PRESENCE OF A NON-SULPHATED GLUCOSAMINOGLYCAN IN EMBRYONIC CORNEA

R.PRAUS and I.BRETTSCHNEIDER

Laboratory of Physiology and Pathology of the Eye, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

Received 5 January 1970

An analtical evidence has been obtained for the presence of a non-sulphated glucosaminoglycan in the young embryonic White Leghorn chicken cornea, consisting of nearly equimolar amounts of glucosamine and glucuronic acid. The polysaccharide is supposed to be identical with hyaluronic acid.

1. Introduction

Adult vertebrate cornea contains two sulphated glycosaminoglycans: keratan sulphate, which predominates, and chondroitin sulphate. Mayer et al. [1] and Davidson and Meyer [2] reported the presence of a very low sulphated galactosaminoglycan in bovine corneal stroma, which they called chondroitin because of identical composition of the carbohydrate moiety with chondroitin sulphate, and because of almost complete absence of ester sulphate groups. Recently, we have obtained evidence on the presence of a non-sulphated glycosaminoglycan, other than chondroitin, in the developing chicken cornea. This paper deals with its chemical characterization.

2. Materials and methods

Fertilized White Leghorn eggs were incubated in an aerated thermostat at 38°C. On the ninth day of embryonic development, 100 μCi of radioactive sulphate (carrier-free $Na_2{}^{35}SO_4$, product of ROTOP, Dresden, DDR) was inoculated under sterile conditions into the allantoic cavity of each egg. The openings in the shells were sealed with wax and after another 24 hr, the incubation was stopped. Corneas were excised and dehydrated in acetone at 4°C over night. The drying was completed *in vacuo* over P_2O_5 at 60°C, and the dry weight of the pooled material was determined. Dry corneas were digested with papain (3.4.4.10, PAP 5607, Worthington Biochemical Corp., New Jersey, U.S.A.) and the digest was chromatographed on a cellulose column, in microscale, using cetylpyridinium chloride (CPC) and a stepwise increasing gradient of KCl in 0.05% CPC to separate glucosaminoglycans and galactosaminoglycans. The procedure is described in details elsewhere [3]. All glycosaminoglycan fractions were assayed for hexosamine by a modified Elson-Morgan reaction [3], and for radioactivity. Sulphate was precipitated as a barium salt from reaction mixtures remaining after the determination of hexosamine. In order to increase the sensitivity of the method, a constant amount of sodium sulphate was added to each sample before precipitation of barium sulphate. The precipitate was collected by filtration and its radioactivity measured on a decadic counter (Vakutronic, Dresden, DDR) using GM-tube VA-Z-310.

The peak fractions were analysed for glucuronic acid according to Bitter and Muir [4] and for sulphate according to Antonopoulos [5]. Paper chromatography of hexosamines of the peak fractions was performed using a mixture of pyridine, water, ethyl acetate and acetic acid (5:3:5:1 v/v) as a separatirg solvent in a tank saturated with a mixture of pyridine, ethyl acetate and water (11:40:6 v/v) according to Fischer and Nebel [6]. A ninhydrin reagent spray was used for the detection of hexosamine spots on the chromatogram. Pentoses were derived from hexosa-

Table 1
Analysis of main glycosaminoglycan fractions obtained by chromatography of corneal digest on CPC-cellulose.

	Fraction no.		
	I	II	III
Hexosamine (μg)	1.8	11.0	3.7
Sulphate (μg)	1.2	0.0	2.5
Glucuronic acid (μg)	0.0	11.6	4.0
Radioactivity (CPM)	1475	126	2902
Specific radioactivity (CPM/μg hexosamine)	820	12	785
Molar ratio $\frac{\text{sulphate}}{\text{hexosamine}}$	1.18	–	1.27

mines as described by Stoffyn and Jeanloz [7] and identified by paper chromatography with the use of butan-1-ol: ethanol: water (4:1:1 v/v) as solvent. The detection of sugars was made by means of aniline phthalate reagent spray. Keratan sulphate and chondroitin sulphate, both isolated from the adult chicken cornea by the above technique, and potassium hyaluronate were hydrolysed with 8 N HCl at 100°C for three hours, the hydrolyzates were evaporated to dryness and submitted to paper chromatography as described for hexoamines.

