TABLE II

COMPARISON OF SOME CHARACTERISTIC PROPERTIES OF FRUCTOSE– AND TAGATOSE–AMINO ACIDS[a]

Compound	Total sugar: Parent sugar	Total sugar: Hot reduction of ferricyanide, glucose	Total sugar: Anthrone, % of glucose	Reduction of ferricyanide at room temp.,[3] % of fructose–glycines	Stimulation of incorporation *in vitro* of leucine into reticulocyte proteins, % of blank: Concn., *M* 5×10^{-4}	Stimulation of incorporation *in vitro* of leucine into reticulocyte proteins, % of blank: Concn., *M* 1×10^{-4}
Fructose		90	95	3	108	..
Glucose		100	100	≈0	109	..
Galactose		74	71	≈0	100	..
Fructose–L-alanine	Glucose	99	20	129	132	117
Fructose–L-alanine	Mannose	100	21	129	139	115
Fructose–L-aspartic acid	Glucose	82	45	89	135	132
Fructose–L-glutamic acid	Glucose	81	35	114	118	108
Fructose–glycine	Glucose	103	21	100	139	126
Fructose–glycine	Mannose	104	20	103	139	127
Fructose–L-leucine	Glucose	81	11	104	126	120
Fructose–L-phenylalanine	Glucose	70	13	108	127	121
Fructose–L-serine	Glucose	100	23	142	140	128
Fructose–L-threonine	Glucose	81	6	143	135	118
Fructose–L-valine	Glucose	87	12	110	132	118
Tagatose–L-alanine	Galactose	68	11	225	143	131
Tagatose–L-glycine	Galactose	71	16	210	143	130

[a] The following sizes of aliquots were used: hot reduction of ferricyanide, 1.0 ml. of 4×10^{-5} *M*; anthrone 1.0 ml. of 4×10^{-3} *M*; room temperature reduction of ferricyanide, 0.1 ml. of 4×10^{-3} *M*. The tests on the incorporation of leucine were carried out at 38° for four hours.

was in peptide or other combination was not determined.[10,11] Evidently the 1-amino-1-deoxy-2-ketose configuration may occur in some mucoproteins. Hodge and Rist[9] have implicated this type of linkage of sugar with proteins in the browning of dried foods during storage.

It is interesting in view of the finding that fructose– and tagatose–amino acids stimulate amino acid incorporation into rabbit reticulocyte proteins that Rogers, *et al.*,[12] found that N-D-glucosylglycine stimulated the growth of *Lactobacillus gayoni*.

(10) A. Gottschalk and P. E. Lind, *Nature*, **164**, 232 (1949).

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(12) D. Rogers, T. E. King and V. H. Cheldelin, *Proc. Soc. Exptl. Biol. Med.*, **82**, 140 (1953).

PASADENA, CAL.

[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS AND THE EDWARD DANIELS FAULKNER ARTHRITIS CLINIC OF THE PRESBYTERIAN HOSPITAL]

Structural Studies on Chondroitin Sulfuric Acid. II. The Glucuronidic Linkage[1,2]

BY EUGENE A. DAVIDSON AND KARL MEYER

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The structure of chondrosine, the repeating unit of chondroitin sulfate of cartilage, tendon and umbilical cord and of chondroitin, a sulfate-free analog, has been established previously as β-D-glucopyranosyluronic acid 2-deoxy-2-amino-D-galactose. In this paper the conversion of the disaccharide into a glucopyranosyluronic acid pentitol *via* the crystalline methyl ester hydrochloride by oxidative deamination with ninhydrin is described. This was converted to a glucopyranosylpentitol, presumably a β-D-glucopyranosyl-D-lyxitol isolated as a crystalline octaacetate. Periodate studies on the amorphous free sugar demonstrated the consumption of four moles of periodate with the formation of two moles of formic acid and one mole of formaldehyde. This indicated a 1,2-link for the glucosidolyxitol, necessitating a 1,3-glucuronidic linkage in chondrosine and thereby in chondroitin sulfate and other polymers which contain chondrosine as the major repeating unit. The hexosaminidic linkage is discussed.

