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The Chondroitin Sulfates of Healing Skin Wounds

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The hexosamine containing substances of wound granulation tissue from rats have been investigated. Chondroitin sulfate A and chondroitin sulfate B were isolated and characterized.

These two mucopolysaccharides, together with possible traces of hyaluronic acid account for less than 20 % of the total hexosamine of the tissue. The remaining portion is considered to be associated with unidentified glycoproteins.

Various roles have been proposed for mucopolysaccharides (MPS) in tissue repair processes or in the formation of new connective tissue. Thus, it has been suggested that MPS synthesis is a necessary prerequisite for the formation of collagen in healing wounds,[1] and in cotton pledget granulomas.[2] Studies by Dunphy and Udupa [3] and later by Rosenberg [4] indicated that the early phase of wound healing was associated with a relatively high tissue concentration of hexosamine, but that this is an extremely poor estimate of MPS concentration was shown by Grillo *et al.*[5] who found that the hexosamine content of wound tissue closely parallelled that of serum. Indeed, Jackson *et al.*[6] found that less than 3 % of the tissue hexosamine could be accounted for by the presence of MPS and that this largely consisted of chondroitin sulfates.

It is well known that the content of the various mucopolysaccharides differs greatly amongst connective tissues [4] and it has also been shown that the types of MPS present in a tissue vary with age.[8] Variation has also been described between histologically defined zones of a single tissue.[9,10] It was thus felt that if the relationship between MPS and collagen formation was to be elucidated, then an analysis of the MPS present in a tissue, which has previously been extensively used as a model for collagen biosynthesis studies, was necessary. Such a tissue is that formed in an open cutaneous wound. The elaboration of techniques by Antonopoulos *et al.*,[10] whereby this could be carried out on the small amounts of material available from wound tissue made the analysis possible.

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EXPERIMENTAL

Wound granulation tissue. 25 male Sprague-Dawley rats, weighing between 170 and 190 g, were weighed daily until they were gaining weight at a uniform rate whereupon they were wounded by the removal of a circular piece of dorsal skin about 4 cm in diameter. The rats were again weighed daily and it was found that they continued to gain weight for the duration of the experiment. On the eighth day after wounding, the rats were decapitated and the wound granulation tissue was dissected free. Care was taken to ensure that skin did not contaminate the samples which were then pooled and weighed (wet weight 24.9 g). The tissue was frozen in liquid nitrogen, crushed in a steel mortar and dried in several changes of acetone. Following defatting by means of chloroform/methanol it was reduced to a powder in a Wiley mill, 3.2 g of dry powder being obtained.

Tissue digestion. 2.5 g of the wound tissue powder was digested with papain in 60 ml of buffer as described by Antonopoulos *et al.*[11] Following digestion the mixture was clarified by high speed centrifugation and dialyzed for eight hours against distilled water. Aliquots were analyzed for hexosamine before and after dialysis and no losses were observed. Mucopolysaccharide was precipitated from the clear dialysate by the addition of ethanol to a concentration of 90 % together with 10 ml of 25 % sodium acetate. The precipitate was washed in ethanol and ether and dried in vacuo.

A small residue (69 mg) of undigested material was found upon centrifugation of the digestion mixture, but since it contained no hexosamine, it was not investigated further.

Fractionation procedures

ECTEOLA cellulose chromatography. 136.2 mg of the alcohol precipitable material was adsorbed in a column (2.5 × 30 cm) of ECTEOLA Cellulose prepared according to Anseth and Laurent[12] and eluted with 600 ml of 0.02 N HCl. Fractions of 12 ml were collected mechanically and were assayed for hexosamine and uronic acid content. The optical density at 280 mμ was also determined.

The column was then washed with 300 ml of 2 N NaCl, a procedure which has been shown to elute all mucopolysaccharides.[12]

Following dialysis this latter fraction was alcohol precipitated as before. Nine mg of a white powder, subsequently referred to as MPS powder was obtained.

