Short Communications

SC 2260

Chromatographic separation of sulfated oligosaccharides

For the separation of the sulfated oligosaccharides, a chromatographic method using ion exchange has been developed by HOFFMAN *et al.*[1]. This method involving the use of strong mineral acids is unfavourable for unstable sulfate esters and can not be applied on a smaller scale because of the broadness of the peaks.

We found that neutral sodium salicylate solution could effect a chromatographic separation of an incomplete hyaluronate lyase, (EC 4.2.99.1) hyaluronidase, digest of chondroitin sulfate on Dowex-1. This separation is understandable since the salicylate ion possesses a much higher combination constant to anion exchangers of the polystyrene type compared to monovalent inorganic anions.

480 mg of chondroitin sulfate C of shark cartilage (a product of Seikagaku Kogyo Co. Ltd., Tokyo, Japan) dissolved in 120 ml of 0.02 M phosphate buffer (pH 6.2) containing 0.45 % NaCl were digested with testicular hyaluronate lyase at 37°.

After 3 days' incubation when the reducing power as measured by the method of NELSON[2] and SOMOGYI[3] reached 7.7 μequiv of glucose per ml of the digestion mixture, fractionation of the digestion products with ethanol was carried out under the conditions of MEYER *et al.*[4]. The supernatant from the 50 % ethanol precipitation was passed through a column of Dowex-50 X8 (H form, 30–50 mesh). The effluent was neutralized and concentrated to one tenth the volume of the digestion mixture. 10 vol. of ethanol were added and the resulting precipitate amounting to 300 mg was used as the material for the chromatography.

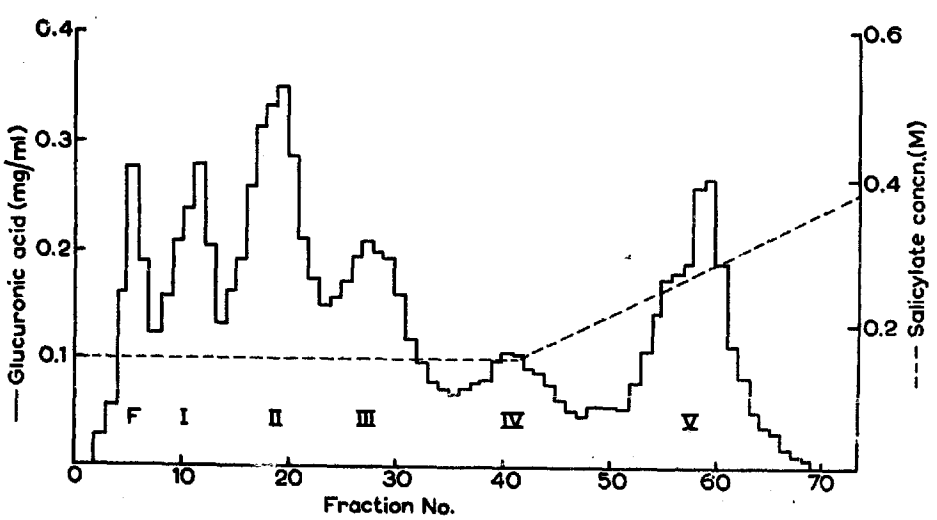

Fig. 1. Chromatography of the hyaluronate lyase digest of chondroitin sulfate. 10 ml were collected in each tube and the presence of glucuronic acid in the effluent was determined according to DISCHE[6]. Up to the Peak V, 70 % of the material was recovered and a further 10 % could be eluted with 0.4 M salicylate.

The material in 30 ml water was applied to a column (1.6 × 30 cm) of Dowex-1 X2 (salicylate form, 200–400 mesh) and the elution with sodium salicylate (pH 6.5) was carried out with the results shown in Fig. 1. Each peak of the chromatogram was concentrated and acidified to pH 2–3 with HCl. Salicylic acid was then repeatedly extracted with ether and after neutralization of the aqueous layer with NaOH, the saccharides could be recovered as an ethanol precipitate. Alternatively, the effluent was treated with Dowex-50 X8 (H form, 30–50 mesh) and after extraction of salicylic acid followed by neutralization, the saccharides could be recovered either by precipitation with ethanol or by lyophilization.

