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Changes in Molecular Weight of Acid Mucopolysaccharides in Connective Tissue Due to Hormone Treatment, Dehydration and Age

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Acid mucopolysaccharides have been isolated from animal skin and characterized by means of viscosimetry, chromatography and metachromasia. It is shown that the molecular weight of these substances decreases with age. A depolymerization of these substances apparently also takes place during severe dehydration and under the influence of cortisone, while oestradiol treatment results in a polymerization. It is concluded that the size of the hyaluronic acid molecule is an important factor in the waterbinding mechanism of connective tissue.

It is generally accepted that in the connective tissue there is a close relationship between the amount and physical state of the acid mucopolysaccharides on one side and the water content on the other side. Oestrogenic treatment has for some time been known to increase the amount of mucopolysaccharides as well as the content of water in the skin (Taylor and Sprunt [1], and Schmidt [2]), and severe dehydration causes a minor decrease in the hexosamine content in the skin of mice and rats, (Hvidberg [3,4]). Further it has been observed (Boas and Foley [5], Hvidberg [4]) that both the hexosamine and the water content of the connective tissue decrease during growth in rats. From these and other investigations the amount of the acid mucopolysaccharides seems to be important for the waterbinding mechanism of connective tissue. However, we also found it worth while to study the possible rôle played by the degree of polymerization of the mucopolysaccharides. We therefore have determined the molecular weights of these substances isolated from animals of different ages and after different treatments, in order to provide some information on the waterbinding mechanism of connective tissue.

MATERIALS AND METHODS

As experimental animals were used white male mice, 7—8 weeks old, weighing about 25 g, and white female rats of different ages, namely: 2 days, 10 days, 26 days, 6 weeks, about 3 months, and about 6 months old. In the experiments with mice the groups contained about 40 animals, and in the experiments with rats the groups contained from about 60 to about 25 animals depending on the age. The animals were kept under constant environmental conditions with free access to commercial food-stuff and also to water, except the group of dehydrated mice.

Groups of mice treated in different ways: 1. Control group. 2. Group deprived of water for 7 days. 3. Group treated with oestradiol monobenzoate (Ovex ® Leo), 10 μg in 0.1 ml of vegetable oil, injected subcutaneously in the occipital region on the 1st and the 3rd day; killed on the 7th day. 4. Group treated with cortisone acetate (Organon ®) 1 mg in 0.1 ml (diluted with physiological saline) given intraperitoneally daily from the 1st to the 15 th day, and killed just after.

Only skin from the distal part of the back was used. After depilation the skin and underlaying connective tissue were removed, freeze-dried, and defatted carefully according to the method described by Hvidberg [3]. Then all the tissue pieces from each group were pooled.

Isolation processes. In order to isolate mucopolysaccharides from the dry and defatted tissue the following methods were employed. It appeared necessary to use different techniques depending upon the origin of skin. *Mice:* The minced skin was stored under acetone for a fortnight in order to denature proteins thus rendering these substances insoluble (Schütte and Greiling [6], Jensen [7]). Then the acetone was removed by soaking in distilled water for 2 h. The broth was then treated with acetic acid for 2 days in order to remove iron, and then the broth was neutralised with potassium hydroxide to pH 8—9. The broth was filtered througth 8 layers of gauze, and dialysed against running tap water for three days. To the dialysate were added 3 volumes of absolute alcohol. The precipitate was partly stringy, and partly flocculent. After rinsing with alcohol and ether a white powder was obtained. *Rats*: The above method did not appear to be successful regarding isolation of mucopolysaccharides from rat skin, as the tissue would not disintegrate. Therefore we employed enzyme digestion using a crude extract from pancreas (Pancreatin ® Novo). In agreement with Gardell [8] we found that crystalline trypsin and chymotrypsin were less effective than the pancreatic enzymes. The pH was adjusted to 7—8 and the digestion lasted for three days at room temperature. The broth was then filtered through gauze. The protein split products were precipitated with 10 % trichloroacetic acid after the method of Lundquist [9]. After neutralisation and dialysis the mucopolysaccharides were obtained and rinsed as described above. During the isolation processes care was taken that the temperature did not exceed room temperature and the pH was less than 9. This was done as both high temperature and alkaline reaction involve the danger of degrading effects of mucopolysaccharides (Snellman [10]).

