4. The hypothesis is advanced that a diet high in sucrose stimulates the processes of mineralization by maintaining a maximum level of glycogen in the developing tooth.

The author is indebted to Prof. R. A. Morton, F.R.S., and Prof. H. H. Stones for their helpful criticism, and to Dr D. Riding of The Evans Biological Institute, Runcorn, for supplies of Hepamino.

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Chemistry of Tissues

4. METHYLATION STUDIES ON HYALURONIC ACID

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Despite the many investigations on hyaluronic acid there is no precise knowledge of the structural units of which the complex polysaccharide is constituted. Accordingly, our investigation was undertaken in order to apply chromatographic technique, methylation studies, etc. to gain knowledge of hyaluronic acid structure.

Hyaluronic acid is extracted from human umbilical cords with water. Acidification of the extract yields a precipitate or mucin clot containing the hyaluronic acid in conjunction with protein. Most of the various procedures which have been employed for the isolation of hyaluronic acid have differed in the method of removal of this protein.

In early methods of isolation (Meyer & Palmer, 1934) both the cords and the mucin clot obtained therefrom were subjected to rather drastic acid treatment so that products of low viscosity and with low nitrogen and ash contents were usually obtained. Later (Meyer & Palmer, 1936), it was found that the Sevag chloroform-amyl alcohol method for protein denaturation (Sevag, 1934) could be applied to the dissolved mucin clot from which a hyaluronic acid product could then be precipitated from aqueous solution by acetic acid. Further treatment with zinc acetate and alkali effectively removed the protein residue. However, this treatment also reduced the viscosity of the product and it was found difficult to remove the zinc completely. It was necessary also to use dilute solutions owing to the danger of precipitating zinc hyaluronate.

Two general methods have been used to redissolve this clot: (*a*) 10% calcium chloride at pH 8, and (*b*) enzymic digestion at a more alkaline pH. Use of method (*a*) gave a product of slightly higher viscosity but in lower yield (Meyer & Chaffee, 1940*a*). McClean & Hale (1941) removed the protein residue by tryptic digestion followed by precipitation of the trypsin with trichloroacetic acid. Blix & Snellman (1945) extracted dissected cords with water in an atmosphere of nitrogen six or eight times for 12 hr. each. They found it necessary to apply the Sevag process many times before the last traces of protein could be removed and a product of high viscosity obtained.

Most of the procedures aimed at obtaining, on a small scale, a product of the highest possible viscosity. Since our work was completed (1947),

Hadidian & Pirie (1948) have reported yet a further modification in which they claim to have separated several fractions of hyaluronic acid having varying viscosities. Meyer (1948) has also described a method for preparing highly viscous sodium hyaluronate.

For the present chemical investigation a highly viscous preparation was not necessary, but a comparatively large quantity of material was required, and methods have been developed which are easily applicable on a large scale for the preparation of material for methylation.

EXPERIMENTAL

Preparation of hyaluronic acid and umbilical cord mucin. Human umbilical cords, preserved in 1% aqueous phenol solution, were washed thoroughly with cold water to remove blood clots, minced coarsely and extracted four or five times with hot water at 65° (total 5 l./30 cords) and filtered through cheese cloth. This solution was cleared by centrifuging and precipitated with glacial acetic acid (final concn. 0·2%) stirring vigorously. The fibrous precipitate was collected on a glass rod or by centrifuging and was dried over P_2O_5 in a vacuum. It contained N 14·8, ash 0%, and is termed the 'clot'. It was reprecipitated from an alkaline solution (pH 8·5) several times, the N content remaining constant at 15·0%; ash 1·1%. This preparation is regarded as umbilical cord mucin, a protein-polysaccharide complex (20 g. from 24 g. of clot). When dry it was very hard and brittle.

Hyaluronic acid. Method A. The mucin preparation (30 g.) was dissolved with stirring in a slight excess of aqueous K_2CO_3 (1 l., pH 8·5). $CHCl_3$ (1·25 vol.), containing 5% of *n*-butanol, was added and the mixture shaken vigorously for 4 hr. or longer (cf. Sevag, 1934). The two phases were separated in the centrifuge (2500 rev./min.). The aqueous layer was diluted to 2 l. with water and solid matter removed in the Sharples centrifuge (24,000 rev./min.). The supernatant liquid was poured, with stirring, in a fine stream into dry ethanol (2–3 vol.) and the white, fibrous precipitate was collected on a rod and dried in a vacuum over P_2O_5. This product (14 g.) contained N 10·8, ash, 3·3%.