In this chromatogram, the overlapping of the neighbouring fractions is apparent. Chromatography of a smaller amount of the material on the same column or rechromatography of each peak could bring about complete separation of each fraction except the Peaks F and V. The former contained the disaccharide, the repeating unit of chondroitin sulfate, as the main component, but was contaminated with some unidentified sugars. The latter appears to be a mixture of the hexamer and heptamer of the repeating unit. The further rechromatography of these fractions is necessary for obtaining a homogeneous preparation.

The analytical values on each purified peak are shown in Table I. The values show that these saccharides belong to a homologous series of the repeating unit of chondroitin sulfate C. The conclusion was also supported by a linear relationship of $\log(1/R_F - 1)$ *versus* the degree of the polymerization[5].

TABLE I

CHARACTERIZATION OF THE PEAKS I–IV

The following methods were used for the analysis. Carbazole reaction of DISCHE[6] for uronic acid, SVENNERHOLM modification of Elson–Morgan reaction[7] for amino sugar after hydrolysis in 2 N HCl at 100° for 16 h, benzidine method of DODGSON AND SPENCER[8] for sulfate after hydrolysis in 6 N HCl at 100° for 20 h, and ferricyanide method of PARK AND JOHNSON[9] for reducing power. In the calculation of the reducing power, the experimental values obtained by using glucose as the standard were divided by a factor of 1.67 which had been adopted by SUZUKI AND STROMINGER[10] for the analysis of the sulfated oligosaccharides from chondroitin sulfate A. R_F values are those in paper chromatography using isobutyric acid–0.5 N aq. ammonia (1:1). R_F value of the disaccharide, the repeating unit, was 0.52. Identical infrared spectra of these saccharides were observed with the characteristic absorption at 775, 820 and 1000 cm^{-1} indicating that these saccharides possess sulfate group at C-6 of the galactosamine moiety[11].

	Peak			
	I	*II*	*III*	*IV*
Yield from 300 mg (mg)	28	56	49	19
Uronic acid (%, as free acid)	39.5	37.2	37.7	39.6
Aminosugar (%, as free base)	32.5	34.6	35.5	32.4
Sulfate (%, as SO_4)	16.7	19.6	19.3	20.8
Molar ratio uronic acid/aminosugar	1.12	1.00	0.98	1.13
Molar ratio sulfate/aminosugar	0.96	1.06	1.03	1.20
Mol. wt. from reducing power	1010	1670	2490*	3250*
Mol. wt., calculated	1024	1527	2030	2533
R_F values	0.40	0.35	0.28	0.26

* The deviation from the calculated values is apparently due to the fact that the factor 1.67 is too large for the higher homologues.

The yield of a single chromatography for the peaks up to V was 80 % or more, but dropped as the molecular weight of the saccharides increased. The undigested chondroitin sulfate could be eluted with 0.4–0.5 M sodium salicylate, but the yield was only 40–60 %.

Since this method can be applied also to a few mg or less of the material, it will be a useful procedure for purification of the degradation products of sulfated mucopolysaccharides or of the naturally occurring sulfated oligosaccharides and also of other acidic substances of various types which are adsorbed on the resin as strongly as sulfate esters. Of equal value, the desalting of the effluents is very easily achieved. We are applying this process for studies on biogenesis of cartilage of chicken embryo with the aim of finding out the sulfated oligosaccharides which might exist as intermediates for the biosynthesis of mucopolysaccharides.

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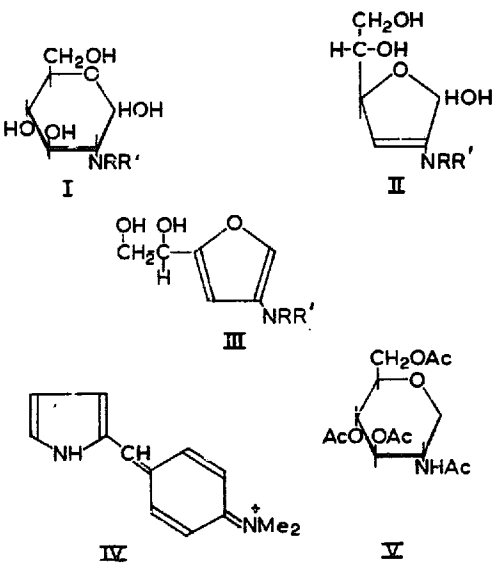
On the Morgan-Elson reaction

The Morgan–Elson procedure[1] has been used extensively for the estimation[2,3] and detection[4] of 2-acetamido-2-deoxy-D-hexoses. In this procedure a solution of the sugar, after heating for a short time under alkaline conditions, is treated with an acid dimethylaminobenzaldehyde reagent and the resultant purplish colour measured.