Mucopolysaccharide separation. 2.15 mg MPS powder was dissolved in 300 μl of water and 25 μl aliquots were placed in each of nine cellulose micro-columns prepared according to Antonopoulos *et al.*,[10] and which had been previously washed with a 1 % aqueous solution of cetyl pyridinium chloride * (CPC). The mucopolysaccharide cetyl pyridinium (CP) complexes which thus formed were separated by eluting the column with 1 ml each of the following solutions: 1 % CPC, 0.3 M NaCl, 0.75 M $MgCl_2$ in 0.1 % acetic acid, 0.75 M $MgCl_2$ in water, and finally with 2 M $MgCl_2$ all containing 0.05 % CPC. Uronic acid was determined on the eluates from five of the columns and hexosamine on those from the remaining four columns.

A larger (1 × 15 cm) cellulose column was prepared and 6 mg of the MPS powder separated according to the above scheme. MPS were recovered from the eluates of this column by dilution with water so that the salt concentration was below the critical solubility[13] of the CP complexes.

The precipitates which formed in the fractions eluted with 0.75 M $MgCl_2$ in acid and 0.75 M $MgCl_2$ in water, were purified by redissolving in 50 % propanol, followed by ethanol precipitation. The material thus obtained was subjected to digestion with testicular hyaluronidase as described below. Infra red spectrometry was carried out in KBr pellets in a Unicam SP 200 spectrometer.

Paper chromatography of the ninhydrin oxidation products of the constituent amino sugars was also performed.[14]

Insufficient material was obtained from the 0.3 M NaCl eluate to permit anything but chromatographic analysis to be carried out.

Analytical procedures. Hexosamine was determined by the Blix modification of the Elson and Morgan procedure[15] or by the Boas method[16] depending upon the expected

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amount of contaminants. Uronic acid was assayed by the Dische procedure.[17] Sulfate was determined according to Antonopoulos.[18] Glucosamine/galactosamine separation was achieved using Dowex 50 columns as described by Gardell.[19]

Digestion with testicular hyaluronidase was carried out in a pH 7.0 Mc Ilvaine buffer at 37°C using about 3 I.U. hyaluronidase per microgram of MPS. Completeness of digestion was determined by CPC precipitation after dilution of buffer salts. The hyaluronidase was obtained through the courtesy of Dr B. Högberg, AB Leo, Hälsingborg, Sweden.

RESULTS AND DISCUSSION

ECTEOLA cellulose separation. It can be seen from Fig. 1 that 0.02 N HCl eluted hexosamine containing material in two peaks and that the second of

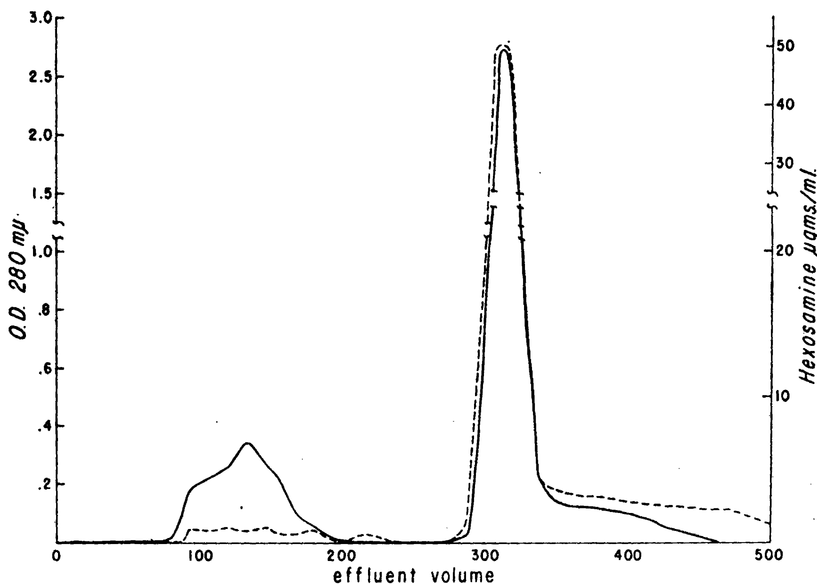

Fig. 1. Elution pattern of the 0.02 N HCl effluent from an ECTEOLA cellulose column. The solid line represents hexosamine concentration whilst the broken line represents optical density.