The reaction of amino sugars with ninhydrin has been made the basis of their qualitative and quantitative estimation and separation.[3] Conversion of glucosamine and chondrosamine to arabinose and lyxose, respectively, is accomplished rapidly and in good yield.

The repeating unit of umbilical cord hyaluronic acid, hyalobiuronic acid, has been established as β-D-glucopyranosyluronic acid-2-deoxy-2-amino-D-glucose.[4] This was based on conversion to β-D-glucopyranosyl)-D-arabinose *via* the corresponding hexosaminic acid which was oxidatively deaminated with ninhydrin.[5]

(1) This work was supported by grants from the National Institutes of Health and the New York Chapter of the Arthritis and Rheumatism Foundation.

(2) Taken in part from a thesis to be submitted by Eugene A. Davidson in partial fulfillment of the requirements for the Ph.D. degree, Faculty of Pure Science, Columbia University.

(3) S. Gardell, F. Heijkenskjold and A. Rochnorlund, *Acta Chem. Scand.*, **4**, 970 (1950).

(4) B. Weissmann and K. Meyer, THIS JOURNAL, **76**, 1753 (1954).

(5) It has been pointed out to us that a choice between the structure proposed by us[4b] and that proposed by Wolfrom, *et al.*,[8] may appear ambiguous. We wish to re-emphasize that the experimental data presented by the latter are compatible with both structures. However, the data presented in our previous paper are only compatible with the structure proposed by us. Furthermore, Prof. Wolfrom according to a personal communication has obtained additional evidence which supports the structure proposed by us.

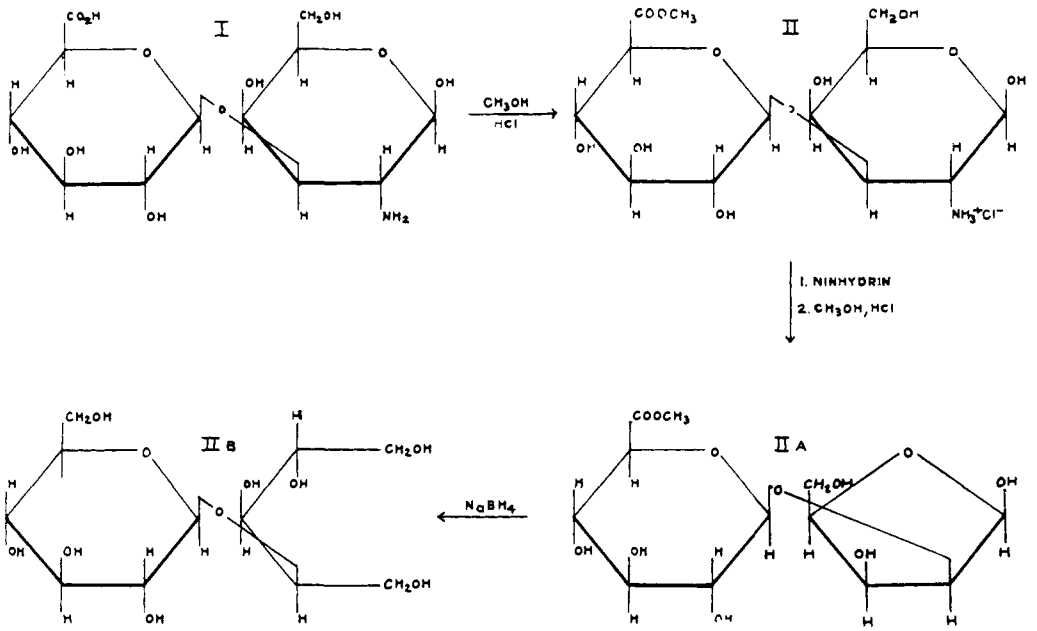


Fig. 1.—Ninhydrin degradation of chondrosine.