One half of this material (7 g.) in water (800 ml., pH 8·5) was denatured with $CHCl_3$ repeatedly until no more protein was coagulated and the solution no longer gave a biuret test. After working up as described, and drying, a pure white, fibrous product, potassium hyaluronate, was obtained (2·3 g.) which gave viscous, water-clear, aqueous solutions: N (Kjeldahl) 3·2, acetyl (Elek & Harte, 1936) 12·3, uronic acid (Burkhart, Baur & Link, 1934), 44·9%. The hexosamine estimation by the method of Elson & Morgan (1933) was not satisfactory, high results frequently being obtained, namely, 52·9%; other values: 35·4, 35·0%. No *N*-methyl or *O*-methyl groups could be detected; $[\alpha]_D^{18°} - 68°$ in water (*c*, 0·22). The viscosity was determined in 0·05 M-NaCl and 0·05 M-phosphate buffer at pH 7·0. At 0·15 g./l. the relative viscosity (see Hadidian & Pirie, 1948) was 1·7; at 1 g./l. it was increased to 1·8. This value is low compared with some preparations (cf. Hadidian & Pirie, 1948), but it is the usual value for hyaluronate obtained from cords which have been extracted with hot water rather than with cold (Kaye, 1949). The electrophoretic pattern with one main peak, shown in Fig. 1, was exhibited in 0·05 M-Na_2CO_3 solution (total ionic strength increased to 0·2 with KCl), at pH 11·36. (The small peak on the left of Fig. 1 is due to the building up of a salt boundary.) A current of 10 m.a. was applied for 180 min. The rate of migration was slow, suggesting a molecule of low net charge. All analytical results are expressed on a dry, free acid basis. The sample contained ash 20·0% (K, 11·3%); moisture (air-dry sample), 10·5% (cf. Kaye, 1948). It contained no glycogen (cf. Meyer & Palmer, 1936) or phosphate, and was non-reducing.

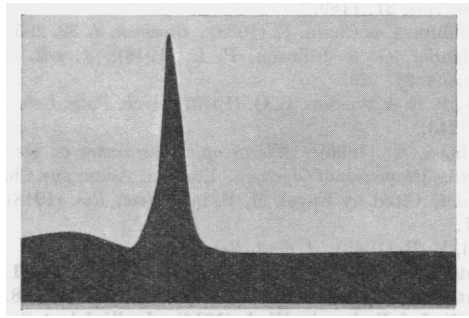

Fig. 1. Electrophoretic behaviour of the potassium hyaluronate preparation in 0·5% solution at pH 11·36 (0·05 M-Na_2CO_3, with KCl added to raise the ionic strength to 0·2) showing a symmetrical and homogeneous boundary after 9,420 sec. (Migration is from left to right.) The small peak is due to a salt anomaly.

This material (0·5 g.) was dissolved in water (200 ml.) and passed through a column of 'Zeo-Karb 215' to remove K ions. The rate of flow through the column was very slow, and if the original solution was too concentrated gel formation was found to prevent flow completely. The free acid could not be precipitated by pouring into ethanol, but the freeze-drying technique was found expedient and yielded hyaluronic acid as a tough, fibrous, spongy material (0·3 g.). Ash, 1·4; N, 3·6%. $[\alpha]_D^{17°} - 67°$ in water (*c*, 0·3). The relative viscosity (see above) at 0·15 g./l. was 1·5.

Hyaluronic acid. Method B. The umbilical cord mucin preparation (22 g.) was dissolved in dilute aqueous KOH (700 ml., pH 8·5). The solution was boiled for a few minutes with $BaCO_3$, cooled and centrifuged. The clear supernatant liquid was poured into ethanol (4 vol.), the precipitate separated in the centrifuge and dried in a vacuum over P_2O_5. It was a yellowish white powder (18 g.); N, 7·9%, $[\alpha]_D - 56°$ in water (*c*, 0·3). This material was heated again in water (600 ml.) with $BaCO_3$ (25 g.) for a further period of 6 hr. at about 80°. The insoluble matter was separated in the centrifuge and the supernatant liquid was poured into ethanol (4 vol.). The white, fibrous precipitate was collected on a rod, and dried in a vacuum over P_2O_5. It was re-extracted with water, precipitated and dried again. This preparation (10·5 g.) showed $[\alpha]_D - 30·6°$ in water (*c*, 0·72); N, 3·7; ash, 19·1%. The relative viscosity (see above) was 1·3 at both 0·15 and 1·0 g./l. This preparation was regarded as depolymerized hyaluronic acid and was a typical product.

