phelps (1967*a*). The reason for the discrepancy may be in the greater sensitivity of the micro-modification (with a reaction volume of 1·2 ml.) employed here. The sensitivity of the anthrone reaction was increased fivefold by lowering proportionately the volumes of both the reagent and the sample. It should also be noted (Table 1) that in the Trevelyan & Harrison (1952) modification of the anthrone reaction galactose has approx. 70% of the colour yield of glucose, in agreement with the data of Yemm & Willis (1954), whereas in other commonly used anthrone procedures (Ashwell, 1957) the extinction value produced by galactose is only 54% of that given by glucose.

Uronic acid was analysed by the modification by Bitter & Muir (1962) of the carbazole reaction (Dische, 1947). Galambos (1967) has shown that in this modification the extinction values given by neutral sugars at 530 mμ are depressed, but not completely abolished, in the presence of borate. Appropriate corrections were therefore made for the contribution of neutral sugar in this reaction according to the data given in Table 1. Amino sugars, although not giving rise to the same characteristic spectra as uronic acids, likewise contribute to the extinction value at 530 mμ, as shown in Table 1.

RESULTS

Several preliminary attempts were made to determine the principal types of acid mucopolysaccharides in whole retina by means of specific colorimetric reactions. However, none of these determinations was considered reliable because of chromogenic substances in the tissue that interfere with nearly all the colour reactions used. Apart from large amounts of lipid (which cannot be extracted by acetone alone), the major interfering substances are nucleoproteins and glycogen, the former causing brown colorations in the carbazole reaction and the latter giving values for neutral sugar that are artificially high for substances presumed to be either keratan sulphate or 'neutral heteropolysaccharides'. Amino sugar estimations do not reflect the actual mucopolysaccharide content of the retina because of the uncertainties as to the amount that may be associated with glycoproteins.

These problems were circumvented by dividing the tissue into two 'anatomical' areas according to the fractionation procedure shown in Scheme 1. The first consists of the filtrate fractions F-1, F-2 and F-3, which, for reasons pointed out above, represent the matrix substances liberated together with the rod and cone outer segments. These fractions (after centrifugation and dialysis) are almost completely free of interfering materials, thereby simplifying the problem of isolation and identification of the mucopolysaccharides present. The other 'anatomical' area, the residue fraction R, accounts for 85–90% of the retina on a weight basis and consists for the most part of all the tissue layers except the photoreceptor cells. It is in this portion that the chromogenic substances are mainly concentrated, and the isolation of the mucopolysaccharides from this area requires selective extraction and purification procedures, different from those used for the filtrate fractions.

Apart from these two 'anatomical' areas, the mucopolysaccharides originating in the pigment epithelial-cell layer, which *in situ* lies adjacent to

the photoreceptor cell layer (see the Appendix), were also analysed. Their composition, together with that of the filtrate fractions, is considered first.

Composition of crude preparations. The analytical data on all the retinal fractions, expressed as mg./100 cattle retinas, are summarized in Table 2. The amino sugar/uronic acid (corrected value)/neutral sugar/sialic acid molar proportions in the soluble filtrate fractions F-1, F-2 and F-3 are approximately 1·0:0·7:2·0:0·2. Although galactosamine is the dominant amino sugar liberated in the first saline agitation (fraction F-1), these fractions become progressively richer in glucosamine in the second and third agitations (fractions F-2 and F-3), suggesting that the glucosamine-containing components are more firmly bound to the insoluble elements of the tissue than the galactosamine-containing mucopolysaccharides. That this is actually so is revealed by the fact that 85–90% of the amino sugar in the residue consists of glucosamine. Moreover, this is the principal amino sugar found in the soluble extract (fraction WE) derived from it. In addition to glucosamine, the residue (fraction R) is exceedingly rich in both sialic acid and neutral sugars. Because of interfering materials present, the uronic acid content could only be estimated by the combined use of sample blanks and internal standards. Even then, a precise value is difficult to determine, but it has been tentatively concluded that only traces are present, compared with the amount found in the filtrate fractions. These findings confirm, on a more quantitative basis, previously published data on the distribution of this acid mucopolysaccharide component in the retina (Berman, 1965).

The soluble fraction associated with the pigment epithelial-cell layer (Table 2) is similar qualitatively to the retinal fractions F-1, F-2 and F-3, but quantitatively there is about five times more uronic acid-containing mucopolysaccharide in the retinal fractions. The similarity in chemical composition is not surprising in view of the close apposition of these two cell layers in the intact eye.

Purification and fractionation. In a typical experiment with 200 cattle retinas as starting material, the filtrate fractions F-1, F-2 and F-3 were combined and the excess of protein was removed by passage through columns of CM-Sephadex. Nucleic acids were removed by precipitation with 10% (w/v) trichloroacetic acid. The soluble preparation from the pigment epithelial-cell layer was treated in a similar manner, but to conserve material complete analyses were not carried out during the various steps of the purification. The analytical data for the filtrate fractions from the retina (Table 3) show that, after passage through this cation-exchange gel and treatment with 10% trichloroacetic acid, over 99% of the protein originally present was removed. At the same time, about two-thirds of the carbohydrate components was recovered.

