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Note

Neutral sugars from the hyaluronate–peptide of vitreous humor

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Whereas the structure of the protein–carbohydrate linkage regions of the sulfated mucopolysaccharides has been elucidated, hyaluronate–peptide presents a difficult problem because of its complex structure, high molecular weight, and low content of neutral sugars. Recently, a helical structure having two antiparallel chains has been assigned to hyaluronate[1], but the nature of all the neutral sugars present in the hyaluronate–peptide is still not known. Workers in this laboratory reported the presence of arabinose as a component of hyaluronate–peptide from cerebral tissue[2,3] and vitreous humor[4], and these results were confirmed by other workers for the hyaluronate–peptides from brain[5] and urine[6]. A further report from this laboratory suggested the involvement of arabinose in the linkage of hyaluronate with the peptide through threonine or serine, or both[7].

This Note further confirms the involvement of arabinose, and reports the presence of two additional neutral sugars, fucose and a 7-deoxyheptose (7-deoxy-L-*glycero*-D-*manno*-heptose or 7-deoxy-L-*glycero*-D-*gluco*-heptose), as components of the hyaluronate–peptide of vitreous humor. The presence of fucose has been reported in other mucopolysaccharides; L-fucose is a minor component of keratan sulfate[8], and Ciffonelli and Mathews[9] reported the presence of L-fucose in spisulan (an acid mucopolysaccharide isolated from clams). A "methylpentose" has been mentioned as a component of an acid mucopolysaccharide isolated from the liver of squid[10], and Kao *et al.*[11], in a study of the neutral sugars in the protein–polysaccharides from human and rat costal-cartilage, reported the presence of L-fucose and the possible involvement of this sugar in the linkage as a minor component. Deoxyheptoses are not known to occur commonly. Hellerqvist *et al.*[12] reported the presence of a "*manno*-6-deoxyheptose" in the acid hydrolyzate of a polysaccharide from *Yersinia* (Pasteurella) *pseudotuberculosis* Type IIA.

The hyaluronate–peptide of vitreous humor that was prepared in our laboratory from bovine eyes was very pure; chemical analysis of the preparation was performed as described earlier[3], and the results are given in Table I. It was free from sulfated mucopolysaccharides, as the content of 2-amino-2-deoxygalactose was negligible, and its infrared spectrum showed no absorption at 851–818 cm^{-1} (C–O–S stretching band) or at 1252–1228 cm^{-1} (S=O stretching band), regions characteristic of sulfated

TABLE I

CHEMICAL ANALYSIS OF THE HYALURONATE–PEPTIDE OF VITREOUS HUMOR

Serine (%)	*Threonine* (%)	*Total amino acids* (%)	*2-Amino-2-deoxy-glucose* (%)	*2-Amino-2-deoxy-galactose* (%)	*Total hexosamines* (%)	*Glucuronic acid* (%)	*Molar ratio of hexosamine to glucuronic acid*
0.063	0.015	0.28	40.5	<0.23	40.73	43.94	1.00

mucopolysaccharides. The hyaluronate preparation was also free from glycoproteins, as the molar ratio of hexosamines to glucuronic acid was unity (the presence of glycoproteins would increase this ratio considerably). Electrophoresis on cellulose acetate in barbiturate buffer of pH 8.6 showed the absence of bands for sulfated mucopolysaccharides. The absence of any glycoprotein was further shown by the lack of additional electrophoretic bands upon staining with Alcian Blue after periodate oxidation by the method of Wardi and Allen[13]. Also, paper and gas–liquid chromatography (g.l.c.) of a hyaluronate–peptide hydrolyzate did not reveal any mannose or galactose (normally present in glycoproteins).