Hydrolysis of potassium hyaluronate by acid. A portion of the potassium hyaluronate preparation (1·0 g.) in 0·1 N aqueous HCl (60 ml.) was heated in a water bath for several hours. There was no change in rotation during the first 8 hr. and then only a slow change after a further 8 hr. $[\alpha]_D \rightarrow -12°$. At this stage the acid concentration was increased to 1·0 N. Reaction then proceeded more rapidly: $[\alpha]_D - 12° \rightarrow +24°$

(max.) in 50 hr. The hydrolysate gave positive tests for carbohydrate, reducing sugar, amino sugar (Elson & Morgan, 1933) and uronic acid (naphthoresorcinol). White crystals of an amino sugar were isolated from the hydrolysate. These were separated and recrystallized from methanol-acetone mixture (0·25 g.) (cf. Meyer & Palmer, 1936). (Found: $[\alpha]_D^{21°}+67°$ (constant after 18 hr.) in water; c, 1·82.) Glucosamine hydrochloride requires $[\alpha]_D^{20°}+72·5°$, equilibrium value in water. It was identified by formation of *N*-acetyl glucosamine (decomp. about 190°; $[\alpha]_D-40·9°$ in water) and also by an X-ray powder photograph (cf. Cox & Jeffrey, 1939).

approx.). The hydrolysate was examined by the filter paper strip method as used by Partridge (1946) for reducing sugars. Two papers containing a drop (0·01 ml.) of a 2% aqueous solution of each of the following were irrigated for 12 hr., one with *n*-butanol-ethanol and the other with *n*-butanol-acetic acid. Both were dried at 105°, developed with ammoniacal $AgNO_3$ and heated at 105° for 10 min.: (i) D-glucosamine, (ii) D-glucurone, (iii) D-glucosamine + D-glucurone, (iv) hydrolysate (see Fig. 2). On both strips the hydrolysate gave rise to a zone corresponding with that given by authentic D-glucosamine. Authentic D-glucurone gave rise to an upper zone (*a*) due to the free acid formed from the lactone and to

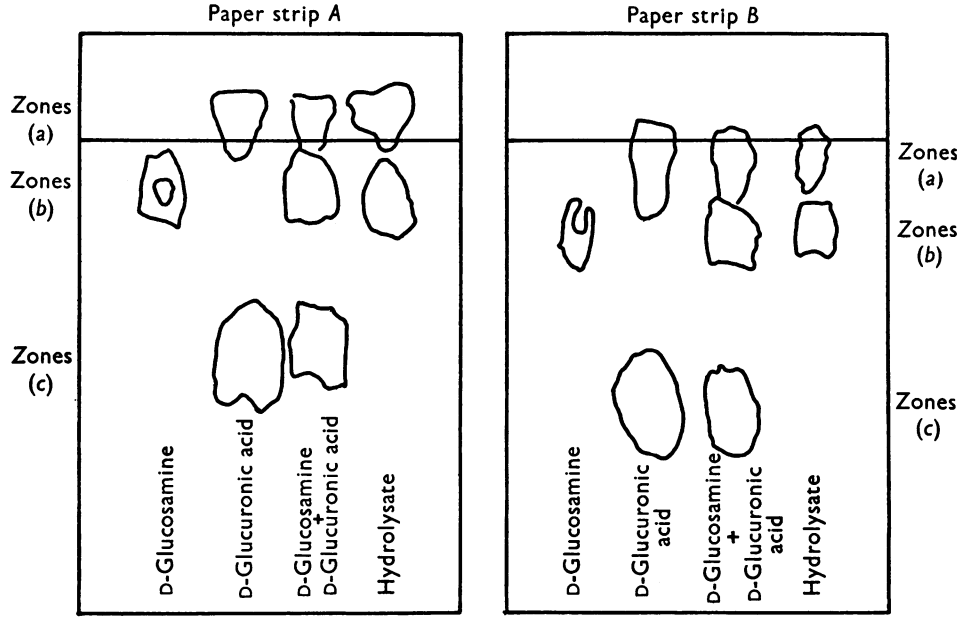

Fig. 2. A diagrammatic reproduction of the paper-strip chromatograms comparing a hydrolysate of potassium hyaluronate with authentic substances. Both strips were irrigated for 12 hr. (*A* with *n*-butanol-ethanol, *B* with *n*-butanol-acetic acid) and developed with ammoniacal $AgNO_3$.

