ANALYSIS OF A SULFATED SIALOFUCOGLUCOSAMINOGALACTO-MANNOSIDOGLYCAN FROM CORNEAL STROMA*

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Glycoproteins containing hexosaminoglycans have been demonstrated in various forms of connective tissue. The ability of this type of compounds to organize soluble collagen into typical collagen fibrils and their accumulation in connective tissues of scorbutic animals suggest a role of such glycoproteins in the synthesis and maturation of collagen. The corneal stroma contains collagen fibrils which are considered as transitional between the embryonal and mature ones. It appeared, therefore, of interest to demonstrate and analyze hexosaminoglycans in this tissue. Balazs and Dohlman (1957)[1] demonstrated the presence in corneal stroma of a water soluble glycan containing mannose, galactose, glucose, glucosamine and fucose. We report here evidence that the corneal stroma contains a glycoprotein not extractable by water or salt solution with a sulfated glycan containing as constituents mannose, galactose, glucosamine, fucose and derivatives of neuraminic acid as its carbohydrate moiety. This glycoprotein appears linked by weak bands to the collagen of the stroma.

Preparation of the Glycoprotein Fraction. 1. Removal of acid mucopolysaccharides. One hundred to two hundred fresh corneas were stripped of epithelium, homogenized with five times their weight of crushed ice in a Waring Blender and the mixture left overnight at 4° C. Next day, an equal volume of 20% $CaCl_2$ solution was added and the mixture adjusted with 1 M tris-citrate

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buffer to pH 8, again homogenized and left with gentle shaking for 24 hours at 4° C. The insolub residue was centrifuged off, thoroughly washed with 10% $CaCl_2$ solution, pH 8, and again extracte for 24 hours with the same solution. This procedure was repeated six times until no significant amounts of hexuronic acids or hexose were found in the last tenfold concentrated extract. In two experiments the extraction with $CaCl_2$ was preceded by two extractions with 0.16% NaCl and washing with H_2O after each NaCl extraction. Experiments I and II in Table.

TABLE I.

MOLAR RATIOS OF CONSTITUENTS OF THE CARBOHYDRATE MOIETY OF THE GLYCOPROTEIN FRACTION FROM THE CORNEAL STROMA (a) AND ITS PART EXTRACTED BY ALKALINE ETHANOL

Gal=Galactose Man=Mannose Ham=Hexosamine F=Fucose S=Neuraminic Acid as Sialic Acid

Exp. No.	$\frac{Gal}{Man}$	$\frac{Ham}{Man}$	$\frac{F}{Man}$	$\frac{S}{Man}$	Remarks
I. a.	0.50	1.08	0.06		Stroma Extracted with NaCl 0.16 M H_2O, $CaCl_2$, 10% pH 8 and for 90' with TCA at 90°.
b.	0.50	1.08	0.120	0.235	
II. a.	0.66	1.17	0.03		"
b.	0.50	1.01	0.113	0.225	"
III. a.	0.50	0.94	0.07		Stroma Extracted with $CaCl_2$ and for 150' with TCA at 90°.
IV. a.	0.69	1.25	0.0		"
b.	0.50	1.04	0.114	0.209	"

a.) Ratios in the ethanol insoluble hexosaminoglycan fraction obtained after incubation with alkaline ethanol.
b.) Ratios calculated for the total hexosaminoglycan containing mannose.

2. Removal of the collagen. The residue of the $CaCl_2$ extraction was washed with H_2O, brought to 90°, suspended in a fivefold amount of a .25 M TCA solution (v/w), preheated to 90° C, and extracted during three time intervals of 5, 25, and 60 minutes each respectively. The residue was washed after each extraction with .25 M TCA. The final residue was divided by weighing in two approximately equal parts, one of which was again treated with .25 M TCA at 90° C for 60 minutes. This treatment removed more than 99% of the collagen, and a certain amount of hexuronic acid, not extractable with 10% $CaCl_2$ because apparently it is loosely bound to collagen. For complete removal of collagen ethanol was added to the residue of the 90 minutes and 150 minutes TCA extraction to a final concentration of 80% and enough 50% KOH solution to a final concentration of KOH of 5%. The final volume of the mixture correspond the original wet weight of the corneas. The mixture was then incubated with gentle shaking at R. T. for 48 hours. The insoluble small residue was dissolved in a small volume of water and reprecipitated 3 times at 4° from a 5% KOH solution in 80% ethanol. This fraction consisted of about 50% carbohydrate and 50% protein. The first 80% ethanol extract was saturated with CO_2, the precipitate of $KHCO_3$ centrifuged off and the ethanol removed in vacuo from the supernatant.

Analytical Procedures. Sugar constituents of individual fractions were tentatively identified by subjecting their HCl hydrolysates, freed of HCl, to chromatography on Dowex 50 according to Gardell.[2] Hexosamines were identified by the elution pattern and after appropriate concentration of the eluates by paper chromatography with pyridine butanol water as solvent, neutral sugars (freed from HCl by evaporation in vacuo of the suitably concentrated first effluent) by paper chromatography with pyridine butanol water and pyridine ethyl acetate water. Quantitative determination of hexoses were carried out by the three cysteine H_2SO_4 reactions;[3] fucose by the cysteine H_2SO_4 reaction.[3] Ninety-two percent of the derivatives of neuraminic acid (N.A.) were extracted by TCA at 90° in the first 30 minutes. These extracts were neutralized and dialyzed against an equal volume of H_2O. N.A. was determined in the dialysate in terms of sialic acid by two methods: according to Svennerholm and by the thiobarbituric acid reaction according to Aminoff.[5] Hexuronic acids were determined by the carbazole reaction[3] and total hexosamines by the HCl indole reaction[3]

after two hours hydrolysis with 2 N HCl. Hexosamine values were corrected for 8% loss during hydrolysis. Ester sulfate was determined by the nephelometric method of Nalefski and Takane[6] after six hours hydrolysis in 5.5 N HCl and appropriate concentration of the hydrolysate in vacuo. Hydroxyproline was determined according to Newman and Logan.[7]