KUHN AND KRUGER[5,6] have shown that under alkaline conditions, 2-acetamido-2-deoxy-D-glucose (I: R=CH_3.CO., R′=H) is partly converted into an anhydro sugar (which probably has the structure II, R=CH_3.CO., R′=H) together with smaller amounts of other materials including a furan derivative III (R=CH_3.CO., R′=H). It has been suggested[7] that under the acid conditions of the DMAB reagent, the anhydro sugar II (R=CH_3.CO., R′=H) loses another molecule of water to form the furan derivative III (R=CH_3.CO., R′=H) which reacts with DMAB to form the

Abbreviation: DMAB, dimethylaminobenzaldehyde.

characteristic colour of the assay technique. There are analogies for each of these steps: treatment of hexoses with dilute alkali produces anhydro sugar intermediates similar to II by a β-elimination mechanism[8]; acid treatment of such intermediates results in dehydration to furan derivatives[9]; the reaction of pyrroles with an acid DMAB reagent to give coloured compounds of type IV (see ref. 10) is analogous to that postulated for the furan derivative III. A difficulty with this theory is that different colours were apparently obtained[6] from the anhydro sugar II (R=CH_3.CO., R'=H) and the furan derivative III (R=CH_3.CO., R'=H) after reaction with DMAB under acid conditions.



In the present work the pure anhydro sugar II (R=CH_3.CO., R'=H) and the furan derivative III (R=CH_3.CO., R'=H), prepared by modifications of the methods of KUHN AND KRUGER[5,6], have been shown to react directly with an acid DMAB reagent[2] to give, at similar rates, similar amounts of a colour with absorption maxima at 545 and 584 mμ. The colour resembled closely that formed from *N*-acetylglucosamine by the Morgan–Elson technique[2]. The slow fading from purple to red, which was observed in each case, has been attributed to instability of the furan ring[7]. Quantitative studies indicated that approx. 20 and 50 % respectively of the acetamido compound I was converted to the corresponding anhydro sugar II by the carbonate and borate treatments of Morgan–Elson procedures[2,3] and that under the acid conditions of the DMAB reagent[2] the anhydro sugar II (R=CH_3.CO., R'=H) was converted almost quantitatively to the furan derivative III (R=CH_3.CO., R'=H); the efficiency with which the latter coupled with DMAB to form the coloured compound is not known. It was established that the colour development from the acetamido compound II was dependent upon the concentrations of DMAB, water and acid in the DMAB reagent.

Preparations of 2-acetamido-3:4:6-tri-O-acetyl-2-deoxy-D-glucose[11–13] (V), shown by paper chromatographic methods to contain neither acetamido compound II or III, gave a direct colour with the DMAB reagent. This colour, with absorption maxima at 545 and 584 mμ and an unresolved shoulder at 510–520 mμ, developed and faded more slowly than that derived from the anhydro sugar II (R=CH_3.CO., R'=H). Since *N*-acetylglucosamine gives no colour directly with the DMAB reagent, it was

concluded that the 3-O-acetyl group in the tetra-acetate V must favour anhydro sugar formation under acidic conditions (as has already been shown under basic conditions[6,14]).