The hyaluronate–peptide of vitreous humor was hydrolyzed with M hydrochloric acid for 4 h at 100°. Paper chromatography of this hydrolyzate in two solvents on "regular" size paper (11 × 9 in.) did not separate the 7-deoxyheptose from 2-amino-2-deoxyglucose; this behavior probably explains its nondetection in previous studies. However, when a longer paper (17 × 7.5 in.) was used for this chromatographic separation, the 7-deoxyheptose appeared as a spot on spraying with *p*-anisidine hydrogen phthalate[14] or alkaline silver nitrate[15]. This spot (see Fig. 1) was slightly faster-moving than that for 2-amino-2-deoxyglucose; it was not revealed by spraying with ninhydrin or *p*-dimethylaminobenzaldehyde, indicating that it was not a hexosamine. A spot was also obtained for arabinose. Fucose was not detected under the aforementioned conditions of hydrolysis with M acid, but we have now found that maximal release and minimal destruction of fucose occur when 0.16M hydrochloric acid is used for the hydrolysis of the hyaluronate–peptide. Arabinose was also released under these conditions, but a negligible amount of the 7-deoxyheptose was observed. When the hydrolysis was conducted with 0.5–0.6M hydrochloric acid, the presence of fucose, arabinose, and the 7-deoxyheptose was detected (see Fig. 1).

In the solvent chosen (see Experimental section), the R_F value of fucose was slightly higher than that of xylose (see Fig. 1). Also, fucose and the 7-deoxyheptose gave a brownish color with *p*-anisidine hydrogen phthalate[14], and, like other pentoses, arabinose gave a pink color with this reagent. Paper-chromatographic separation of fucose from arabinose was conducted on a preparative scale with hydrolyzate from treatment of the hyaluronate–peptide with 0.16M hydrochloric acid. The two upper strips, corresponding to fucose and arabinose, respectively, were eluted, and the solid residues obtained by evaporation of the eluates were separately derivatized to (*a*) the acetates and (*b*) the alditol acetates[17], which were identified by thin-layer

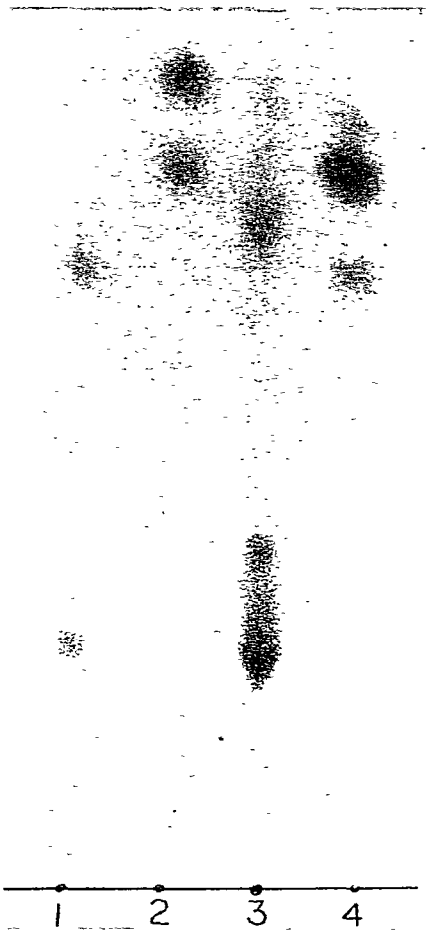

Fig. 1. Paper chromatogram of the reference sugars (lanes 1, 2, and 4) and of the acid hydrolyzate of hyaluronate–peptide (lane 3) in solvent *A*. [Spots (from top to bottom): lane 1, 2-amino-2-deoxy-D-glucose and D-glucuronic acid; lane 2, L-fucose and L-arabinose; lane 3, fucose, arabinose, 7-deoxy-L-*glycero*-D-*manno*-heptose, and glucuronic acid; and lane 4, D-xylose, L-arabinose, and D-galactose.]

chromatography and g.l.c.[17]. Further confirmation of the presence of fucose was obtained, and the determination of neutral sugars in the hyaluronate–peptide was conducted, as their aldononitrile acetates, by g.l.c., as described by the Varmas *et al.*[18–20]. In this method, the neutral sugars are released from the hyaluronate–peptide as their methyl glycosides by methanolysis[20], thereby minimizing the possibility of side reactions. The hexosamines and amino acids were removed, the methyl glycosides were hydrolyzed to the free sugars, and these were derivatized to their aldononitrile acetates[18–20] and subjected to g.l.c. analysis[20] (see Fig. 2).