these two peaks is associated with high optical density at 280 mμ. Sufficient material could be recovered from this peak by lyophilization to permit separation of the constituent amino sugars [19] and both glucosamine and galactosamine were found to be present. This galactosamine does not, however, necessarily originate from chondroitin sulfate (CSA) since glycoproteins isolated from serum,[20] and from granulation tissue [21] are also known to contain this amino sugar. The lyophilized material was also analyzed for sulfate content and 11.57 μg of SO_4/mg of powder was found. Even if this small amount of sulfate was exclusively associated with a sulfated MPS such as keratosulfate it would only account for about 7 % of the weight of material obtained. No uronic acid was found in any of the fractions eluted with 0.02 N HCl so the presence

of hyaluronic acid is unlikely even though it has been shown to be very weakly bound to ECTEOLA cellulose at low pH.[22] It is thus felt, as suggested by Anseth and Laurent [12] that the material eluted with 0.02 N HCl, is glycoprotein in nature.

If the amount of material eluted with 0.02 N HCl is compared with that eluted with 2 M NaCl, then 80 % (1.45 mg) of the total hexosamine was found in the glycoprotein fraction whilst only 19 % (0.34 mg) was eluted with 2 N NaCl. These findings confirm the work of Jackson *et al.*[6] who showed that MPS of granulation tissue represent a relatively minor proportion of the total hexosamine content of this tissue.

Mucopolysaccharide separation. Table 1 summarizes the data obtained from analysis of the various fractions eluted as CP complexes from cellulose columns and it is apparent that nothing could be eluted with either 1 % CPC or with 2 M $MgCl_2$ which have been previously shown to dissolve the CP complexes of keratosulfate and heparin, respectively.[10,27 *] The absence of the latter may seem somewhat surprising in view of its demonstrated presence in skin.[23] It is known, however, that at the time of wounding local mast cells degranulate and the heparin granules are removed by macrophage activity (Jackson, personal communication) so that little or nothing would be expected to remain on the eighth day after wounding.

0.75 M Aqueous $MgCl_2$ soluble fraction. This solvent has previously been shown to elute the CP complex of chondroitin sulfuric acid B (CSA-B) [24] and in the present experiment also, material having the characteristics of CSA-B was isolated. Thus, Table 1 shows that the amino sugar moiety is exclusively galactosamine, as demonstrated by the presence of lyxose after ninhydrin oxidation. The apparent ratio uronic acid/hexosamine is 0.54 and this is consistent with the presence of iduronic acid which gives only 50 % of the color produced by glucuronic acid in the Dische procedure. The inability of testicular hyaluronidase to digest this material is further evidence of its identity with CSA-B.

The infra red spectrum, Fig. 2, shows absorbancies at 710 cm^{-1}, 840—855 cm^{-1}, 930 cm^{-1}, and at 1050 cm^{-1}, and is in agreement with previously published spectra of CSA-B.[25] The differences between the spectra of CSA-B and CSA-A are, however, very small.

0.75 M $MgCl_2$ (in acid) soluble fraction. Table 1 shows that this material has a uronic acid to hexosamine ratio very close to 1.0 and that the amino sugar is galactosamine, it is also digested with testicular hyaluronidase. All these data suggest its identity with either CSA-A or CSA-C which is also indicated by its solubility in this salt concentration.[24] These two isomers can be easily distinguished by infra red spectrometry, particularly if the ratio of absorbance at 800 cm^{-1} to that at 850 cm^{-1} is determined.[26] The infra red spectrum of this material, Fig. 3, shows absorbancies at 710 cm^{-1}, and 1040—1050 cm^{-1}, and the ratio A_{800}/A_{850} is 0.78. This is consistent with the material being CSA-A.[25,26]

0.3 M NaCl soluble material. It was not possible to isolate sufficient of this material to perform infra red spectrometry or to investigate its susceptibility

* Concentrations of $MgCl_2$ referred to in this publication [27] should be divided by a factor of four (Gardell, *personal communication*).

Table 1. MPS Separation on cellulose columns.