Chromatography of the hydrolysate. The conditions were determined which gave the maximum hydrolysis of potassium hyaluronate with the minimum decarboxylation of the uronic acid. Accordingly, potassium hyaluronate (0·14 g.) in N-H_2SO_4 (10 ml.) was maintained just below boiling point for 8 hr. It was exactly neutralized with N-NaOH (using a volume predetermined by a control titration) and concentrated under reduced pressure to small volume (1 ml. a lower zone (*b*) due to the lactone itself (cf. Partridge, 1948). The hydrolysate gave rise to an upper zone only, corresponding with that from D-glucuronic acid. Table 1 indicates the similarity of the R_F values of the hydrolysate zones to those of D-glucuronic acid and D-glucosamine respectively.

By irrigating a strip containing two separate drops of hydrolysate, cutting the strip longitudinally so that each drop then occupied a separate narrow strip, and by de-

Table 1. *Comparison of R_F values of the zones on the paper chromatograms*

	Paper A (solvent boundary 20·1 cm.)				Paper B (solvent boundary 26·4 cm.)			
	Zones (*a*)		Zones (*b*)		Zones (*a*)		Zones (*b*)	
	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$	Distance (cm.)	$R_F \times 10$
D-Glucosamine	—	—	1·2	0·60	—	—	2·5	0·95
D-Glucurone	−0·5	−0·25	—	—	0·7	0·27	—	—
D-Glucosamine + D-Glucurone	−0·5	−0·25	1·2	0·60	0·6	0·23	2·4	0·91
Hydrolysate	−0·6	−0·30	1·5	0·75	0·5	0·19	2·4	0·91

veloping only one of these narrow strips, the position of the zones on the undeveloped strip was determined by comparison with the developed strip. These undeveloped zones were cut out and eluted with water. In this way the upper zone (*a*) was found to give a positive naphthoresorcinol test confirming uronic acid and the lower zone (*b*) gave a positive Elson-Morgan test confirming hexosamine.

Methylation of hyaluronic acid. The depolymerized hyaluronic acid preparation (10·2 g.) was dissolved in water (70 ml.), mixed with CCl_4 (300 ml.) and 35 % aqueous NaOH (21 ml.) and stirred vigorously at room temperature. Methylation was effected by addition of dimethyl sulphate (80 ml.) and 35 % NaOH (149 ml.) at 10 min. intervals over a period of 1 hr., vigorous stirring being maintained throughout. The temperature was raised to 35° and dimethyl sulphate (75 ml.) and 35 % NaOH (160 ml.) were added at 10 min. intervals over a period of 2 hr., stirring continuously for a further 5 hr. Excess dimethyl sulphate was then destroyed by boiling and the CCl_4 distilled off. Stirring was continued while the mixture was cooled to 0° and neutralized with 25 % H_2SO_4. The Na_2SO_4 was separated by crystallization and precipitation with ethanol and the supernatant liquid was concentrated to a thin brown syrup. This product was stirred at 50° with CCl_4 (260 ml.) and 35 % NaOH (11 ml.). Methylation proceeded with addition of dimethyl sulphate (40 ml.) and 35 % NaOH (74 ml.) at 10 min. intervals over a period of 1 hr. Stirring was continued at 50° for 6 hr. The methylation process was repeated and the mixture cooled and neutralized and the product isolated as described. Six further methylations at 50° were effected similarly in pairs.

To protect free amino groups, the methylated product was acetylated with acetic anhydride (12 ml.) in dry methanol (120 ml.) at room temperature for 48 hr. The solvents were removed at 40° and the product 'dried' in a vacuum over P_2O_5 and KOH for several days. This material, in methyl iodide (30 ml.), containing just sufficient dry acetone (5 ml.) to effect solution, reacted vigorously in the cold upon the addition of Ag_2O (1 g.), due to the rapid methylation of free carboxyl groups. After refluxing for 8 hr. the Ag residues were removed in the centrifuge and the supernatant liquid was concentrated. (4·18 g. OMe, 25·6; N, 2·6 %. Mol. wt. 720 approx., in camphor.) This molecular weight is essentially a minimum value owing to the poor solubility of the product in camphor.