Chromatography of each of the preparations on columns of DEAE-Sephadex (Fig. 1) yielded three principal fractions from the retina and two from the pigment epithelium. All of the uronic acid-containing material applied was eluted at sodium chloride concentrations in the range 0·15–0·40 M. The yields were nearly quantitative when calculated directly from the effluent fractions. Although the chromatographic profiles of the two preparations were for the most part similar, a further comparison of the two

Table 2. *Analyses of crude retinal and pigment epithelial-cell preparations*

All results are given as mg./100 cattle retinas (average wet wt. of a single retina 600 mg.)

Fraction	Uronic acid		Amino sugar		Sialic acid	Neutral sugar	Protein
	Observed	Corrected*	Glucosamine	Galactosamine			
Retina							
Filtrate (F-1)	7·25	6·15	3·72	4·85	1·55	13·2	503
Filtrate (F-2)	1·36	1·20	0·96	0·82	0·30	2·8	144
Filtrate (F-3)	0·73	0·52	0·43	0·30	0·12	1·5	112
Water extract (WE)	0·23	0·12	1·40	Trace	0·45	5·4	98
Residue (R)	†		22·90	3·8	11·8‡	93·2	950
Pigment epithelium							
Soluble	1·72		0·85	1·22	0·38	3·2	115
Residue	0·14		§	§	§	§	13

* These values are corrected for neutral sugar, which has approx. 10% of the extinction value of uronic acid in the carbazole reaction.

† Uronic acid could not be measured in this fraction (for details, see the text).

‡ Sialic acid was assayed enzymically with neuraminidase (Behringwerke A.-G.).

§ These measurements were not made owing to insufficient material.

Table 3. *Purification of crude retinal preparations*

All results are given in mg. The starting material consisted of combined filtrates (fractions F-1, F-2 and F-3) from approx. 200 cattle retinas, obtained according to the procedure outlined in Scheme 1.

Step no.	Description	Amino sugar: Glucosamine	Amino sugar: Galactosamine	Sialic acid	Uronic acid	Neutral sugar	Protein
I	Original preparation	8·0	9·6	3·6	15·2	30·0	1240
II	After adsorption of protein on CM-Sephadex	6·2	7·8	3·0	13·6	26·6	112·4
III	Supernatant after precipitation with 10% (w/v) trichloroacetic acid	5·8	7·0	2·5	11·4	18·4	18·0

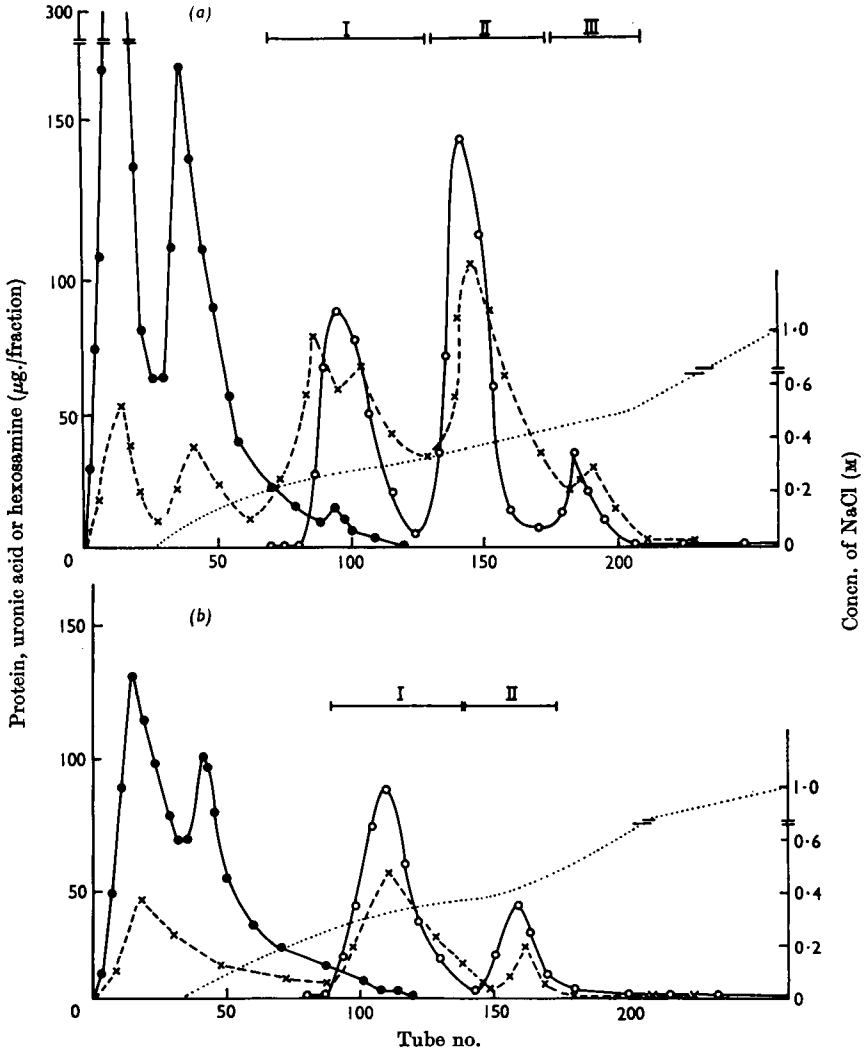


Fig. 1. DEAE-Sephadex chromatograms of soluble preparations from the matrix area of the retina (*a*) and pigment epithelium (*b*). The elutions were carried out with NaCl (in 5 mM-phosphate buffer) at the concentrations indicated by the dotted lines. The components analysed in the effluent fractions were: protein (●), uronic acid (○) and hexosamine (×).

