[CONTRIBUTION FROM THE STUDY GROUP ON RHEUMATIC DISEASES AND THE DEPARTMENTS OF CHEMISTRY AND MEDICINE, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

A Method for the Desulfation of Chondroitin Sulfate[1]

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Treatment of dry potassium chondroitin sulfate with dilute HCl in methanol at room temperature yields a non-dialyzable product with no ester sulfate and with methylated carboxyl groups. This on hydrolysis with alkali yields a second product which appears to be desulfated chondroitin sulfate. Neither of these products has any significant metachromatic properties. These observations probably account for the observation of Fisher and Lillie that treatment of cartilage with acid methanol destroys its property of staining metachromatically.

Fisher and Lillie[3] described a technique for the suppression of metachromatic staining in cartilage by treatment with acid–methanol. Since the metachromatic staining of cartilage is mainly due to its content of chondroitin sulfate, the effect of acid–methanol might plausibly be sought in an effect on chondroitin sulfate, particularly in a reaction leading to the disappearance of its anionic groups. While acid–methanol would be expected to methylate the carboxylate groups of chondroitin sulfate it did not seem likely that it would methylate the ester sulfate groups. The ester sulfate groups of chondroitin sulfate are chiefly responsible for the intense metachromasia it produces. Therefore a study was made to determine what happens to these groups on treatment with acid–methanol.

Methods and Results

Potassium chondroitin sulfate was made from crystallized calcium chondroitin sulfate by the use of the potassium salt of Dowex 50. The acid–methanol solution was made by passing dry HCl into methanol and diluting with methanol to 0.06 *M* HCl as determined by titration. It could also more conveniently be prepared by adding acetyl chloride (5 ml.) to methanol (1 l.), and allowing the solution to age at least a day before use to complete the methanolysis of acetyl chloride. Finely powdered, well dried potassium chondroitin sulfate (2.40 g.) was shaken with acid–methanol (400 ml.) for a day. Both the starting material and the main product are insoluble in methanol. The mixture was centrifuged and the residue was similarly shaken twice more with fresh acid methanol (400 ml.) each time for a day. The combined separated acid methanol was clear and colorless and was worked up as described below. The residue was perfectly white and differed from the starting material in settling out from methanol much more slowly. It was dissolved in water (50 ml.) and after dialyzing the solution against running water the product was precipitated by addition of ethanol (300 ml.), separated by centrifuging, washed with ethanol and then with ether, and dried *in vacuo* over $CaCl_2$. The yield varied between 1.29 and 1.48 g., averaging 80% of the theoretical based on the reaction equation below. Analytical data in Table I show that the action of acid methanol on potassium chondroitin sulfate resulted in loss of ester sulfate. At the same time the carboxylate ion appeared to be methylated. As in previous analyses for chondroitin sulfate,[4] both hexosamine and hexuronate were found considerably below the values calculated for the theoretical repeating period.

Following the reaction the centrifuged clear acid–methanol appeared to contain the sulfate as potassium methyl sulfate, since on dilution with water it gave no immediate precipitate with barium chloride. The combined acid–methanol was evaporated *in vacuo* almost to dryness and the residue was dissolved in water (30 ml.) to which had been added concentrated HCl (5 ml.). The solution was heated at nearly 100° for 4 hours and then the sulfate precipitated with barium chloride. The yield of $BaSO_4$ was 0.832 g. or 90% of the theoretical.