The isolated substances were tested for metachromasia by preparing dry smears. These were fixed in basic lead acetate, and stained with a 0.05 % solution of toluidine blue (Toluidinblau nach Hoyer Riedel-de Haën A. G., Hannover, Germany) at pH 6.5, beforé and after incubation with hyaluronidases. Testicular hyaluronidase was prepared by Leo Pharmaceutical Products, Copenhangen and bacterial hyaluronidase was prepared by dr. V. Faber, the Danish State Serum Institute.

Viscosimetric investigations were performed in the viscosimeter described by Dalgård-Mikkelsen and Kvorning [11].

Chromatographic investigations were undertaken after the isolated substances had been hydrolysed with 2.0 N HCl for 15 h at 100°C in sealed ampullae. Samples containing 10 mg/ml were placed on Whatman filter paper No. 1 in a volume of 20 μl, and run in 2,6-lutidine:water (65:35) for 24 h using the descending type of chromatography. Standard solutions (10 mg/ml) of D-glucosamine hydrochloride (Nutritional Biochemical Corporation) and D-galactosamine hydrochloride (Mann Research Laboratories) were placed on the paper in a volumn of 5 μl each. For the development was used a 0.1 % ninhydrin solution in acetone. For publication the chromatogrammes, were copied in drawings as shown in Fig. 4.

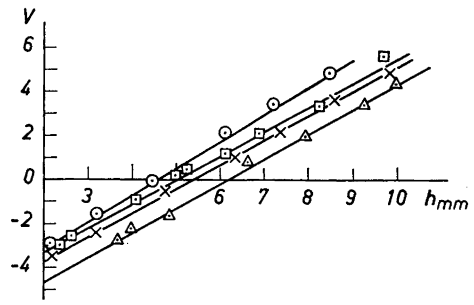

Fig. 1. Osmotic pressure of isolated mucopolysaccharides from skin of mice. The graph shows the relation between height of the air column (abscissae) and the rate of movement of the upper miniscus of the air column (ordinates) in micrometer divisions in 10 min. The values for the equilibrium length are given in Table 1.

⊙ — ⊙ Control (untreated animals)
△ — △ Dehydrated animals
× — × Animals treated with cortisone
⊡ — ⊡ Animals treated with oestradiol

The molecular weight was calculated from osmotic pressure measurements, which were performed by the micro-osmometer described by Christiansen and Jensen [12]. The semipermeable membranes were prepared from a pyroxylin solution (Parlodion "Mallinckrodt") containing 4 g in 100 ml of an alcohol-ether mixture (75:25); to this mixture were added 5 ml of ethylene glycol freshly distilled *in vacuo*. The membranes were dried for about one hour.

RESULTS

From the following reasons we conclude that the substances isolated from the skin of the experimental animals really are mucopolysaccharides, more or less contaminated with impurities. The powders were 1) white or nearly white, 2) highly hygroscopic, and 3) easily soluble in water. 4) The aqueous solutions were viscid, and 5) the viscosity decreased considerably upon incubation with both hyaluronidases. 6) Further more, the substances were strongly metachromatic with toluidineblue, this metachromasia being highly sensitive to testicular hyaluronidase and partly sensitive to the streptococcal enzyme. 7) Chromatography of the hydrolysates showed spots corresponding to authentic glucosamine and galactosamine. 8) Although being not a proof the calculat-

Table 1. The molecular weight of mucopolysaccharides isolated from skin of mice, calculated from the measurement of the osmotic pressure (w = weight of the material in mg per ml, and h = equilibrium length in cm) M means the molecular weight. 1 and 2 refer to two seperate experiments with the same material.

Treatment	w 1	w 2	h 1	h 2	$M \times 10^{-3}$ 1	$M \times 10^{-3}$ 2	$M \times 10^{-3}$ mean	± % of control group	Hexosamine and Water contents * Hex. mean	Hex. sem	Water mean	Water sem
Control	7.02	5.80	0.54	0.46	321	320	321	—	521	± 9	316	± 8
Dehydrated	4.48	4.92	0.55	0.62	201	196	198	−40	481	± 8	211	± 5
Oestradiol	9.01	9.40	0.48	0.50	463	464	464	+44	1 065	±24	540	±15
Cortisone	5.04	5.50	0.51	0.54	251	244	247	−20	528	± 9	261	± 7

* The figures of these columnes are taken from completely equal series published by Hvidberg and Schmidt [16], and Hvidberg [3]. The content of hexosamine is in mg, and the content of water is in g, both per 100 g dry, defatted tissue. sem = Standard error of the mean.