This product, in dry methanol (50 ml.), was acetylated again with acetic anhydride (5 ml.) in the presence of silver acetate (1 g.) for 48 hr. at room temperature. The Ag salts were removed in the centrifuge and the supernatant liquid was concentrated and dried over KOH in a vacuum. This product was methylated in methyl iodide (20 ml.) in the presence of Ag_2O (2 g.) added in two portions during 8 hr. The product was isolated in the usual manner and the methylation repeated (OMe, 32·9 %.) This figure was not increased after further methylation. Yield, 2·75 g.

Fractionation and methanolysis of methylated hyaluronic acid

The methylated polysaccharide in 1 vol. of $CHCl_3$ (10 ml.) was fractionally precipitated by light petroleum. Four fractions were obtained (see Table 2).

Fraction *F*4 (1·27 g.) was refluxed with 3 % methanolic HCl (100 ml.) (see Fig. 3, curve *b*). After 34 hr. the solution was neutralized with Ag_2CO_3 and the filtered solution concentrated to a thin syrup, 1·10 g. (OMe, 37·3 %.)

Table 2. *Fractional precipitation of methylated depolymerized hyaluronic acid from chloroform solution by light petroleum* (60–80°)

Fraction	Precipitant volume	Wt. (g.)	Ash (%)	OMe (%)	$[\alpha]_D$
*F*1	1	0·12	55·4	24·1*	+12·4°* in $CHCl_3$ (*c*, 0·57)
*F*2	6 (+5)	0·59	0·0	29·0	+17·9° in $CHCl_3$ (*c*, 1·06)
*F*3	21 (+15)	0·29	0·0	29·0	+91·9° in $CHCl_3$ (*c*, 1·03)
*F*4	∞	1·29	0·0	33·7	+ 8·9° in methanol (*c*, 1·29)

* Calculated on ash-free basis.

Fractions *F*1 and *F*2 were combined (0·61 g.) and refluxed with 3 % methanolic HCl (60 ml.) (see Fig. 3, curve *a*). After 30 hr. the product was isolated in the usual way and concentrated to a syrup, 0·47 g. (OMe, 33·3 %.)

Fractionation of the methanolysates. Fraction *F*4 methanolysate (1·10 g.) was distilled in a high vacuum and yielded two fractions: (*a*) a mobile, ether-soluble fraction, *F*4*a*, 0·41 g. at 120–140° and 0·01–0·007 mm. Hg (OMe, 46·2; N, 0·2 %; n_D 1·4560; $[\alpha]_D^{17.5°}$ +3·8° in $CHCl_3$ (*c*, 1·31)); and (*b*) a viscous, ether-insoluble fraction, *F*4*b*, 0·26 g. at 210–240° and 0·005–0·004 mm. Hg (OMe, 27·3; N, 0·9 %; n_D 1·4835; $[\alpha]_D^{17.5°}$ +7·4° in $CHCl_3$ (*c*, 0·94)).

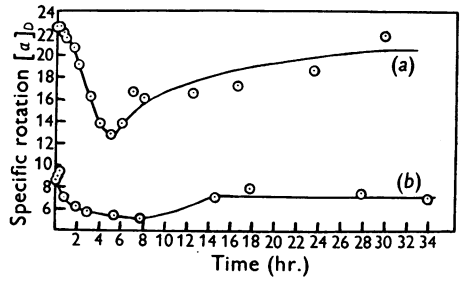

Fig. 3. Hydrolysis of the methylated depolymerized hyaluronic acid. (*a*) Fraction *F*2; (*b*) fraction *F*4.

Fraction *F*4*a* was methylated with methyl iodide and Ag_2O (×6) to OMe 50·2 % (constant). The product, in acetone (5 ml.), was passed through a column of activated Al_2O_3 and the eluted solution collected in 2 ml. portions which were grouped into fractions according to the optical rotation. The first fraction was concentrated and re-esterified with diazomethane in ether to ensure that no carboxyl groups, liberated by the catalytic action of the Al_2O_3, remained free (cf. Strain, 1942). The ether was removed and the amide formed from the ester in methanolic NH_3. The solvents were removed at 0° and the product (20 mg.) dissolved in acetone from which a small crop of crystals was obtained. $[\alpha]_D^{20°}$ +136° in acetone (*c*, 0·5); m.p. and mixed m.p. with authentic 2:3:4-trimethyl-α-methyl D-glucopyruronoside acid amide 179°.