Table 4. *Analyses of mucopolysaccharide fractions after DEAE-Sephadex chromatography*

All results are given as mg./fraction.

Preparation	Amino sugar: Glucosamine	Amino sugar: Galactosamine	Uronic acid	Sialic acid	Neutral sugar	Sulphate	Protein
Retina							
Fraction I	1·60	1·96	2·56	0·72	5·62	0·48	1·30
Fraction II	0·50	2·20	3·24	0·30	1·70	0·70	0·84
Fraction III*	0·44†		0·68	0·04	0·23	—	—
Pigment epithelium							
Fraction I	0·62†		0·60	0·14	1·70	0·13	0·48
Fraction II*	0·15†		0·24	—	0·64	—	—

* Insufficient quantities were available for complete chemical analyses.
† The values given are the total amino sugars (galactosamine + glucosamine).

patterns showed that the retina contains a somewhat more complex mixture of mucopolysaccharides than the pigment epithelium. The chromatograms also suggested an incomplete resolution of the mucopolysaccharides, and chemical analyses (Table 4) of the principal fractions obtained after combining and concentrating the uronic acid-containing peaks substantiated this. The galactosamine/glucosamine molar ratio in fraction I from the retina differs very little from that in the original preparation before chromatography. However, some resolution of the amino sugars is achieved in fraction II, where the galactosamine/glucosamine molar ratio is approx. 4:1. A consideration of the relative amounts of uronic acid, sialic acid and neutral sugar found in these fractions justifies the assumption that (at least) two different amino sugar-containing mucopolysaccharides are present, but their ionic characteristics are too similar to allow an effective separation by ion-exchange chromatography alone.

Resolution of the retinal mucopolysaccharides. The following techniques were used in an attempt to separate the glucosamine- and galactosamine-containing polymers isolated after DEAE-Sephadex chromatography.

(*a*) Ethanol fractionation. It was not possible to separate the amino sugars in either fraction I or fraction II by this method, since both components were precipitated at approximately the same ethanol concentration, namely 45–55% (v/v).

(*b*) Gel filtration. This technique was likewise unsuccessful because all of the material applied was eluted as a single symmetrical peak. Amino sugar analyses of the initial, intermediate and final fractions indicated no differences in their ratio at any stage of the elution.

(*c*) Ammonium sulphate precipitation. In the presence of 80%-saturated ammonium sulphate, approx. 70% of the glucosamine-containing polymers of fraction I were precipitated; at the same time 80% of the galactosamine-containing material remained in solution. Most of the neutral sugar and sialic acid were found in the glucosamine-rich precipitate, whereas most of the uronic acid and sulphate were associated with the galactosamine-rich supernatant fractions. Although ammonium sulphate is not a commonly used agent for separating the various classes of mucopolysaccharides, Pusztai & Morgan (1961) found it extremely effective for the purification of a sialomucopolysaccharide from ovarian-cyst fluid. The similarity in behaviour of the ammonium sulphate-insoluble mucopolysaccharide of the retina and the sialomucopolysaccharide isolated from cyst fluid thus provided an important clue as to its possible identity. Nevertheless, ammonium sulphate treatment alone was not sufficient to separate completely the mixture of glucosamine- and galactosamine-containing mucopolysaccharides.

(*d*) Cetylpyridinium chloride fractionation. Addition of cetylpyridinium chloride to the mixture of mucopolysaccharides isolated after DEAE-Sephadex chromatography also resulted in a partial resolution of the two species. In this case the uronic acid-containing material formed an insoluble complex whereas most of the sialic acid-containing polymers did not. The observations of Brunngraber & Brown (1964, 1967) are pertinent here, since the sialomucopolysaccharides found in extracts of rat brain likewise did not form insoluble complexes with cetylpyridinium chloride. However, with the retinal preparations, the separation between the two classes of mucopolysaccharides was not as complete as that found in brain extracts. It was tentatively concluded that cetylpyridinium chloride fractionation alone, like ammonium sulphate precipitation, resulted in only a partial resolution of these mucopolysaccharides.