Table 2. The molecular weight (M) of mucopolysaccharides isolated from skin of rats of different ages, calculated from the measurement of the osmotic pressure (w = weight of material in mg per ml, and h = equilibrium length in cm).

Age	w	h	$M \times 10^{-3}$	Hexosamine and Water contents *			
				Hex.		Water	
				mean	sem	mean	sem
2 days	7.58	0.61	266	676	±14	571	±7
10 days	6.01	0.62	239	540	±6	400	±2
26 days	5.59	0.62	219	538	±7	318	±2
6 weeks	5.88	0.74	196	488	±7	272	±4
3 months	5.68	0.84	167	410	±6	228	±2
6 months	6.68	1.15	143	342	±6	218	±2

* The figures of these columnes are taken from an other investigation using equal groups of rats (Hvidberg [4]). Hexosamine and water contents are given in mg and g, respectively, per 100 g dry, defatted tissue. sem = Standard error of the mean.

ed molecular weights do not exclude the anticipation that the substances are mucopolysaccharides.

As seen from Table 1, which shows the molecular weights of substances isolated from mice skin, the molecular weight is considerably lower, when the mice had been dehydrated for seven days. Also cortisone treatment seems to cause a marked fall in the molecular weight, while the oestrogenic treatment causes a considerable increase. According to our experience on the relationship between substrate concentration and osmotic pressure (Jensen [7], Jensen and Marcker [13]), the differences between the molecular weights are likely still greater than the figures apparently indicate. An example of the osmotic measurements are given in Fig. 1.

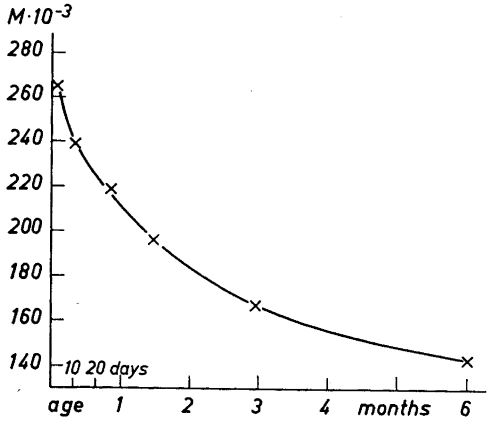

Fig. 2. The relation between age (abscissae) and molecular weight (ordinates) of mucopolysaccharides isolated from skin of rats of different ages.

Table 3. Metachromasia of the isolated mucopolysaccharides from skin of mice and rats.
Intensities: + + + + : very strong reaction
+ + + : strong reaction
+ + : medium reaction
+ : weak reaction
O : no or practically no reaction
The quoted intensities of the metachromatic reactions are based only on the subjective impressions of the authors and involves no exact measurements.

Animals	Group	Intensity of metachromasia		
		Genuine metachromasia	After treatment with bacterial hyaluronidase	After further treatment with testicular hyaluronidase
Mice	Control	+ + +	+	O
—	Dehydrated	+ +	+	O
—	Oestradiol	+ + + +	+	O
—	Cortisone	+ (+ + ?)	+	O
Rats	2 days old	+ + +	+	O
—	10 days old	+ + +	+	O
—	26 days old	+ + +	+	O
—	6 weeks old	+ + +	+ +	O
—	3 months old	+ + +	+ +	O
—	6 months old	+ + +	+ +	O

Table 2 gives the results of the osmotic measurements and the calculated molecular weights of the substances isolated from skin of rats of different ages. It is obvious from Table 2 and from Fig. 2, that the molecular weight decreases with age. The decrease is greatest during the first weeks of life.

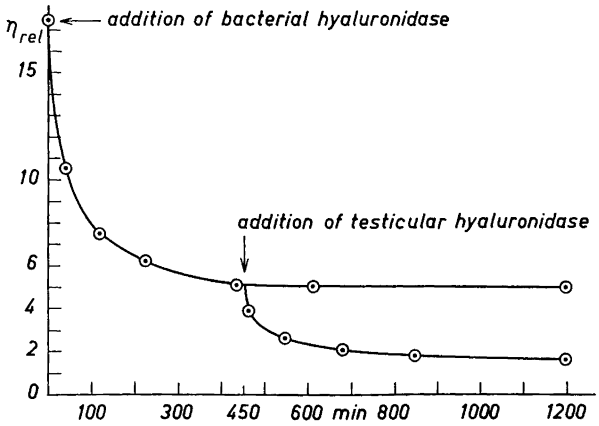

Fig. 3. Degradation of isolated mucopolysaccharides from skin of control mice, after enzymatic incubation, shown by the fall in viscosity. Abscissae: Times after incubation. Ordinates: Relative viscosities.