Table 2.
Content of hexosamine in main glycosaminoglycan fractions of three corneal preparations.

Fraction	Preparation		
	1	2	3
I	0.8	0.9	0.9
II	5.1	4.0	4.5
III	1.7	1.1	1.5

Hexosamine content is given in μg/mg dry weight.

3. Results

The results of fractionation of corneal glycosaminoglycans of 10 days old chicken embryos are presented in fig. 1. It is evident that the main portion of hexosamine is distributed in three peak fractions: a small amount is present in fractions I and II, and more than 50% of the total hexosamine is present in fraction II. Radioactivity was found, on the other hand, in the fractions I and II, whereas a very low radioactivity only was recorded in the fraction II. More analytical data of all three fractions are summarized in table 1. It follows from the table that, in agreement with the distribution of radioactive sulphate, fractions I and III contain besides hexosamine an esterified sulphate. The sulphate-to-hexosamine molar ratio of the fraction

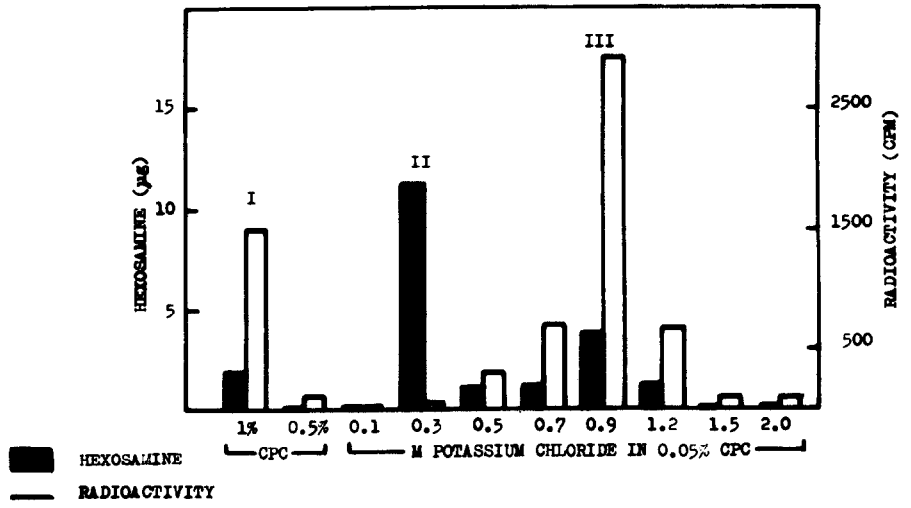

Fig. 1. Fractionation of corneal glycosaminoglycans of 10 days old chicken embryo. Total amounts of hexosamine and radioactivity per fraction are presented.

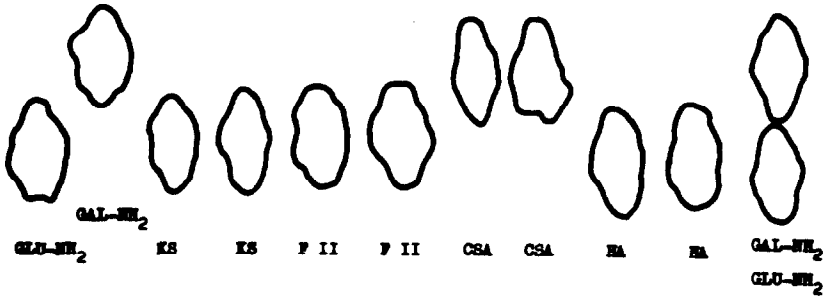

Fig. 2. Schematic drawing of the detail of paper chromatograph of hexosamines liberated from glycosaminoglycans by hydrochloric acid hydrolysis. Abbreviations used: GLU-NH_2 = glucosamine, GAL-NH_2 = galactosamine, KS = keratan sulphate, CSA = chondroitin sulphate, HA = hyaluronic acid, F II = fraction II.