TABLE I

ANALYTICAL DATA FOR POTASSIUM CHONDROITIN SULFATE AND TWO OF ITS DERIVATIVES

	Chondroitin sulfate Potassium salt		Desulfated chondroitin sulfate Methyl ester		Potassium salt	
Empirical formula per period	$C_{14}H_{19}NSO_{14}K_2 \cdot 4H_2O$		$C_{16}H_{23}NO_{11} \cdot 2H_2O$		$C_{14}H_{20}NO_{11}K \cdot 2H_2O$	
Period weight	607		429		453	
	Calcd.	Found	Calcd.	Found	Calcd.	Found
Hexosamine	29.5	25.4	41.7	35.0	39.5	32.1
Hexuronate	32.0	30.0	45.3	34.0	42.8	33.5
Nitrogen	2.3	2.5	3.3	3.0	3.1	3.2
Sulfur	5.3	5.2	0	<.1	0	<.1
Potassium	12.8	11.0	0	1.3	8.6	9.4
Methoxyl	0	1.4	7.2	9.6	0	1.2
Acetyl	7.1	8.1	10.0	9.8	..	....
Moisture	11.9	9.3	8.4	9.3	8.0	5.4
$[\alpha]^{25}D$, 2.5% H_2O	..	−25.0°	..	−14.6°	..	−22.2°

If the repeating unit of chondroitin sulfate be represented by KOOC–R–OSO_3K, the reaction appears to be

$$\begin{array}{l} \text{KOOC—R—OSO}_3\text{K} + 2\text{CH}_3\text{OH} + \text{HCl} \longrightarrow \\ \qquad\qquad \text{CH}_3\text{OOC—R—OH} + \text{KO}_3\text{S·OCH}_3 + \text{KCl} + \text{H}_2\text{O} \end{array}$$

The transesterification of the sulfate group from R to CH_3 might be expected also to occur with an alkaline catalyst. However, treatment of potassium chondroitin sulfate at room temperature with methanol containing KOH produced no change.

The methyl ester of desulfated chondroitin sulfate is soluble in water and its solution gives no precipitate with either hexamminecobaltic chloride or with the complex called hexol nitrate, either of which can precipitate chondroitin sulfate easily from dilute solution.[5] A comparison of its chromotropic properties with those of potassium chondroitin sulfate was made by a study of the variation of the optical densities of methylene blue at 665 mμ (α-band) and 570 mμ (μ-band) with chromotrope concentration. This was carried out over a wide range of chromotrope concentration since it has been shown that at too high or too low a concentration metachromasia may not be apparent.[6] The results shown in Fig. 1 are only for the α-band for which the effects are most prominent. At no concentration did the methyl ester of desulfated chondroitin sulfate (curve B) show any significant depression of the α-band or elevation of the μ-band. The small depression in the α-band produced at high concentration could be due to remaining traces of chondroitin sulfate.

Methanolic HCl at higher temperature and HCl concentration is used for methanolysis of polysaccharides. Since

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(2) Aided by a fellowship from the Arthritis and Rheumatism Foundation.

(3) E. R. Fisher and R. D. Lillie, *J. Histochem. & Cytochem.*, **2**, 81 (1954).

(4) J. Einbinder and M. Schubert, *J. Biol. Chem.*, **191**, 591 (1951).

(5) M. Vouras and M. Schubert, THIS JOURNAL, in press.

(6) A. Levine and M. Schubert, *ibid.*, **74**, 5702 (1952).

the desulfated product described above was isolated in good yield after dialysis it appears that under the conditions used no extensive decrease in chain length due to methanolysis could have occurred. An attempt to detect change in chain length was made by measuring reducing end-groups by the method of Meyer, Noelting and Bernfeld.[7] At the outset difficulty was met since chondroitin sulfate gave apparent chain lengths of only about seven periods when galactosamine or glucuronolactone were used as standards. The desulfated product showed a higher chain length, about 16 periods. Neither of these results appears plausible and a further study of the reaction seems needed before it can be applied to mucopolysaccharides.