Isolation of the individual components. By utilizing the differences in behaviour of the polysaccharides towards ammonium sulphate and cetylpyridinium chloride, the components were ultimately resolved in the following way. After removal of most of the unbound protein from the filtrate fractions (F-1, F-2 and F-3) by CM-Sephadex chromatography and precipitation with 10% trichloroacetic acid, the preparation was divided into two equal portions. One was treated with 80%-saturated ammonium sulphate solution and the resulting supernatant (containing galactosamine and glucosamine in a molar ratio approx. 4:1) was dialysed exhaustively and chromatographed on DEAE-Sephadex. The other half was treated with cetylpyridinium chloride and the sialic acid-rich supernatant likewise chromatographed on DEAE-Sephadex. These chromatograms (Fig. 2), which have been aligned according to the eluting salt concentrations used, reveal that the two mucopolysaccharides not only are polydisperse but also have similar ionic characteristics. This precludes the possibility of resolving them on anion-exchange columns without partially separating the principal components beforehand. The chromatograms show that only the first fractions (eluted up to salt concentrations of approx. 0·1 M-sodium chloride) are free of uronic acid and only the last fractions (eluted at salt concentrations higher than 0·25 M-sodium chloride) are free of sialic acid. The intermediate fractions obviously contain a mixture of both. The uronic acid-containing peak (fraction A) in Fig. 2(*a*) and the sialic acid-containing peak (fraction B) in Fig. 2(*b*) were each then pooled and analysed.

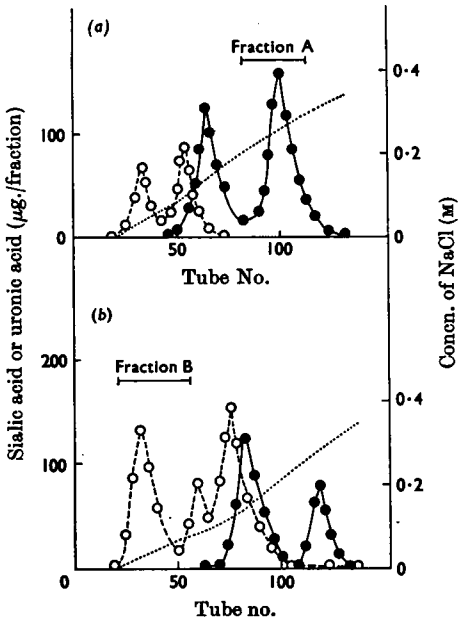


Fig. 2. Chromatography of ammonium sulphate-treated (*a*) and cetylpyridinium chloride-treated (*b*) fractions of the retina on DEAE-Sephadex. The elutions were carried out with NaCl (in 5mM-phosphate buffer) at the concentrations indicated by the dotted lines. The components analysed in the effluent fractions were sialic acid (○) and uronic acid (●).

The data shown in Table 5 are consistent with the assumption that the principal galactosamine-containing mucopolysaccharide of the retina (fraction A) is a 'half-sulphated' chondroitin sulphate. It contains uronic acid, galactosamine and sulphate in the molar proportions 1·27:1·00:0·54. The ratio of uronic acid to amino sugar was greater than 1:1 in all the preparations examined; the reason for this is not obvious. One possibility is that

Table 5. *Analyses of mucopolysaccharide fractions A and B isolated after DEAE-Sephadex chromatography*

Fractions A and B were isolated from the pooled samples shown in Fig. 2. Molar proportions for fractions A and B are relative to a value of 1·00 for galactosamine and for glucosamine respectively.

	Fraction A		Fraction B	
Component	μmoles	Molar proportions	μmoles	Molar proportions
Uronic acid	3·63	1·27	0*	
Galactosamine	2·85	(1·00)	0·59	0·29
Glucosamine	0·38	0·13	2·05	(1·00)
Galactose	0·58	0·20	4·95	2·40
Sulphate	1·53	0·54	0·10	—
Sialic acid	0		0·62	0·30
Nitrogen	5·88		5·18	
Hyaluronidase sensitivity	Degraded		Not degraded	

* Although a yellow–brown colour was obtained in the carbazole reaction, the absorption spectrum showed no peak at 530 mμ (Mathews & Cifonelli, 1965).

the uronic acid component is not glucuronic acid but galacturonic acid, which has a higher colour yield in the carbazole reaction. Other explanations may be either the incomplete release or the partial destruction of some of the amino sugar under the hydrolytic conditions used. Also noteworthy is the presence of both neutral sugar (which has been shown to be mainly galactose by thin-layer chromatography) and glucosamine, as well as a slight excess of nitrogen over the amount expected from the amino sugars. Rechromatography of the preparation on DEAE-Sephadex did not decrease the relative concentration of these two carbohydrate components, nor did Pronase treatment result in any lowering of the nitrogen content.

The mucopolysaccharide isolated in fraction B may be a member of the recently defined class of acidic polymers, termed sialomucopolysaccharides, that have been isolated from a number of other tissues. Although the principal carbohydrates found in them bear more resemblance to glycoproteins than to mucopolysaccharides, the low nitrogen content of fraction B argues against the possibility that it is a glycoprotein. Galactose, glucosamine and sialic acid are present in the molar proportions approx. 2·4:1·0:0·4. The small amount of sulphate found may not be a true value since the calculations were made on extinction values only 10% higher than the sample blank. Some galactosamine was detected in all of the purified sialomucopolysaccharide preparations isolated from the retina, but since these fractions contained no uronic acid it seems improbable that it represents contamination with chondroitin sulphate. The presence of ganglioside (whose only amino sugar is galactosamine) has likewise been excluded, partly on the basis of its different elution position in DEAE-Sephadex chromatography (E. R. Berman, unpublished work) and partly on the low sialic acid/neutral sugar molar ratio in this material. Since galactosamine is present as a minor component in sialomucopolysaccharides isolated from other sources as well, it is reasonable to assume that this particular mucopolysaccharide may contain variable amounts of both amino sugars.