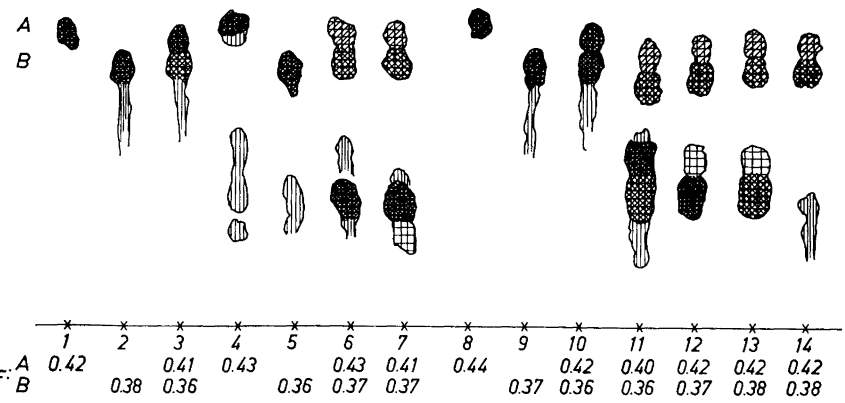

Fig. 4. Drawing of paper chromatogrammes of hydrolyzed mucopolysaccharides isolated isolated from skin of rats and mice, showing the spots of glucosamine (A) and galactosamine (B). Further the R_F-values are given. Other spots are impurities, probably hydrolysates of protein.

1. Authentic glucosamine
2. Authentic galactosamine
3. Authentic glucosamine and galactosamine
4. Hydrolysate of hyaluronic acid (from human umbilical cords)
5. Hydrolysate of chondroitin sulphuric acids (from ox nasal septa)
6. 2 days old rats
7. 10 days old rats
8. As No. 1
9. As No. 2
10. As No. 3
11. 26 days old rats
12. 3 months old rats
13. 6 months old rats
14. Control mice (untreated animals)

From Table 2 it is further seen that hexosamine as well as water content also decrease with age (compare Hvidberg [4]).

Table 3 shows the metachromasia of the isolated substances. We have to point out that the quoted intensities are based only on subjective impressions and involve no exact measurements. The most remarkable observations are that the intensities of the metachromatic reactions in the dehydrated and the cortisone treated animals are weaker than in the normal and especially than in the oestradiol treated mice. As seen the intensities do not appear to change with age. Further it is seen that practically all metachromasia is abolished by treatment with testicular hyaluronidase. When the substances were incubated with streptococcal hyaluronidase an obvious decrease in intensity is seen, but this decrease is not so marked in the older groups of rats.

Fig. 3 gives an example of the viscosimetric investigation showing the degradation of the isolated substance following incubations partly with testicular, partly with streptococcal hyaluronidases. The curves indicate that the preparation contains both hyaluronic acid and chondroitin sulphuric acids.

The chromatographic investigations are shown in Fig. 4 which gives a drawing of the paper chromatogrammes. From this study it might be concluded that the isolated powders contain substances which upon hydrolysis yielded both glucosamine and galactosamine. Perhaps the investigation indicates that the ratio between hyaluronic acid and chondroitin sulphuric acids decreases slightly with increasing age. Unfortunately, we had not so great amounts of the substances which allowed us to perform chromatographic investigations on dehydrated and hormone treated mice and on the 6 weeks old rats. As references were used, besides authentic glucosamine and galatosamine, hydrolysates of hyaluronic acid prepared from human umbilical cords according to Jensen [14] and chondroitin sulphuric acid isolated from ox nasal septa according to Blix and Snellman [15]. As seen most preparations are contaminated with impurities, probably protein split products.

DISCUSSION

As we think it most valuable to know the physico-chemical behaviour of the acid mucopolysaccharides in the state in which they occur in the living connective tissue, we have made use of very mild isolation procedures. However, we are well aware, indeed, that this demand is not obtainable, the substances being always altered during the isolation procedures. But we think that the experiments in this study with the isolated material, help us to give a fairly good picture of the real conditions. The quantitative extraction of the acid mucopolysaccharides is a matter of considerable difficulty, and considering the mild methods here used, we are of the opinion, that only a relative small part of the total amount of the mucopolysaccharides has been isolated at all. This might be considered as a source of error too.