Treatment of the methyl ester of desulfated chondroitin sulfate (250 mg.) with titrated aqueous sodium hydroxide (10 ml., 0.1 *M*) at room temperature for 1 or 2 days followed by back titration with standard HCl showed the liberation of free acid which in one day amounted to 0.93, and in two days amounted to 1.03 equivalents per period. From the neutralized solution, after dialysis, addition of potassium acetate and precipitation with four volumes of alcohol, the hydrolysis product was obtained as the potassium salt in a yield of 90%. Analytical data for this product are included in Table I. This product is analogous to hyaluronate in having one carboxylate ion per period. Like hyaluronate it is not precipitated from aqueous solution by hexamminecobaltic chloride but is precipitated by hexol nitrate.[5] A study of its metachromatic properties is included in Fig. 1, curve C, and shows a slightly greater effect on the α-band of methylene blue than did the methyl ester. Hyaluronate has a weak chromotropic effect[8] which is, however, not so feeble as that of the potassium salt of desulfated chondroitin sulfate. The difference could be due to the much higher molecular weight of the hyaluronate compared to chondroitin sulfate and consequently to desulfated chondroitin sulfate.

Discussion

The method described is a convenient one for the complete desulfation of chondroitin sulfate. It may be of some value in the determination of structure in the group of sulfated mucopolysaccharides since, as has often been pointed out,[8] methylation techniques applied to sulfated polysaccharides have generally not been successful. The desulfation method of Wolfrom and Montgomery[9] involves treatment of such polysaccharides at $-10°$ with almost absolute sulfuric acid followed by acetylation. From chondroitin sulfate a yield of about 30% was reported of the desulfated acetylated product. The method described in the present work is simpler and gives larger yields.

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(8) A. B. Foster and A. J. Huggard, *Adv. Carbohydrate Chem.*, **10**, 356 (1955).

(9) M. L. Wolfrom and R. Montgomery, THIS JOURNAL, **72**, 2859 (1950).

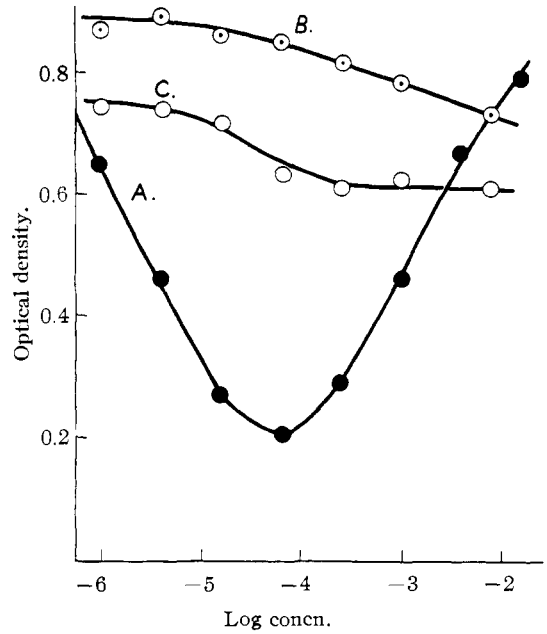

Fig. 1.—The effect of increasing concentration of chondroitin sulfate and its derivatives on the optical density of methylene blue at 665 mμ (α-band). Concentration of methylene blue constant in all solutions at 1.5×10^{-5} *M*. Abscissa, log concentration of chondroitin sulfate or its derivatives in periods per l. Ordinate, optical density of solutions at 665 mμ in 1 cm. cells: A, potassium chondroitin sulfate; B, methyl ester of desulfated chondroitin sulfate; C, potassium salt of desulfated chondroitin sulfate.

A point of nomenclature requires notice. It would be convenient to refer to desulfated chondroitin sulfate simply as chondroitin. The two products described here could then be called methyl chondroitin and potassium chondroitin, respectively. However, the word chondroitin has already been used by Davidson and Meyer[10] to describe a product isolated from cornea, containing 2% sulfate, and considered to be a precursor of chondroitin sulfate. To avoid confusion it seems best for the present to refer to the products described in the present work as desulfated chondroitin sulfate methyl ester and potassium salt, respectively.

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(10) E. A. Davidson and K. Meyer, *J. Biol. Chem.*, **211**, 605 (1954).