Susceptibility to hyaluronidase. Neither chemical nor histological studies in other Laboratories have clarified the question whether the mucopolysaccharides surrounding the photoreceptor cells of the retina are degraded by hyaluronidase. In the present study fractions A and B (see Table 5) isolated from this area of the tissue were tested separately. Studies on the time-course of the reaction (Fig. 3) showed that at pH 6·0 the 'half-sulphated' chondroitin sulphate is degraded by hyaluronidase, but at a rate somewhat lower than either hyaluronic acid or chondroitin sulphate. Under the same conditions, and with equivalent amounts of substrate, the sialic acid-containing mucopolysaccharide is not attacked at all. It has since been found that the optimum pH for the degradation of the 'half-sulphated' chondroitin sulphate of the retina lies between 4·5 and 5·5, and under these conditions it is degraded at approximately the same rate as hyaluronic acid. On the other hand, no activity could be detected towards the sialomucopolysaccharide at any pH in the range 2·5–7·0.

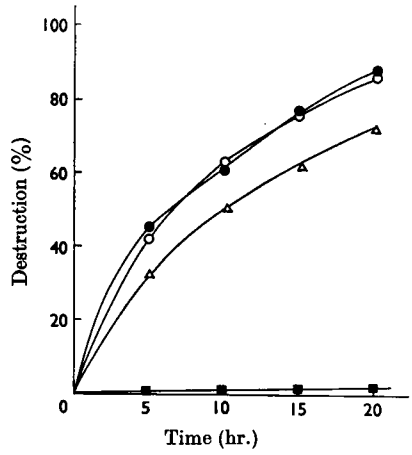
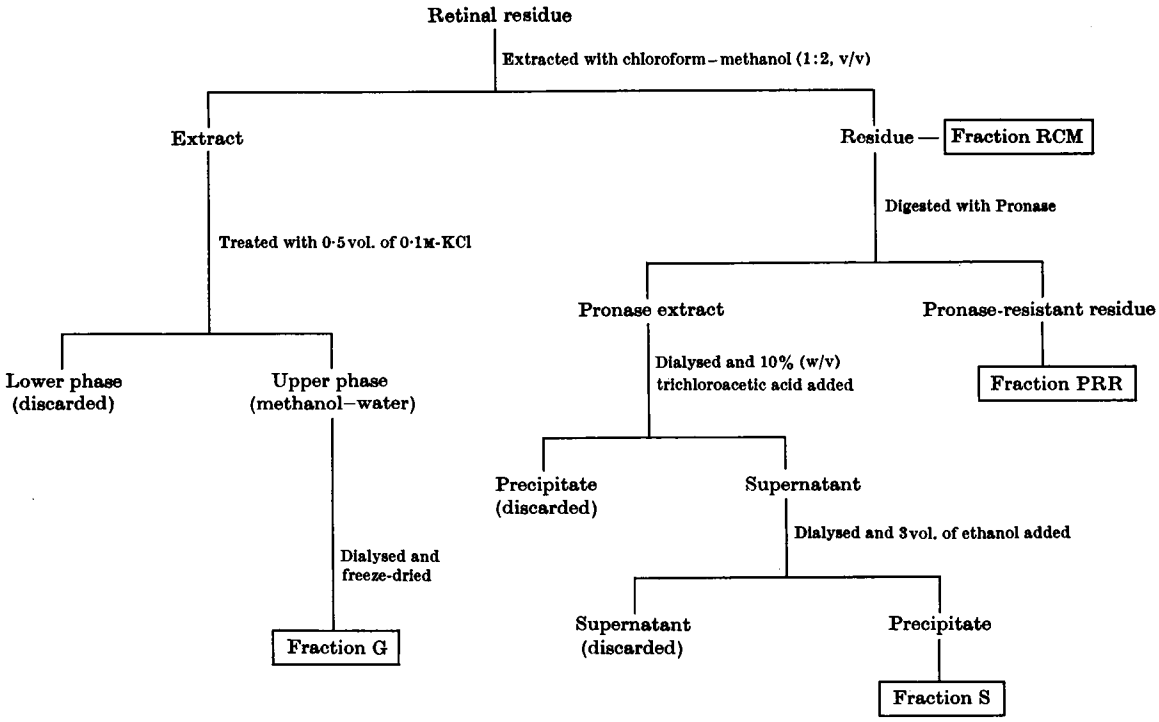


Fig. 3. Degradation of hyaluronic acid (●), chondroitin sulphate (○), retinal fraction A (△) and retinal fraction B (■) by testicular hyaluronidase. For details see the text.

Amino sugar-containing macromolecules of the residue fraction R. The chemical analyses shown in Table 2 indicate that only a small amount of soluble material (fraction WE) could be extracted from the residue by homogenization in water or buffer. However, because of the high lipid content of the retina it was suspected that non-polar solvents might be far more effective as extracting agents. Although no systematic studies have been made on the total lipid composition of this tissue, a fraction whose chemical composition is nearly identical with that of a ganglioside has been isolated by Schmidt (1961). The possibility that other sialic acid-containing polymers are also present in the retina was not considered and it therefore seemed important to investigate this question on a more quantitative basis.