The peaks having retention times of 5.9 and 9.1 min were identified as the aldononitrile acetates of fucose and arabinose, respectively. A third peak, having a retention time (16.9 min) greater than that of xylose but less than that of mannose

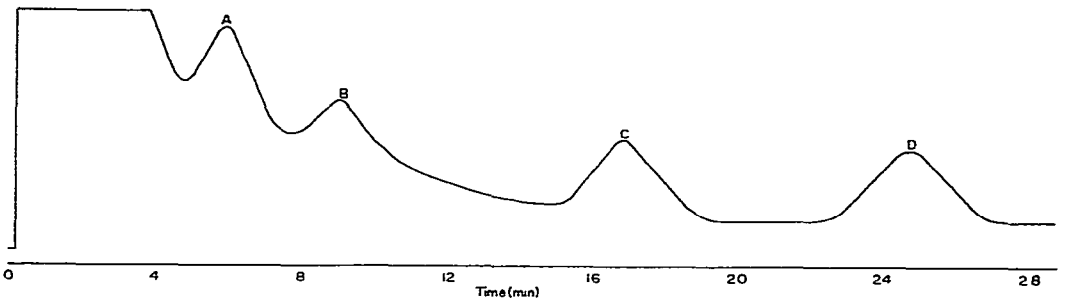

Fig. 2. Gas chromatogram of the aldononitrile acetates from the neutral sugars from hyaluronate–peptide. [The peaks were identified as follows: A, fucose; B, arabinose; C, 7-deoxy-L-*glycero*-D-*manno*-heptose; and D, D-mannose (internal standard).]

was, presumably, that of the 7-deoxyheptose derivative. To identify this sugar, various possibilities were checked. As the aldononitrile acetate method[18–20] does not detect ketoses, hexosamines, or hexuronic acids, the possibility that the heptose was any of these was eliminated. Furthermore, g.l.c. of the aldononitrile acetates of the eight isomeric aldohexoses and 2-deoxy-*arabino*- and -*lyxo*-hexose showed that it was none of these. Moreover, the aldononitrile acetate of the 3,6-anhydrogalactose obtained by hydrolysis of agarose had a much shorter retention time, and so it, also, was eliminated. From a comparative study of the retention times of the various sugars, the possibility that the sugar was a 7-deoxyheptose was investigated. Standard deoxyheptoses are not available commercially, but can be readily synthesized as their aldononitriles (cyanohydrins) from the corresponding 6-deoxyhexoses by the procedure of Varma and French[21]. Thus, starting from a 6-deoxyhexose, controlled Kiliani–Fischer synthesis would give two epimeric 7-deoxyheptononitriles, the proportion of one being much higher than that of the other. The retention time (16.9 min) of the aldononitrile acetate from the unknown sugar did not agree with that of either of the two acetylated, epimeric 7-deoxyheptononitriles from L-rhamnose, but agreed with that of one of the two acetylated, epimeric 7-deoxyheptononitriles (7-deoxy-L-*glycero*-D-*manno*-heptononitrile and 7-deoxy-L-*glycero*-D-*gluco*-heptononitrile)[22] obtained by controlled, cyanohydrin synthesis starting from L-fucose.

The aforementioned two epimeric 7-deoxyheptononitriles do not separate on paper (see Fig. 3) and their per(trimethylsilyl) derivatives have the same retention time in g.l.c. (see Fig. 4). However, their acetylated derivatives are well resolved by g.l.c. (see Fig. 5). The components of one part of the cyanohydrin reaction-mixture from L-fucose were separated on paper (see Fig. 3). The components of the second part were derivatized to the per(trimethylsilyl) ethers, and these were subjected to g.l.c.[21] (see Fig. 4). The paper-chromatographic and g.l.c. patterns (see Figs. 3 and 4) were very similar to those obtained by Varma and French[21] for the components of the cyanohydrin reaction-mixture from D-arabinose; they[21] identified the various components with the help of readily available standards. Our identification is based on analogy with the results of those authors[21], as no standards were available for the