Fraction *F*1–*F*2 methanolysate (0·47 g.) was acetylated and remethylated several times in the usual manner without increasing the methoxyl content (33 %). The product was distilled in a high vacuum yielding two fractions, *F*2*A* (81 mg.) and *F*2*b* (23 mg.), which resembled the fractions *F*4*a* and *F*4*b* respectively in solubility and viscosity.

Fraction *F*2*a* was methylated twice with methyl iodide and Ag_2O and the amide formed in methanolic NH_3. The solution was concentrated to a syrup at 0° and dissolved in dry acetone (1 ml.) when a small quantity of crystalline material separated, resembling in crystalline form the fraction *F*4*a* and the authentic 2:3:4-trimethyl-α-methyl D-glucopyruronoside acid amide: m.p. and mixed m.p. with the authentic material 174–175°.

The ether-insoluble fractions. Fractions *F*2*b* and *F*4*b* were combined, acetylated and methylated to constant methoxyl content (×4) with methyl iodide and Ag_2O. The product (0·29 g.) contained OMe, 37·4; N, 1·8 %. This material would not crystallize and the low N content suggested that the main part of the glucosamine residue had remained undistilled; therefore the two still residues were combined and extracted with $CHCl_3$. The extract was a dark, viscous liquid (0·18 g.). OMe 19·1; N, 6·1 %. Mol.wt. 327 ± 10. (Calculated for a dimethyl *N*-acetyl glucosamine: OMe, 25; N, 5·6 %; mol.wt. 283.) The methoxyl content could not be increased by further methylation or by methanolysis. It was non-reducing to Fehling's solution.

RESULTS AND DISCUSSION

It is interesting to compare the physical nature of the umbilical cord mucin and potassium hyaluronate preparations. Both form a stringy meshwork in the wet state when precipitated from solution, but when dried the hyaluronate assumes a flexible, fibrous nature whilst the mucin shrinks to a very hard and compact mass which is very tough and brittle and which can be ground to a granular powder. The depolymerized hyaluronate is precipitated in flocks, not fibres, and dries to a fluffy powder of very open texture. It is well known that considerable cross-linking between long chains leads to a brittleness of the product. It is suggested that the physical nature of the mucin is due to considerable cross-linking of the hyaluronic acid and protein to form a fairly rigid three-dimensional structure. On this account it might be thought feasible that such a complex will have a greater viscosity than any one component separated from it (cf. Meyer & Chaffee, 1940*b*): Hadidian & Pirie (1948) found that removal of protein lowers the viscosity. Recently, Ogston & Stanier (1950) reported the isolation of a hyaluronic acid-protein complex from synovial fluid by an ultrafiltration procedure, having the same viscosity as the fluid, but removal of protein was found to cause a lowering of the viscosity.

Although the viscosity of the potassium hyaluronate was low, the relative viscosity increased slightly with increase in concentration, which phenomenon is characteristic of the more highly polymerized samples (cf. McClean & Hale, 1941; Blix & Snellman, 1945; Hadidian & Pirie, 1948). On the other hand, the relative viscosity of the depolymerized preparation bore a linear relationship to the concentration.

The hyaluronic acid obtained from the potassium salt was submitted to Prof. W. T. Astbury for X-ray examination, but the X-ray photographs were unsatisfactory and the substance showed no crystalline form. This is in accordance with the conclusions of Blix & Snellman (1945) that hyaluronic acid has no very highly ordered structure. However, the relative viscosity was found to have been decreased by contact with the 'Zeo-Karb 215' and further samples are being prepared suitable for X-ray examination.

To the authors' knowledge, the paper chromatographic results provide the first direct evidence other than that of a colour test for the presence of uronic acid in an acid hydrolysate of hyaluronic acid. Meyer has isolated and characterized its lead salt from an enzymic hydrolysate (Meyer, Smyth & Dawson, 1939) and its presence in hyaluronic acid has been inferred also from the isolation of a saccharic acid derivative as an oxidation product (Meyer & Palmer, 1936; Kendall, Heidelberger & Dawson, 1937). It seems that many uronic acids are very slow-moving when irrigated on paper. Also, their R_F values are very close, so that the chromatographic results cannot be regarded as providing conclusive evidence for the presence of glucuronic acid specifically.