Results. The carbohydrate moiety of the glycoprotein fraction remaining after the incubation of the residue of the TCA extraction with alkaline ethanol (AEEGP) consists of galactose, mannose, traces of glucose (Fig. 1), glucosamine, and very small amounts of methylpentose which could be demonstrated only by the cysteine H_2SO_4 reaction. AEEGP contained also hexuronic acid and galactosamine in amounts corresponding to about 1/10 of that of hexose. It finally contains ester sulfate far in excess of the amount equivalent to hexuronic acid and galactosamine. In AEEGP the galactose to mannose ratio was 0.5 and the ratio of hexosamine (corrected for the amount linked to hexuronic acid) to mannose was 1.08 and 0.93 in experiments I and III, Table line a. In experiments II and IV, the galactose to mannose ratios were significantly higher. There appear in TCA extracts of the stroma, in addition to the residual hexuronic acids and the carbohydrate firmly linked to collagen, significant amounts of mannose and of fucose, Fig. II. As these sugars are characteristic for AEEGP, it can be assumed that the latter is partly extracted during the incubation with TCA. Small amounts of mannose and fucose were also found in the supernatant of the KOH ethanol extraction. As fucose and derivatives of neuraminic acid are extracted by TCA at a faster rate than the other sugar constituents of the AEEGP, it appeared necessary for the determination of the composition of the mannosidoglycan to determine the amounts of its sugar constituents in TCA extracts and in the KOH ethanol supernatant and add these amounts to those found in AEEGP. Galactose and hexosamine could not be accurately determined in TCA extracts because of the possible presence of keratosulfate. In experiment I the ratios of galactose and hexosamine to mannose remained unchanged after the second 60' extraction with TCA. This indicates an equal rate of extraction of these three sugars by TCA. Therefore, for the determination of the composition of the total mannosidoglycans of the stroma, it was assumed that the ratios galactose and hexosamine to mannose, because of the equal extraction rates of the three sugars by TCA, are

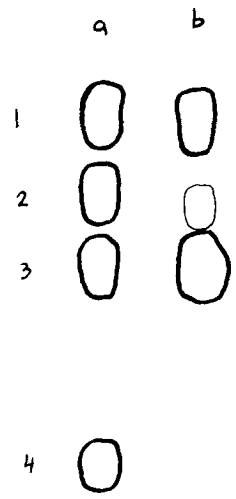

Fig. I. Paper chromatogram of neutral sugar constituents of the ethanol insoluble glycoprotein fraction of the corneal stroma obtained by alkaline ethanol extraction of the TCA extraction residue. a) standards, b) sample. 1.) galactose, 2.) glucose, 3.) mannose, 4.) fucose.

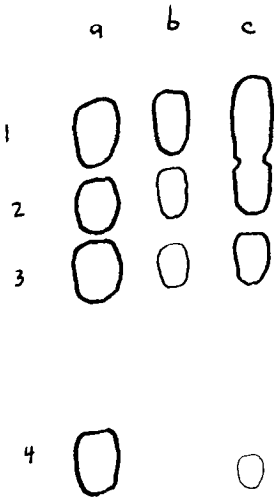

Fig. II. Paper chromatogram of neutral sugar constituents extracted by TCA at 90° from the glycoprotein fraction of the corneal stroma not extractable by H_2O and salt solutions. a) standards, b) sample, c) sample in double amount. 1.) galactose, 2.) glucose, 3.) mannose, 4.) fucose.

identical with those found by direct determination in AEEGP in I and III. The excess of galactose and hexosamine over these ratios in experiments II and IV was assumed to be due to contamination of AEEGP by keratosulfate. This appears confirmed by the fact that where the amount of hexosamine in these two experiments equivalent to the excess in galactose over its ratio to mannose of 0.5 is subtracted from the hexosamine found directly. The true ratio hexosamine to mannose in the mannosidoglycan does not significantly differ from that in AEEGP in experiments I and III, (Table line b). As far as the neuraminic acid is concerned, it was assumed that the total amount of this constituent extracted by TCA during the first 30' is derived from the glycoprotein as no significant amounts of gangliosides were found in the stroma and no neuraminic acid could be found in the carbohydrate of the collagen fibrils. In the table the molar ratios of sugar constituents of the total mannosidoglycan are listed on line b. A characteristic feature of the glycan of AEEGP which differentiates it from any other compound of this type from tissues or body fluids so far analyzed is its content in ester sulfate. The molar ratio of SO_4 in excess of that equivalent to hexuronic acid to mannose was 0.65 in experiment I and 0.9 in experiment III. The total

amount of the sialofucoglucosaminogalactomannosidoglycans in the cornea amounts to 600 µg per g wet weight, that is about 1/10 of the amount of acid mucopolysaccharides.

REFERENCES

1. Dohlman, C. H., and Balazs, D. A., Acta Ophth. 35: 454, 1957.

2. Gardell, S., Acta Chem. Scand. 7: 207, 1953.

3. Dische, Z., in "Methods of Biochemical Analysis" (Glick, ed.) Vol. II, p. 313. Interscience Publ., New York, 1955.

4. Svennerholm, L., Biochim. et Biophys. Acta 24: 604, 1957.

5. Aminoff, D., Biochem. J. 81: 384, 1961.

6. Nalefski, L. A., and Takano, F., J. Lab. and Clin. Med. 36: 468, 1950.

7. Neuman, R. E., and Logan, M. A., J. Biol. Chem. 184: 299, 1950.