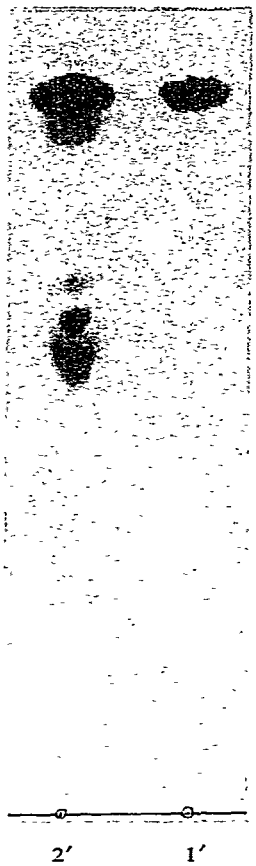

Fig. 3. Paper chromatogram of the cyanohydrin reaction-mixture (lane 1′) and of L-fucose (lane 2′) [Spots (from top to bottom): lane 1′, 7-deoxy-L-*glycero*-D-*manno*-heptono- and 7-deoxy-L-*glycero*-D-*gluco*-heptono-imidolactones, 7-deoxy-L-*glycero*-D-*manno*-heptono- and 7-deoxy-L-*glycero*-D-*gluco*-heptononitriles, L-fucose, 7-deoxy-L-*glycero*-D-*manno*-heptonamide and 7-deoxy-L-*glycero*-D-*gluco*-heptonamide, 7-deoxy-L-*glycero*-D-*manno*-heptono -and 7-deoxy-L-*glycero*-D-*gluco*-heptonolactones, potassium 7-deoxy-L-*glycero*-D-*manno*-heptonate and potassium 7-deoxy-L-*glycero*-D-*gluco*-heptonate.]

present study. G.l.c. of the acetylated, cyanohydrin reaction-mixture from L-fucose showed only the presence of unchanged starting-sugar and the two epimeric 7-deoxy-heptononitriles (see Fig. 5), unlike the results from g.l.c. of the per(trimethylsilyl) derivatives of this reaction mixture. Our g.l.c. studies showed that the two epimeric 7-deoxyheptono-imidolactones are converted into the corresponding 7-deoxy-heptononitrile acetates during the process of acetylation (treatment with a mixture of pyridine and acetic anhydride for 15 min at 100°), and thus their presence goes undetected. The two 7-deoxyheptonic acids do not appear in the chromatogram, because their free carboxylic groups make them nonvolatile. The absence of peaks for the 7-deoxyheptonolactones and the 7-deoxyheptonamides may be due to their presence in very small, undetectable amounts.

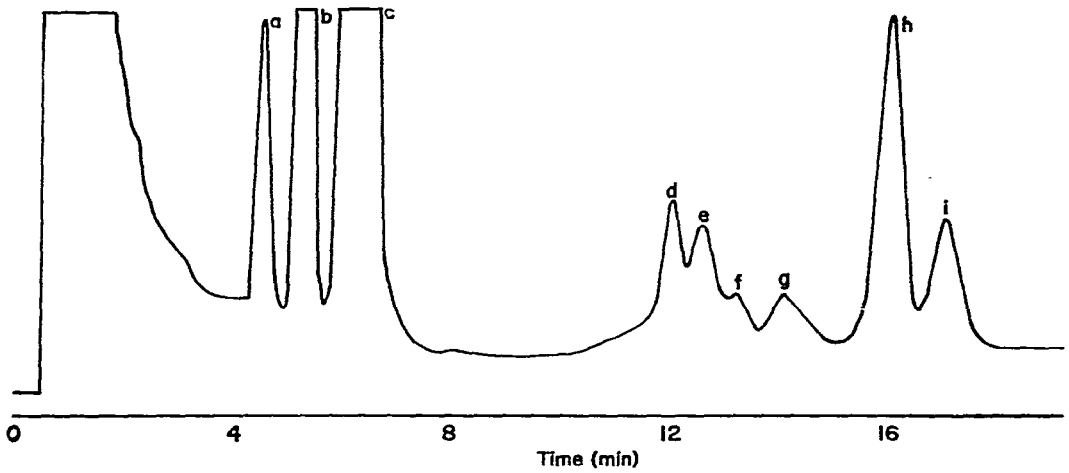

Fig. 4. Gas chromatogram of the per(trimethylsilyl) derivatives of the components of the cyanohydrin reaction-mixture. [The peaks were identified as follows: a, b, c: L-fucose; d: 7-deoxy-L-*glycero*-D-*manno*- and 7-deoxy-L-*glycero*-D-*gluco*-heptono-imidolactones; e: 7-deoxy-L-*glycero*-D-*manno*- and 7-deoxy-L-*glycero*-D-*gluco*-heptononitriles, f: 7-deoxy-L-*glycero*-D-*manno*- and 7-deoxy-L-*glycero*-D-*gluco*-heptonolactones; g: potassium 7-deoxy-L-*glycero*-D-*manno*-heptonate; h: potassium 7-deoxy-L-*glycero*-D-*gluco*-heptonate; and i: 7-deoxy-L-*glycero*-D-*manno*- and 7-deoxy-L-*glycero*-D-*gluco*-heptonamide.]