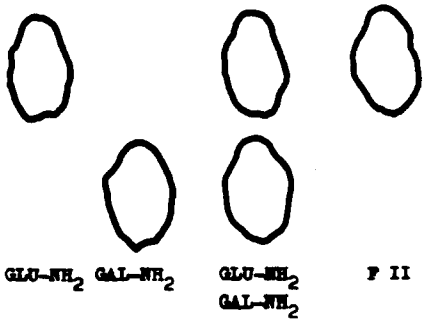

Fig. 3. Schematic drawing of the detail of paper chromatograph of pentoses derived from hexosamines by ninhydrin reaction. Abbreviations used: see fig. 2.

I is 1.18 and that of the fraction III 1.27. Fraction II does not contain any detectable amount of sulphate whereas the hexosamine content is high. Glucuronic acid is present both in fractions II and III in an amount nearly equimolar to the content of hexosamine. The values of specific radioactivity indicate that glycosaminoglycans of the fraction I and II are sulphated at similar rates. The specific radioactivity of fraction II is negligible.

Results of paper chromatography of hexosamines liberated from the keratan sulphate, chondroitin sulphate, hyaluronic acid and fraction II and of pentose derived from the hexosamine of fraction II are shown in figs. 2 and 3. It is evident that both hexosamine and pentose originating from the glycosaminoglycan present in the fraction II reveal similar mobility on the chromatograms as glucosamine and/or pentose derived from glucosamine by the ninhydrin reaction. Hexosamine liberated from keratan sulphate and hyaluronic acid was proved to be glucosamine, and that liberated from chondroitin sulphate was identified as galactosamine.

From three groups of 10 days old chicken embryos, fractionation of corneal glycosaminoglycans was performed, and the content of hexosamine of the peak fractions was compared (table 2). It appears from the table that the concentration of the main glycosaminoglycan fractions in the corneal dry weight of all three tissue preparations is very similar.

4. Discussion

It was found that most of hexosamine of the young chicken embryonic cornea is contained in the fraction which is eluted with 0.3 M KCl in 0.05% CPC when glycosaminoglycans are chromatographed on CPC-cellulose. Evidence was obtained that the glycosaminoglycan of this fraction contains glucosamine and uronic acid in practically equimolar amounts, but no ester sulphate. When corneas were labelled with radioactive sulphate, radioactivity was present in fractions I and III, whereas fraction II contained only a trace of sulphate radioactivity. On the basis of analytical data, fraction I corresponds to keratan sulphate, and fraction III to chondroitin sulphate. As far the glycosaminoglycan of the fraction II is concerned, it might be characterized as a non-sulphated glucos-

aminoglycan, most likely hyaluronic acid. It is known that some of the embryonic connective tissues contain a relatively high concentration of hyaluronic acid. Thus Loewi and Meyer [8] found that hyaluronic acid represented about 80% of the total glycosaminoglycans in the human embryonic skin, but only 3% in the adult human skin. A question arises whether the non-sulphated glucosaminoglycan is regular constituent of the embryonic cornea, or whether it infiltrates into the cornea from the surrounding tissues and fluids. Its relatively constant concentration in the dry weight of three corneal preparations supports the former presumption. The vitreous, which is the most probable source of contamination, contains an extremely low hyaluronic acid concentration in early stage of embryonic development [9].

References

[1] K.Meyer, A.Linker, E.A.Davidson and B.Weissman, J. Biol. Chem. 205 (1953) 611.
[2] E.A.Davidson and K.Meyer, J. Biol. Chem. 211 (1954) 605.
[3] R.Praus and J.N.Goldman, Invest. Ophthalmology (1969) in press.
[4] M.Bitter and H.M.Muir, Anal. Biochem. 4 (1962) 330.
[5] C.A.Antonopoulos, Acta Chem. Scand. 16 (1962) 1521.
[6] F.C.Fischer and H.G.Nebel, Z. Physiol. Chem. 302 (1955) 10.
[7] P.J.Stoffyn and R.W.Jeanloz, Arch. Biochem. Biophys. 52 (1954) 373.
[8] G.Loewi and K.Meyer, Biochim. Biophys. Acta 27 (1958) 453.
[9] E.A.Balazs and R.W.Jeanloz, in: The aminosugars, Vol. 2A (Academic Press, New York, London, 1965) pp. 401–460.