Chondrosine, the repeating unit of cartilage, tendon and umbilical cord chondroitin sulfate and of chondroitin, an isomer of hyaluronic acid,[6a] has been established as a glucuronidochondrosamine.[6b] Unequivocal evidence for the position of the glucuronidic linkage has not been reported previously.

The extreme lability of amino sugars to alkaline conditions, coupled with the unavailability of the required trimethyl chondrosamine reference compounds, made methylation studies impractical. In view of this, it was decided to investigate the conversion of the disaccharide to one of simpler structure following the general procedure utilized for the structural determination of hyalobiuronic acid.

This method was modified by direct ninhydrin degradation of the aldehyde. Avoidance of the prior conversion to the hexosaminic acid resulted in a greatly improved over-all yield.

The reaction scheme is shown in Fig. 1. Chondrosine, isolated as described in a previous report,[6b] was converted to the crystalline methyl ester hydrochloride, II. Treatment of this material with ninhydrin in pyridine yielded an amorphous glucuronidolyxose, not further purified. Re-esterification and reduction with sodium borohydride in cold methanol–borate buffer yielded a glucosidopentitol which, precluding any unlikely inversions, would be glucosidolyxitol IIb isolated as the crystalline octaacetate. Deacetylation of this material with dry sodium methoxide in chloroform yielded the amorphous free sugar which, in the absence of any nitrogenous functions, lent itself well to periodate studies.

Periodate oxidation of this material resulted in the consumption of four moles of periodate with the formation of one mole of formaldehyde and two moles of formic acid, as shown in Fig. 2. These data require a 1,2-linkage for the glucosidolyxitol and thereby a 1,3-glucuronidic linkage in chondrosine. If the uronidic linkage in chondrosine were 1,4-, then a 1,3-linked glucosidolyxitol would be obtained. This product, on periodate oxidation, would still consume four moles of periodate but would produce one mole of formic acid and two moles of formaldehyde.

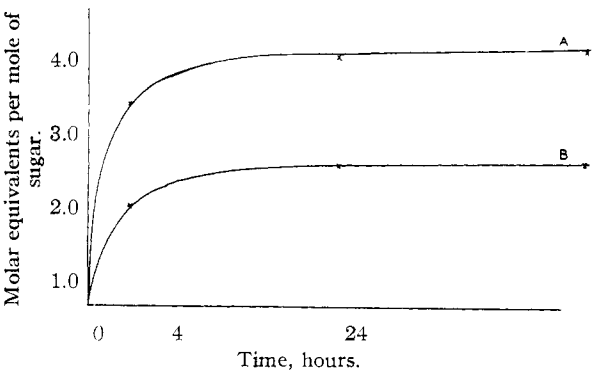


Fig. 2.—Periodate oxidation of 2-(β-D-glucopyranosyl)-D-lyxitol (?). Curve A indicates periodate consumption and curve B formic acid production.

Relatively little is known concerning the hexosaminidic linkage in chondroitin sulfate. Periodate studies have given conflicting results indicating both 1,3- and 1,4-linkages.[7,8] Structural studies carried out on the disaccharide end-product of the action of bacterial hyaluronidase on hyaluronic acid have identified it as a 4,5-unsaturated uronide.[9] The end-product of bacterial enzyme action on the isomeric chondroitin, a sulfate free or nearly sulfate free analog of chondroitin sulfate, appears to be a

(6) (a) E. A. Davidson and K. Meyer, *J. Biol. Chem.*, **211**, 605 (1954); (b) THIS JOURNAL, **76**, 5686 (1954).

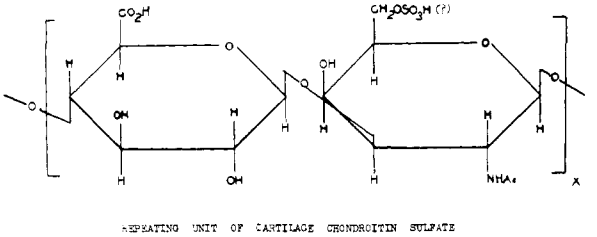
(7) K. H. Meyer, M. E. Odier and A. E. Siegrist, *Helv. Chim. Acta*, **31**, 1400 (1948).