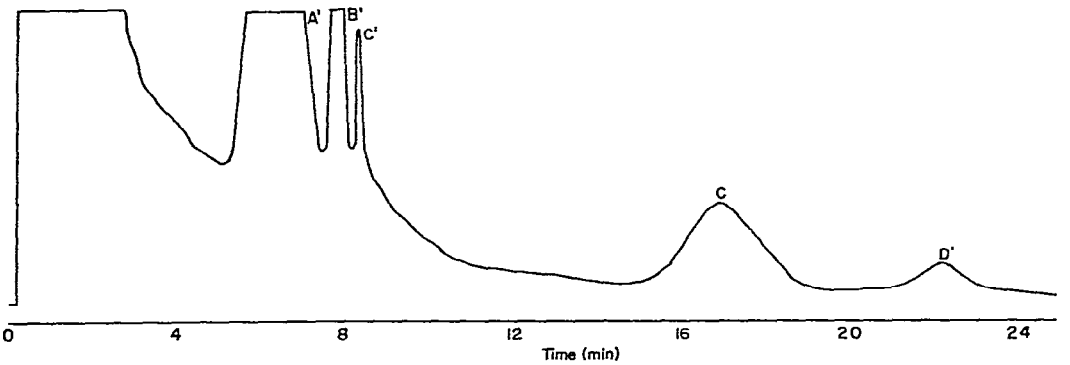

Fig. 5. Gas chromatogram of the aldononitrile acetates from the components of the cyanohydrin reaction-mixture. [The peaks were identified as follows: A', B', C': L-fucose acetates; C: 7-deoxy-L-*glycero*-D-*manno*-heptose; and D': 7-deoxy-L-*glycero*-D-*gluco*-heptose.]

A mixture of the two 7-deoxyheptononitriles (7-deoxy-L-*glycero*-D-*manno*-heptononitrile plus (7-deoxy-L-*glycero*-D-*gluco*-heptononitrile) was separated by preparative paper-chromatography (p.p.c.)[21]. The residue was acetylated, and the product examined by g.l.c.[18]. Two peaks were obtained; the retention time of one of them was the same as that of the aldononitrile acetate of the unknown sugar from the hydrolyzate of the hyaluronate–peptide. Based on the fact that mannose derivatives

are eluted before glucose derivatives in g.l.c., the aldononitrile acetate of 7-deoxy-L-*glycero*-D-*manno*-heptose should be eluted before the aldononitrile acetate of 7-deoxy-L-*glycero*-D-*gluco*-heptose. As the retention time of the aldononitrile acetate of the 7-deoxyheptose from the hyaluronate–peptide corresponded to the earlier peak (16.9 min), it is suggested that the 7-deoxyheptose may be 7-deoxy-L-*glycero*-D-*manno*-heptose, not 7-deoxy-L-*glycero*-D-*gluco*-heptose. The retention times of the aldononitrile acetates of fucose, arabinose, and the 7-deoxyheptose (7-deoxy-L-*glycero*-D-*manno*-heptose), and the percentage of fucose and arabinose in the hyaluronate—peptide are given in Table II. The percentage of the 7-deoxyheptose could not be determined, owing to the nonavailability of this standard.

TABLE II

RETENTION TIMES AND PERCENTAGE OF ALDONONITRILE ACETATES FROM NEUTRAL SUGARS FROM HYALURONATE–PEPTIDE

Sugar	*Retention time (min)*	*Percentage*
Fucose	5.9	0.34
Arabinose	9.1	0.20
7-deoxy-L-*glycero*-D-*manno*-heptose	16.9	—[a]

[a]The percentage could not be calculated, because of nonavailability of this standard.