A protein-free product containing 3·7 % nitrogen could be obtained from the mucin by treatment with barium carbonate. This method of isolation was selected as a milder modification of a more usual, but drastic, procedure of separating polysaccharides from protein matter using sodium hydroxide, since the latter is known to cause severe degradation of hyaluronic acid (cf. Kaye, 1948). The product had a lower specific rotation than the potassium salt obtained by the Sevag procedure and its aqueous solutions were less viscous, suggesting that a certain amount of depolymerization had occurred, which was not apparent in preparations obtained by less drastic methods. However, as the object of the methylation technique for investigating polysaccharides was the recognition of the position of glycoside linkages present, and as these are normally stable to alkaline conditions and not involved in depolymerization brought about by alkali, this product, easily obtained in large quantities, was regarded as suitable for a first investigation.

The Freudenberg methylation procedure (Freudenberg & Boppel, 1938) in liquid ammonia failed to give a satisfactory product and was abandoned. The methylated product obtained in 30 % yield by the Haworth (1915) technique was more satisfactory. The methoxyl content (33 %) corresponds to 4·2 groups/glucuronic acid-*N*-acetyl glucosamine disaccharide anhydro unit of an endless chain. Theory

requires five such groups and 2·7 % nitrogen. Although further methylation could not be effected by the methods used, the following reasoning regarding the fractionation of the product by light petroleum seems to show that it was probably not fully methylated. Fractions *F* 1, *F* 2 and *F* 4 are almost certainly derived from the same molecular species differing slightly in extent of methylation, the least methylated being precipitated first. While fraction *F* 3 is apparently different from the others, probably more degraded, it constitutes only 10 % of the product. Thus, assuming fractions *F* 1, *F* 2, and *F* 4 to be of similar type, it is apparent that fractions *F* 1 and *F* 2 cannot be fully methylated.

Analysis results of the undistilled residue of methanolysed fraction *F* 4 simulate those for a dimethylated glucosamine derivative with possibly a small percentage of dimer. The smaller methoxyl content and higher nitrogen content of fraction *F* 4*b* than *F* 4*a* also suggests that the former contains a greater percentage of a partially methylated glucosamine. Furthermore, these glucosamine units could not be more fully methylated even after further methanolysis and acetylation of the amino nitrogen. It may be noted that glucosamine in a straight chain has two exposed hydroxyl groups available for methylation; methanolysis will produce a third one. Thus, the formation of dimethyl glucosamine derivatives indicates one of three possibilities: (*a*) branching of the chain at the glucosamine unit, (*b*) rupture of the glycoside link without the introduction of a methyl group, although how this will occur in the absence of water is not quite clear, (*c*) only partial methylation of the original polysaccharide itself, in which case, characterization of the partially methylated glucosamine derivatives cannot lead to conclusions regarding the structure of the polysaccharide molecule.

The authors are of the opinion that, for certain reasons, methylation of the hyaluronic acid molecule was incomplete; and, probably for the same reasons, complete methylation of the glucosamine moiety could not be effected. These reasons may be connected in some way with the amino group and its acetylated form. The state of combination of the acetyl group in most naturally occurring polysaccharides containing glucosamine is not yet fully understood. For example, it has been reported (Wolfrom, Weisblat, Karabinos, McNeely & McLean, 1943) that the amino group of heparin is neither free nor acetylated and a recent publication (Jorpes, Boström & Mutt, 1950) suggests an —NH—SO_2OH grouping. Hahn (1946) claims to have isolated *N*-acetyl glucosamine from hyaluronic acid after an enzymic hydrolysis, but it has never been isolated from an acid hydrolysate of the polysaccharide. Furthermore, although it has never been found possible to methylate glucosamine itself owing to the electron-donating properties of the amino group, *N*-acetyl glucosamine can be methylated (see Moggridge & Neuberger, 1938); however, attempts to methylate chitin, the supposed poly-*N*-acetyl glucosamine, have not met with any success (Stacey, 1944). It seems, then, that the position and effects of the acetyl group, or the grouping which will give acetic acid on hydrolysis, in this type of molecule require investigation.