	Results from nine micro columns, mean ± S.E.				Macro column isolated material	
Eluting medium	Uronic acid μg/ml eluate	Hexosamine μg/ml eluate	% Total hexosamine	Ratio of uronic/hexosamine	Sugar present by chromatography	Hyaluronidase sensitivity
1 % CPC	0	0	0			
0.3 M NaCl	1.102 ± 0.11	1.13 ± 0.03	21.5	0.97	Lyxose arabinose	
0.75 M $MgCl_2$ in acetic acid	2.49 ± 0.06	2.68 ± 0.08	51.0	0.93	Lyxose	+
0.75 M $MgCl_2$ in water	0.77 ± 0.08	1.44 ± 0.08	27.4	0.54	Lyxose	−
2 M $MgCl_2$	0	0	0			

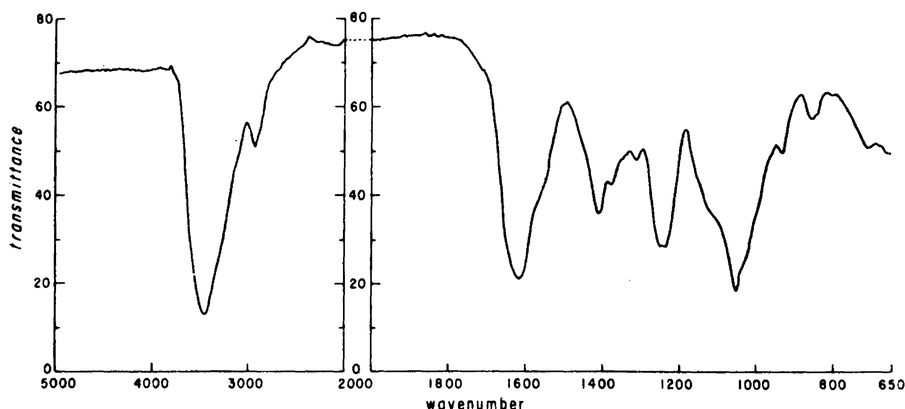

Fig. 2. Infra red absorption spectrum of material eluted with 0.75 M $MgCl_2$ in water (CSA-B). Pellet consists of 150 mg KBr + 500 μg sample.

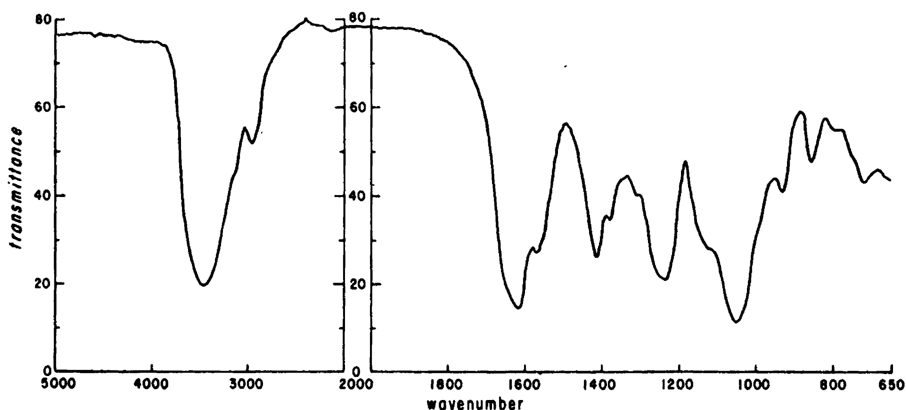

Fig. 3. Infra red absorption spectrum of material eluted with 0.75 M $MgCl_2$ in 0.1 N acetic acid (CSA). Pellet consists of 150 mg KBr + 1 mg sample.

to digestion with hyaluronidase. The presence of both lyxose and arabinose following ninhydrin oxidation indicates the presence of both glucosamine and galactosamine which suggests that it is a mixture, perhaps of hyaluronic acid which is normally eluted with this solvent [24] and of low charge density CSA.[13] If hyaluronic acid were indeed present in this fraction, then the very low critical salt concentration of this MPS [13] would account in part for its failure to precipitate by dilution, even though this fraction contains 21 % of the total hexosamine (Table 1).

The isolation of CSA-A and CSA-B is in agreement with the work of Berenson and Dalferes [28] who reported the presence of these two MPS along with that of hyaluronic acid and heparitin monosulfate, in an analogous tissue produced as a response to turpentine irritation.

The drastic method used here for the liberation of MPS will of course provide no information about their physical state in the tissue. It is felt, however, that the analysis presented is a necessary precursor to investigation of protein-polysaccharide interactions in this connective tissue.

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