The residue fraction R remaining from 100 retinas was extracted with chloroform–methanol (1:2, v/v) and then further fractionated according to the procedure outlined in Scheme 2. The analytical data (Table 6) show that approx. 35% of the sialic acid, 7% of the amino sugar and 5% of the neutral sugar recovered after the chloroform–methanol extraction are found in fraction G. Galactosamine is the



Scheme 2. Flow-sheet for the fractionation of the retinal residue fraction.

Table 6. *Distribution of hexosamine, neutral sugar and sialic acid in retinal fractions after chloroform–methanol extraction and Pronase digestion*

All results are given as mg./100 retinal residues. n.m., Not measured.

Fraction	Neutral sugar	Glucosamine	Galactosamine	Sialic acid	Protein
Upper (methanol–water) phase (fraction G)	2·85	0·02	1·04	3·19	n.m.
Residue after chloroform–methanol extraction (fraction RCM)	62·50	13·40	0·90	6·62	400
Pronase extract	16·35	4·10	—	2·84	290
Pronase-resistant residue (fraction PRR)	32·48	5·05	—	3·00	60

dominant amino sugar present and the sialic acid/neutral sugar/hexosamine molar proportions are approximately the same as those found in gangliosides isolated from other sources (Gatt & Berman, 1963). Although fraction G was not purified further, the data are consistent with the assumption that it contains most of the gangliosides of the retina.

To identify the sialic acid-containing polymers not extractable by chloroform–methanol, the residue (fraction RCM) was digested with Pronase. This step resulted in the solubilization of over 70% of the protein but considerably less of the neutral sugar, sialic acid and amino sugar (Table 6). Similar results were obtained with papain even after prolonged periods of digestion at 65°. The low-molecular-weight fragments (mainly peptides) in the Pronase digest were removed by exhaustive dialysis and the non-diffusible carbohydrate polymers remaining were then purified according to the procedure shown in Scheme 2. The analyses of the final product isolated (fraction S) indicate that it is a heteropolysaccharide containing 16·5% of glucosamine, 21·6% of neutral sugar (as galactose), 18·1%

Table 7. *Composition of a heteropolysaccharide (fraction S) isolated from the retinal residue fraction*

The composition is expressed as percentages of dry weight not corrected for salt or moisture content. Molar proportions are relative to a value of 1·0 for glucosamine.

Component	% of dry wt.	Molar composition: μmoles	Molar composition: Molar proportions
Glucosamine	16·5	2·71	(1·0)
Galactosamine	0		
Neutral sugars			
Galactose	21·6	3·58	1·3
Fucose*	2·7		
Sialic acid	18·1	1·77	0·65
Uronic acid†	0		
Sulphate	0		
Protein	13·0		

* Fucose was assayed by the method of Dische & Shettles (1948).

† Although a yellow–brown colour was obtained in the carbazole reaction, the absorption spectrum showed no peak at 530 mμ (Mathews & Cifonelli, 1965).

of sialic acid and 2·7% of fucose (Table 7). The molar proportions of the various components indicate that this polysaccharide is richer in sialic acid than the soluble sialomucopolysaccharide (fraction B; Table 5) liberated from the retina by agitation in saline. Despite the marked chemical heterogeneity existing among the sialic acid-containing polysaccharides isolated from various sources, it has nevertheless been tentatively concluded that the one isolated from the residue fraction R in all probability belongs to the same family of substances as the soluble one found in the matrix area.

DISCUSSION

An attempt has been made to isolate and identify, on as nearly a quantitative basis as possible, not only the mucopolysaccharides but also other amino sugar-containing macromolecules of the retina. The unique structural organization of this tissue afforded the possibility of dividing it into three major 'anatomical' regions, thus allowing chemical findings to be interpreted in terms of histochemical and radioautographic investigations carried out in other Laboratories. Previous attempts to isolate the mucopolysaccharides of cattle retina by using whole tissue as starting material (Wortman, 1959; Hall *et al.* 1965; Freeman & Wortman, 1966) precluded the possibility of determining their localization. The observation (Berman, 1965) that removal of the rod and cone outer segments by agitation in saline resulted in the liberation of most of the uronic acid-containing mucopolysaccharides greatly facilitated the solution of the problem of identification of these substances in the matrix area. It also provided the basis for the investigations described in the present paper of the approximate distribution of other amino sugar-containing macromolecules in the tissue.

Mucopolysaccharides of the interstitial matrix. On the basis of amino sugar analyses about two-thirds of the mucopolysaccharides in this retinal area were identified as 'half-sulphated' chondroitin sulphate(s) and the remainder as sialomucopolysaccharides. Both are extracellular and apparently not bound to any structural elements in the tissue. These two substances are not known to occur together in any other connective tissue and their presence in the matrix area of the retina suggests that they may have a special function in this region. One such role may be that of promoting the adherence of the neural layers of the retina to the pigment epithelial-cell layer (Zimmerman & Eastham, 1959), since an anatomical union between them has never been proved. It is here that the cleavage occurs in retinal detachment and it is tempting to speculate that changes in the physical state or metabolism of the mucopolysaccharides or both may play a role in the aetiology of the detachment.