There is little information available as to the colour given using the Morgan–Elson procedure upon different N-substituted derivatives of 2-amino-2-deoxy-D-glucose and some confusion as to the conditions under which reaction takes place with *N*-alkyl derivatives. It has been found that, after heating at pH 9–10, a variety of 2-acylamido derivatives of type I (R=H.CO., $Cl.CH_2.CO.$, $NH_2.CH_2.CO.$, $C_2H_5.CO.$, $C_3H_7.CO.$, $C_6H_5.CO.$, $C_7H_7.SO_2.$; R′=H)[14] gave, with either of the usual acid DMAB reagents[2,15], different amounts of a colour indistinguishable from that obtained under similar conditions from the corresponding acetamido derivative. 2-Acetamido-2-methyl-2-deoxyglucose and 2-acetamido-2-carboxymethyl-2-deoxy-glucose, prepared by *N*-acetylation of the corresponding *N*-alkyl derivatives[16], gave high yields of reddish colours with broad absorption maxima at 510 and 518 mμ, respectively. In contrast, *N*-methylglucosamine and *N*-carboxymethylglucosamine gave no colour when treated according to Morgan–Elson procedures but gave low yields of a reddish colour with a broad absorption maxima at about 520 mμ when the alkaline treatment was carried out at about pH 13. Under these more strongly basic conditions *N*-alkyl compounds of type I also liberate some 2-amino-2-deoxy-glucose. This compound can undergo the Elson–Morgan reaction[15], thus accounting for the increased yield of colour with DMAB reagents from *N*-alkyl derivatives I after prior treatment with alkaline 2:4 pentanedione[17,18]. It is known[18,19] that under alkaline conditions, mixtures of hexoses and α-amino-acids ($NH_2.CH.R''.COOH$) give rise to *N*-alkyl compounds of type I (R=CH.R″.COOH, R′=H). The processes described above account for the interference produced by compounds of this type in the estimation of 2-acetamido or 2-amino-2-deoxy-hexoses in crude hydrolysates[3,20].

These results show that, under the conditions of the Morgan–Elson procedure, a wide variety of *N*-acyl groups facilitates chromogen formation (possibly by binding the lone electron pair on the nitrogen atom during the β-elimination process) but that they do not affect the colour formed by subsequent reaction with DMAB. *N*-Alkyl substituents, on the other hand, which do little to promote chromogen formation, have a marked effect upon the final colour. This suggests that acyl, but not alkyl, groups are eliminated during the colour development. It is hoped that this study of the processes taking place under the conditions of Morgan–Elson procedures will lead to a better understanding of the various chromogenic materials which may be present in preparations derived from biological sources.

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SC 2230

Periodate oxidation of hyaluronic acid

Studies by both chemical and enzymic methods have accumulated a body of evidence that supports a structure for hyaluronic acid composed of sequences of 2-acetamido-2-deoxy-3-*O*-[β-D-glucopyranosyluronic acid]-β-D-glucopyranosyl units linked to each other through position 4 of the D-glucuronic acid residues. The details of these studies have been reviewed recently[1,2]. Such a structure should be open to oxidation of the acid residues by periodate but studies of this reaction have been interpreted to indicate uronic units linked through position 3 (see refs. 3, 4) or equally through positions 3 or 4 (see ref. 5). The discrepancy between the results of periodate oxidation and other studies is untenable.

In the present studies the preparation of hyaluronic acid from umbilical cords followed essentially the procedures previously described. These involved digestion with papain[6], elimination of protein by Sevag's method, dialysis, and fractional precipitation as the cetylpyridinium complex[6]. The hyaluronic acid, isolated as the magnesium salt, showed $[\alpha]_D^{23}$ −80° in water[5] (*c* 0.23) and hexosamine, hexuronic acid, and *N*-acetyl in the mole ratios 1.00:1.00:1.05. The material gave very viscous aqueous solutions and contained no ester sulfate groups. Chromatographic analysis on Dowex 1 × 2 (formate form) by the procedure of SCHILLER *et al.*[7] showed only hyaluronic acid.

The general procedure for the oxidation of magnesium hyaluronate with periodate is illustrated by the following particular example. To magnesium hyaluronate (304 mg, by hexuronic acid and hexosamine determination) in water (220 ml) at 5° was added cold 0.35 M sodium metaperiodate (25 ml) and the solution diluted to 250 ml. A blank solution was similarly prepared and both were kept in the dark at 5°. At zero time and periodically thereafter, aliquots of the solution were analyzed for periodate consumption[8,9]. Aliquots (5 ml) were also added to 5% aq. ethylene glycol (1 ml) and after about 30 min a portion (5 ml) of this solution was placed in a well-washed dialysis casing. The solution was dialyzed overnight at 5° against distilled water and then electrodialyzed until free of iodate. The solution was quantitatively washed from the dialysis bag into a volumetric flask to which was added $NaBH_4$ (20 mg). The reduction proceeded overnight and the solution was then acidified with glacial