(8) M. L. Wolfrom, R. K. Madison and M. J. Cron, THIS JOURNAL, **74**, 1491 (1952).

(9) A. Linker, B. Weissmann and K. Meyer, *Federation Proc.*, **13**, 840 (1954).

similarly unsaturated uronide. It has been shown that the failure of the bacterial enzyme to split chondroitin sulfate is due to the inhibitory effect of the sulfate group.[6a] Partially desulfated, partially degraded chondroitin sulfate is hydrolyzed to a considerable extent by the bacterial enzyme with the production of similarly modified end-products. These data are best explained by a 1,4-hexosaminidic linkage for chondroitin sulfate.

The present state of knowledge regarding the structure of chondroitin sulfuric acid may be summarized in the formula shown in Fig. 3.



REPEATING UNIT OF CARTILAGE CHONDROITIN SULFATE

Fig. 3.—Probable repeating unit of cartilage chondroitin sulfate.

Experimental[10]

Chondrosine was prepared from cartilage chondroitin sulfate as described.[6b] Conversion to the methyl ester hydrochloride was effected by treatment with cold 0.02 *N* methanolic hydrochloric acid for 48 hours at ice-box temperature.[11]

2-(Methyl 2-β-D-Glucopyranosyluronate)-D-lyxose (?) (IIa).—Five hundred milligrams of crystalline chondrosine methyl ester hydrochloride was heated with 400 mg. of ninhydrin in 5 ml. of 4% aqueous pyridine for one hour at 100°. A qualitative time study which was followed by colorimetry and paper chromatography indicated these to be the optimum conditions. The yield did not decrease if the heating was carried out for an additional hour. The mixture was cooled to room temperature and extracted twice each with equal volumes of chloroform and of butanol. The pale yellow, aqueous layer was lyophilized to yield 400 mg. of solid.

Analysis at this point showed 52% uronic acid (carbazole[12]), 29% reducing sugar (ferricyanide[13]), 40% pentose (orcinol[14]), nitrogen: less than 0.5% (micro-Kjeldahl). No ninhydrin reacting material was present.

After being dried for 48 hours *in vacuo* over phosphorus pentoxide, the product was treated with 5 ml. of cold, 0.02 *N* methanolic hydrochloric acid and the mixture allowed to stand, with intermittent shaking, for 48 hours at ice-box temperature. The solvent was removed *in vacuo* and the hydrochloric acid removed by evaporation with absolute ethanol. An aliquot taken for analysis at this point showed, by a modification of the hydroxamic acid reaction,[4] better than 90% esterification.

2-(β-D-Glucopyranosyl)-D-lyxitol (?) (IIb) *via* the Crystalline Octaacetate.—Four hundred and ten mg. of compound IIa was dissolved in 5 ml. of cold absolute methanol and added in portions, with occasional stirring, to a solution of 90 mg. of sodium borohydride in 5 ml. of cold 0.1 *M* borate buffer, *p*H 8.1. The mixture was allowed to stand for two hours at 0°, one additional hour at room temperature and, after acidification to *p*H 5 with glacial acetic acid, overnight in the ice-box. The solution was passed through a 25-ml. column of mixed bed deionizing resin[15] and the eluate and water wash were lyophilized; yield 260 mg. of solid.

Analysis at this point showed 49% glucose (anthrone[16]), no reducing sugar, no uronic acid and no pentose.