Chondroitin sulphates of various degrees of sulphation are known to occur in many connective tissues. Since the one isolated from cattle retina contains uronic acid, galactosamine and sulphate in the molar proportions 1·27:1·0:0·54, it is termed a 'half-sulphated' chondroitin sulphate. Minor components found in this fraction include galactose and glucosamine. Whether they are covalently linked to the chondroitin sulphate (Hoffman, Mashburn, Meyer & Bray, 1967; Hoffman, Mashburn & Meyer, 1967) or represent only physically entrapped units of keratan sulphate or glycoprotein or both (Luscombe & Phelps, 1967*b*) could not be determined from the present studies because of the limited amount of material available.

The sialic acid-containing mucopolysaccharide isolated from the matrix bears a close resemblance to substances found in ovarian-cyst fluid (Pusztai & Morgan, 1961), rat brain (Brunngraber & Brown, 1964, 1967) and human gastric juice (Terho, Hartiala & Häkkinen, 1966). Galactose, glucosamine and sialic acid, which represent the major carbohydrate components, are present in the molar proportions approx. 2·4:1·0:0·4. Variable amounts of galactosamine were also detected in the purified samples, but the absence of uronic acid excludes the possibility of any chondroitin sulphate impurity. Despite the fact that these carbohydrate components are similar to the ones found in many well-defined glycoproteins, several lines of evidence

support the view that this material should be classified as a heteropolysaccharide. Apart from its low nitrogen content, the sialic acid-containing polysaccharide has a high anodal electrophoretic mobility at pH 3, and gives a positive staining reaction with cationic dyes but not with periodic acid–Schiff reagent (G. Bach, unpublished work). Many inconsistencies exist at the present time for defining substances of this type. For example, a homogeneous fraction of high electrophoretic mobility, rich in sialic acid and containing approx. 40% of protein has been termed a sialoprotein (Herring, 1964; Andrews, Herring & Kent, 1967). Other sialic acid-containing polymers of lower protein content have been termed sialomucopolysaccharides. It is proposed that the sialic acid-containing heteropolysaccharide isolated from the matrix area of the retina be termed a sialoglycan, in accordance with the systematic nomenclature introduced by Jeanloz (1960) for amino sugar-containing carbohydrate polymers.

Mucopolysaccharides of the pigment epithelium. The mucopolysaccharides isolated from this area, although present in lower concentration than in the adjacent interstitial matrix, are of similar chemical composition. From the ease with which they can be removed from the cells, they are probably located in the intercellular spaces of this tissue layer (see Fig. 1 in the Appendix). It has previously been shown that suspensions of pigment epithelial cells are active in the biosynthesis of the endogenous mucopolysaccharides found in this region of the retina (Berman, 1964), but more recently the inner segments of the photoreceptor cells have also been implicated (Ocumpaugh & Young, 1966). The present findings, which show the similarity in composition of the mucopolysaccharides in both cell layers, may allow a more precise approach to the problem of their biosynthetic site within the tissue.

Enzymic degradation. The 'half-sulphated' chondroitin sulphate is degraded by hyaluronidase, with maximum activity occurring at pH 4·5–5·5. In contrast, the sialoglycan is insensitive to hyaluronidase at all pH values in the range 2·5–7·0. The difference in susceptibility towards hyaluronidase of the two polysaccharides provides a partial explanation of some of the discrepancies reported in the literature. Early histochemical studies (Zimmerman & Eastham, 1959; Fine & Zimmerman, 1963) showed no decrease in the intensity of the alcian blue-staining substances of the matrix area after incubation with either testicular or bacterial hyaluronidase, but later investigations carried out under similar experimental conditions showed in fact that some degradation had taken place (Hall *et al.* 1965). More recently quantitative measurements with [^{35}S]sulphate have shown that about two-thirds of the mucopolysaccharides of the matrix are degraded by hyaluronidase (Ocumpaugh & Young, 1966). Although these experiments are not directly comparable with the ones reported here, a certain parallelism does exist in terms of the differential sensitivities of the polysaccharides toward hyaluronidase.

At present, the only enzyme found that is active on the sialoglycan is neuraminidase. Sialic acid is liberated quantitatively, implying that it occupies a terminal position in the molecule. Proteolytic enzymes, under the conditions used in these experiments, do not cause any detectable degradation of the sialoglycan.