EXPERIMENTAL

Reagents and standards. — Pyridine and acetic anhydride were obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. Standard sugars and trypsin were obtained from Sigma Chemical Co., St. Louis, Missouri. Silver carbonate was obtained from Fisher Scientific Co., Pittsburgh, Pennsylvania. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. Ion-exchange resins [AG-1 X-2 (Cl^-; 200–400 mesh) and AG-50W X-8 (H^+; 200–400 mesh)] were obtained from Bio Rad Laboratories, Richmond, California; and papain was obtained from Worthington Biochemical Corp., Freehold, New Jersey. The ion-exchange resin AG-1 X-2 (HCO_3^-) was prepared by passing 2M sodium hydrogen carbonate through a column of AG-1 X-2 (Cl^-) resin, and washing it thoroughly with water. Bovine eyes were obtained from Pel-Freez Biologicals, Inc., Rogers, Arkansas.

Preparation of hyaluronate–peptide from vitreous humor. — The hyaluronate–peptide (2.1 g) was prepared from bovine eyes (750) by a modification of the procedure of Wardi *et al.*[3]. The acetone powder (100 g) of the bovine, vitreous humor was suspended in 2.5 litres of 0.1M acetate buffer (pH 6.35) containing 2.5 ml of 2,3-dimercapto-1-propanol and 2.5 ml of papain (twice recrystallized), and the mixture was incubated under toluene, with constant stirring, for a total of 36 h at 65°. Additional aliquots (2.5 ml each) of papain were added at 2, 8, and 24 h. The digest was

adjusted to pH 8 with 50% (w/v) sodium hydroxide solution, and, after addition of 200 mg of trypsin (twice recrystallized) was re-incubated for 24 h at 37°, with constant stirring. The digest was then filtered through a bed of Celite, the filtrate was concentrated to one-fifth its original volume in a rotary evaporator at 40°, and the product was precipitated with 1.5 volumes of ethanol. The suspension was centrifuged, the yellow, supernatant liquor was discarded, and the pellet was washed with a washing solution [consisting of 1 volume of sodium acetate (5%; w/v)–acetic acid (3%; v/v) and 1.5 volumes of ethanol), followed by ethanol and then ether. The solid was mixed with sodium acetate (5%; w/v)–acetic acid (3%; v/v), the suspension filtered through a bed of Celite, and the product reprecipitated with ethanol (1.5 vol). The precipitate was washed with the washing solution used earlier, dried, dissolved in water, and the solution clarified by centrifugation, placed on a column (4 × 45 cm) of AG-50W X-8 (H^+; 200–400 mesh) resin, and eluted with water. The pH of the combined effluent and water washings was adjusted to 7 with sodium hydroxide solution, and the solution was concentrated, and fractionated on a column (4 × 45 cm) of AG-1 X-2 (Cl^-; 200–400 mesh) resin by use of a 0–2M gradient of sodium chloride solution. The fraction containing hyaluronate was eluted with 0.5M sodium chloride, the eluate dialyzed, and the dialyzate lyophilized. The residue was dissolved in 0.1M sodium chloride containing 0.02% (w/v) of sodium azide and subjected to gel filtration on a column (2.9 × 150 cm) of Sephadex G-200, eluted with the same solution. The fractions containing hyaluronate were combined, dialyzed, and lyophilized.

Hydrolysis of hyaluronate–peptide. — For maximal release and minimal destruction of fucose, the hyaluronate–peptide (15 mg) was hydrolyzed with 0.16M hydrochloric acid (2 ml) for 4 h at 100°; but, for identification of fucose, arabinose, and the 7-deoxyheptose, the hyaluronate–peptide (15 mg) was hydrolyzed with 0.5–0.6M hydrochloric acid (2 ml) for 4 h at 100°. The hydrolyzates were separately evaporated to dryness under diminished pressure at 35°. Each hydrolyzate was dissolved in water, and the solution passed through a column (1.5 × 35 cm) of AG-50W X-8 (H^+; 200–400 mesh) resin, to remove the amino acids and 2-amino-2-deoxyglucose. The effluent and the washings were combined, and evaporated to dryness under diminished pressure at 35°.