Two hundred forty mg. of this presumed glucosidolyxitol were heated for one hour at 100° with 5 ml. of acetic anhydride and 250 mg. of fused sodium acetate. Cracked ice was added and, after standing overnight in the ice-box, the mixture was neutralized to *p*H 7 with solid sodium carbonate and extracted with three 10-ml. portions of chloroform. The combined chloroform extracts were dried over sodium sulfate and the solvent removed *in vacuo*. The resulting sirup was taken up in hot absolute ethanol and filtered. On cooling, a sirup separated which failed to crystallize. The ethanol was removed *in vacuo* over phosphorus pentoxide and the sirup chromatographed on a 60-g. column of silicic acid–Celite 535 3-1[17,18] using benzene–*t*-butyl alcohol (250:1) as developer.[19] The material was transferred to the column in benzene, 400 ml. of solvent was passed through and the column allowed to run dry. The column was extruded and the zone reacting with alkaline permanganate extracted with chloroform in a Soxhlet extractor for 20 hours. Removal of the solvent *in vacuo* and finally at 78° and 0.1 mm. yielded a colorless sirup. This was dissolved in a minimal amount of hot absolute ethanol, treated with a small amount of decolorizing carbon and filtered. The colorless filtrate was concentrated under nitrogen to the point of persistent turbidity and placed in a desiccator over calcium chloride. Crystals appeared in about three days and were allowed to collect for a week. The fine, colorless needles were recrystallized to a constant melting point from absolute ethanol. The yield of thrice recrystallized material was 201 mg. amounting to 25% over-all from II. Additional material totaling 44 mg. was obtained from the mother liquor; m.p. 108° (cor.), $[\alpha]^{23}D$ −13° (*c* 1.0, chloroform).

Anal. Calcd. for $C_{27}H_{39}O_{18}$: C, 49.80; H, 5.99; CH_3CO, 52.4. Found: C, 50.16; H, 5.94; CH_3CO, 51.5.

A solution of 80 mg. of crystalline acetate in 1 ml. of dry chloroform was cooled to 0°, treated with 1 ml. of freshly prepared, dry, 4.0 *M* sodium methoxide and allowed to stand at 0° for one hour. The mixture was extracted with three 2-ml. portions of distilled water and the combined water extracts neutralized with glacial acetic acid. The solution was deionized by passage through a mixed bed resin and the water removed *in vacuo* and finally with several portions of absolute ethanol. The resulting, nearly colorless sirup was dried to constant weight over phosphorus pentoxide at 78° and 0.05 mm. Final weight of the sirup was 32 mg. Analysis showed 56% glucose equivalent (anthrone), theory is 57%; no reducing sugar.

Periodate oxidation was performed in the dark at 4° in 0.180 *M* sodium metaperiodate solution. Aliquots were withdrawn for measurement of formic acid production and periodate consumption at 4, 24 and 48 hours. Formaldehyde production was measured at 24 and 48 hours.

Periodate consumption was measured by back titration with standard iodine after addition of excess arsenite; formic acid was titrated with standard 0.01 *N* sodium hydroxide, using phenolphthalein indicator after removal of periodate with ethylene glycol; formaldehyde was precipitated as the dimedon complex in buffered solution.[20] After 48 hours, the yield of crystalline dimedon–formaldehyde complex melting at 193–195° was 11.5 mg. from 40% of the total reaction mixture. This corresponds to 0.95 molar equivalent.

NEW YORK, N. Y.

(10) We wish to thank Dr. Alfred Linker for the acetyl determination. Micro-analyses by Micro-Tech Analytical Laboratories, Skokie, Ill.

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(12) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

(13) O. Schales and S. S. Schales, *Arch. Biochem.*, **8**, 285 (1946).

(14) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

(15) Filter-ion Resin, LaMotte Chemical Products, Inc., Baltimore, Md.

(16) W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, **50**, 298 (1952).

(17) Mallinckrodt, 100 mesh Analytical Reagent.

(18) Johns-Manville Co., New York.

(19) J. F. Haskins and M. J. Hogsed, *J. Org. Chem.*, **15**, 1275 (1950).

(20) F. S. H. Head and G. Hughes, *J. Chem. Soc.*, 2046 (1952).