Mucopolysaccharides and gangliosides bound to the retina. Although most of the uronic acid-containing mucopolysaccharides and some of the sialic acid-containing polymers are liberated by agitating the retina in saline, substantial amounts of carbohydrate still remain firmly bound to the residue (see Table 2). It is the glucosamine-containing polymers that are, for the most part, more tightly bound to the tissue and that cannot be released in a soluble form, even by prolonged extraction, if aqueous media are used. When this residue fraction is homogenized with chloroform–methanol by the procedure of Suzuki (1965), about one-third of the sialic acid is partitioned into the upper phase formed after the addition of 0·5 vol. of 0·1 M-potassium chloride (Folch *et al.* 1957). This fraction consisted mainly of gangliosides, and it may be assumed that they were extracted nearly quantitatively from the tissue, since Suzuki (1965) has shown that the recovery of gangliosides is essentially complete if extracting mixtures containing at least 60% methanol are used. Contrary to the findings reported by Schmidt (1961), who concluded that all of the sialic acid of the retina represents gangliosides, the data given in Tables 2 and 6 show that only about one-third of it is bound to gangliosides. A large part of the sialic acid in the residue fraction R is present as a polysaccharide that could only be released from the tissue after Pronase digestion. The product isolated (fraction S; Table 7) contained 16·5% of glucosamine, 21·6% of galactose, 18·1% of sialic acid and 2·7% of fucose. Though differing slightly in chemical composition from the soluble sialoglycan isolated from the matrix area, this heteropolysaccharide has nevertheless been tentatively classified as belonging to the same group of substances.

Since no feasible method has yet been found for dividing the residue fraction (R) into 'anatomical' areas, no conclusions can be made from these studies as to the precise localization of the two principal amino sugar-containing polymers. However, histochemical investigations of the pathological changes occurring in Tay–Sachs disease leave

little doubt that most, if not all, of the gangliosides are localized in the ganglion-cell layer of the tissue (see Fig. 1 in the Appendix), where they may play an important role in nerve transmission (Schmidt, 1964). The location of the sialoglycan(s) *in situ* is less clear but it is not improbable that they are the same substances that have been detected in the plexiform layers and the optic-nerve-fibre layer of the tissue (Ocumpaugh & Young, 1966). Because of their extreme insolubility, some of them may be bound to various types of nerve cell membranes (Brunngraber, Dekirmenjian & Brown, 1967) or to vascular elements of the tissue (Freeman & Wortman, 1966).

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APPENDIX

The retina is a complex nervous structure made up of nine different cell layers. The outermost one, the photoreceptor-cell layer ('rods' and 'cones'), lies directly adjacent to the pigment epithelial-cell layer, but does not form an anatomical union with it. The shaded area in Fig. 1 illustrates the location and (approximate) relative concentration of the mucopolysaccharides in the matrix area surrounding the photoreceptor cells. Apart from the mucopolysaccharides present in this region, small amounts have also been detected by histochemical methods in other areas of the retina, principally the inner and outer plexiform layers (E and G) and the optic-nerve-fibre layer (I) (Hall, Ocumpaugh & Young, 1965; Ocumpaugh & Young, 1966). Chemical studies show that the mucopolysaccharides in these areas are firmly bound to structural elements of the tissue and may actually form an integral part of the blood vessels or cell membranes. The gangliosides are probably localized in the ganglion-cell layer (H).

The 'anatomical' division of the retina described in the main paper may be visualized in the following

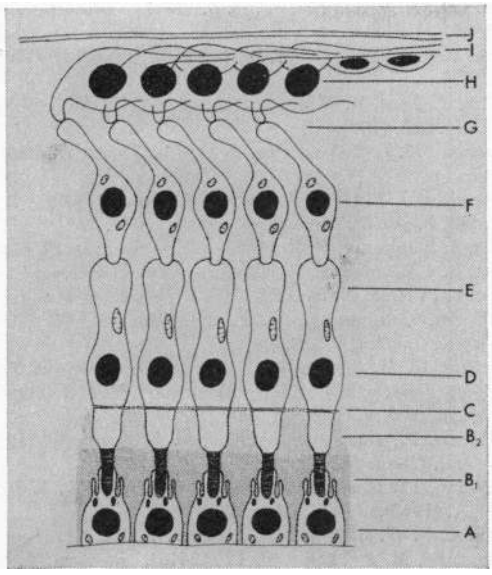

Fig. 1. Schematic representation, adapted from Kuwabara (1966), of the retinal cell layers: pigment epithelium (A); outer segments of photoreceptor cells (B_1); inner segments of photoreceptor cells (B_2); external limiting membrane (C); outer nuclear layer (D); outer plexiform (molecular) layer (E); inner nuclear layer (F); inner plexiform (molecular) layer (G); ganglion cells (H); optic-nerve-fibre layer (I); inner limiting membrane (J).

way. When the retina is lifted out of the optic cup, it is cleaved between the pigment epithelial-cell layer and the outer segments, layers A and B_1 respectively. The pigment epithelial cells (layer A) and the intact retina (layers B_1–J) are then processed separately (see the Experimental section of the main paper). The mucopolysaccharides are released from the pigment epithelial cells simply by agitating them in buffer or saline, but preparation of the two different 'anatomical' areas from the retina is more complex. When the whole retina (layers B_1–J) is shaken in saline, all of the outer segments (layer B_1), and in all probability some of the inner segments (layer B_2), are released into the medium. The soluble extracellular mucopolysaccharides, represented by the shaded area surrounding the photoreceptor cells, are liberated simultaneously. The remaining retina (approximately layers C–J) is, for the most part, still intact and on a weight basis represents close to 90% of the tissue. This has been termed the residue fraction R.

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