Regarding fraction *F* 4*a*, it may safely be assumed that the nitrogen belongs to a glucosamine unit. This being so, presumably it will be more completely methylated than the dimethyl derivative which would not distil under these conditions. If it were a trimethyl *N*-acetyl glucosamine it would constitute only 3·8 % of the fraction, and allowing for this, the remaining glucuronic acid derivative would have a 47 % methoxyl content. This is the value required for a tetramethyl glucuronic acid derivative. This must originally have occupied a central position in a chain, since an end unit of this type (necessarily linked through carbon 1) would yield a fully methylated derivative which would contain 56 % methoxyl. (Upon methylating fraction *F* 4*a*, the methoxyl content was raised to 50·2 % only. This may well explain why only a small quantity of the fully methylated acid amide was obtained.)

The methoxyl content of the combined fractions *F* 2*b* and *F* 4*b* indicates that the glucuronic acid was probably the trimethyl but certainly not the fully methylated derivatives. Thus, 2·75 g. of the methylated product yielded, in four fractions, 2·3 g., of which 2 g. contained non-end-group glucuronic acid. The methylated product was obtained in 30 % yield; therefore, it can be concluded that at least one-third of the glucuronic acid in hyaluronic acid occurs in a 'central' position in the chain and, unless the losses during methylation included selective rupture of end-group glucuronic acid units, it might be suggested as an hypothesis, that only a very few, if any, of the glucuronic acid groups occupy an end position (cf. Kaye & Stacey, 1950).

The type of linkage that combines the protein and carbohydrate molecules in umbilical cord mucin, whether the latter be naturally occurring or artificial, is unknown, it having been presumed to be merely a salt type. Recently, Ogston & Stanier (1950) have reported the existence of a firm linkage between protein and hyaluronic acid in synovial fluid. It was considered, therefore, that the methylation of the cord mucin might provide certain indications of the presence or absence of covalent linkages between protein and polysaccharide. A methylated product was obtained from the mucin containing 18 % methoxyl, 6·6 % nitrogen and 5 % acetyl. Thus, only about one-half of the product could be accounted for by the hyaluronate moiety, the remainder containing about 10 % nitrogen, being

presumably of protein origin. After methanolysis a small unidentified fraction containing 12·5% nitrogen was obtained, in fact no fraction was found to contain less than 6·5% nitrogen. Further treatment failed to produce any nitrogen-free fractions.

Stacey & Woolley (1940) were able to remove all of the protein from ovomucoid during methylation. This was not so in the present case, however, and in view of the recent publications of Gottschalk & Partridge (1950*a*, *b*) reporting the interaction of simple sugars and proteins, we suggest, by way of explanation, that some irreversible reaction may have occurred between the carbohydrate and protein moieties of the methylated product at some stage after the methylation.

Until further information is available concerning the possible interaction between protein and carbohydrate molecules under varying conditions, the mucin must be regarded as an unsuitable starting material for chemical investigations.

SUMMARY

1. Potassium hyaluronate has been prepared from human umbilical cords and the free acid obtained therefrom using 'Zeo-Karb 215' to remove the potassium ions. Both were obtained in fibrous form.

2. The hyaluronic acid preparation has been submitted to X-ray examination, but it gave no satisfactor X-ray photographs and showed no very highly ordered structure.

3. From an aqueous acid hydrolysate of potassium hyaluronate glucosamine hydrochloride was crystallized and identified by an X-ray powder photograph. The presence of glucosamine and a uronic acid in the hydrolysate was confirmed by application of the paper-strip chromatographic technique.

4. A partially depolymerized hyaluronate preparation was obtained and methylated with dimethyl sulphate followed by methyl iodide, to yield a product containing 33% methoxyl. From this, a uronic acid derivative was isolated and identified, after further methylation, as 2:3:4-trimethyl α-methyl-D-glucopyruronoside acid amide. At least one-third of the uronic acid residue in the hyaluronic acid molecule is present in a chain, linked glycosidically through its reducing group and one other hydroxyl group and is not present as an end group. No amino sugar derivatives could be identified from the methylated product, the significance of which is discussed.

5. Methylation with sodium and methyl iodide in liquid ammonia (Freudenberg) was unsatisfactory and the method was considered unsuitable for hyaluronic acid.

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