Paper chromatography. — The components of the hyaluronate hydrolyzates were separated on Whatman No. 1 paper (17 × 6.5 in) with 6:4:3 (v/v) butyl alcohol–pyridine–water (solvent *A*) and 15:10:6 (v/v) ethyl acetate–pyridine–water (solvent *B*). Two ascents were applied to the chromatograms. Preparative paper-chromatographic separation of fucose from arabinose was conducted on Whatman No. 3MM paper (17 × 6.5 in.) with solvent *A*. The spots on the analytical chromatogram were located with *p*-anisidine hydrogen phthalate[14].

Determination of neutral sugars in the hyaluronate–peptide. — The neutral sugars in the hyaluronate–peptide were determined by g.l.c. of their aldononitrile acetates by the procedure of Varma *et al.*[20]; the hyaluronate–peptide (25 mg) was first mixed with 0.05 ml of the (internal standard) solution of mannose (9 mg/5 ml). The mixture was evaporated to dryness under diminished pressure at 35°, the residue

mixed with 0.8M hydrogen chloride in dry methanol (20 ml), and the mixture boiled for 24 h under reflux. The solution was cooled, the pH was adjusted to 4–5 with silver carbonate, and the suspension was filtered, and the solid washed with dry methanol. The filtrate was evaporated to dryness in a rotary evaporator at 35°. The amino acids and hexosamines were removed by passing a solution of the product through a column of AG-50W X-8 (H^+) resin. (Glucuronic acid was not removed, as it does not interfere in this method[20].) The effluent was evaporated under diminished pressure at 35°, and the methyl glycosides in the solid obtained were hydrolyzed with 2M hydrochloric acid (0.5 ml) for 1.5 h at 100°. The pH of the solution was adjusted to 4–5 with AG-1 X-2 (HCO_3^- ; 200–400 mesh) resin, the suspension was filtered, and the filtrate was evaporated to dryness in a rotary evaporator at 35°. The neutral sugars in the residue were transformed into their aldononitrile acetates, and these were analyzed by g.l.c.

Kiliani–Fischer synthesis of the 7-deoxyheptononitriles from L-*fucose.* — The cyanohydrins of 7-deoxy-L-*glycero*-D-*manno*-heptose plus 7-deoxy-L-*glycero*-D-*gluco*-heptose were synthesized from L-fucose by the procedure of Varma and French[21]. An aqueous solution of L-fucose (30 mg), anhydrous sodium carbonate (7.1 mg), and potassium cyanide (13.5 mg) was made to 5 ml with water; the reaction was allowed to proceed for 25 min at 25°, to afford the maximal amount of cyanohydrins, and was then quenched by freezing in Dry Ice–acetone, the solution freeze-dried, and the residue divided into three portions. The components of one portion were per(trimethylsilyl)ated, and the ethers examined by g.l.c. The second portion was subjected to analytical and preparative paper-chromatography. The components of the third portion were acetylated by heating with a mixture of pyridine (5 drops) and acetic anhydride (5 drops) for 15 min at 100°, and the products examined by g.l.c.

Gas–liquid chromatography. — The aldononitrile acetates from the neutral sugars (obtained from the hyaluronate–peptide) were analyzed as described previously[18–20]. This procedure was also used for the separation of the acetates of the two epimeric 7-deoxyheptononitriles obtained by p.p.c., and for the separation of the components of the acetylated, cyanohydrin reaction-mixture obtained from L-fucose.

G.l.c. of the fucose and arabinose obtained by p.p.c. of the hyaluronate–peptide hydrolyzate was conducted on the derived alditol acetates by the procedure of Albersheim *et al.*[17]. G.l.c. of the per(trimethylsilyl) ethers of the components of the cyanohydrin reaction-mixture from L-fucose was performed as described by Varma and French[21].

Thin-layer chromatography of the acetates of sugars and of the alditol acetates from sugars. — Fucose and arabinose, isolated by p.p.c. of the hyaluronate–peptide hydrolyzate, were acetylated by heating with a few drops of 1:1 pyridine–acetic anhydride for 15 min at 80°. The alditol acetates from the sugars were prepared as described by Albersheim *et al.*[17]. The acetates of, and alditol acetates from, the sugars were separated on plates precoated with Silica Gel G by two ascending developments

with 3:2 (v/v) benzene–ethyl acetate. The spots on the dried plate were detected by means of the hydroxamic acid spray-reagent for esters[23].

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