As shown in the experiments with mice the molecular weight of the mucopolysaccharides decreases after intense cortisone treatment. At the same time the relative amount of these substances is not altered as judged from the hexosamine determinations (Hvidberg and Schmidt [16]), but the content of water in the connective tissue is significantly lower. Also in dehydrated mice the molecular weight was found to be considerably lower than in normal animals. According to Hvidberg [3] the relative hexosamine content was somewhat smaller in the dehydrated mice, and naturally the water content of the connective tissue was markedly reduced. On the other hand the molecular weight of the mucopolysaccharides from the oestradiol treated animals was much higher than in normal animals; besides, this treatment nearly doubled the relative amount of mucopolysaccharides, and caused a considerable increase in the water content, (*cf.* Schmidt [2], Hvidberg [3]).

These observations are also interesting when compared to the findings in the metachromatic studies, because the metachromasia of the mucopolysaccharide is closely connected to the degree of polymerization. But it is important to stress once again that we are well aware that these observations are based only on subjective impressions. We have found that the metachromatic intensity is weaker in the cortisone treated animals. Histochemical studies performed by several investigators, *e.g.* Asboe-Hansen [17], have demonstrated that

cortisone treatment decreases the metachromasia of the connective tissue ground substance. This seems to agree with our findings. Dehydrated animals also showed a weaker metachromasia (Table 3). The experiments on these two groups (cortisone treated and dehydrated animals) suggest that a depolymerization of the mucopolysaccharides is followed by a decrease in the metachromasia, and this is, as mentioned above, in agreement with the opinion of most investigators.

In the experiments with rats it is shown that the molecular weight of the mucopolysaccharides is decreasing with age. During the same period a decrease in the water and in the hexosamine content is observed (Table 2, Hvidberg [4]). As may be inferred from the above experiments with hormone treated and dehydrated mice, it is not directly obvious that only the hydrophilic hyaluronic acid is depolymerized with increasing age. The findings with toluidine blue and with chromatography seem to indicate a minor increase in the ratio between chondroitin sulphuric acids and hyaluronic acid. This point of view is supported by the general observation that hyaluronic acid is preferably found in foetal connective tissues (Slack [18]). Furthermore, in experiments on pig skin, Meyer *et al.*[19] found that the ratio of chondroitin sulphate to hyaluronic acid was 1.25 in grown up animals, while this ratio was only 0.2 in the skin of pig embryos.

The fact that connective tissue, containing hyaluronic acid, really binds great amounts of water, is easily explainable when considering the physicochemical properties of hyaluronic acid. This molecule is threadlike (highly asymmetric, Jensen and Carlsen [20]), very large, and carries a great many negative charges (one per disaccharide unit). As it contains many OH-groups there are great possibilities for establishing hydrogen bondings, resulting in a threedimensional network. Thus it is obvious that this mucopolysaccharide is capable of forming a highly hydrophilic gel. It must be anticipated that this gel can bind great amounts of water even when present in low concentrations, as the more asymmetrical the colloidal particles are, the lower is the minimum concentration of the colloid at which gelation occurs (Jirgensons and Straumanis [21]) Although not a proof, it is likely that the water of the connective tissue (also skin) is found in a gel-like structure as no water escapes from the tissue, when it is punctured (Schmidt [2], Gibian [22]). According to our experience hyaluronic acid must be associated with protein in order to form a typical gel (see below).

Thus the ground substance of the connective tissue in which the structural elements are imbedded is a hydrophilic gel. Most probably this gel consists of a protein-hyaluronate complex (Bensley [23], Meyer [24], and Day [25]). The waterbinding mechanism of the connective tissue is most likely closely related to the gel-structure of the ground substance, but this mechanism depends on several, partly unknown, factors. The gel-like structure of the ground substance can be broken and the viscosity destroyed if the hyaluronic acid is depolymerized, (Jensen and Vilstrup [26] and a review by Schmidt [2]). It is a question if this rapid procedure influences the waterbinding mechanism, but from our investigations and the above considerations it might be suggested, that the waterbinding in the gel of the ground substance partly depends on the size of the hyaluronic acid molecules. This theory, already touched by

other investigators (see Altschuler and Angevine [27]), is supported by our findings, that there is a connection between the decrease in the molecular weight and the water content (cortisone treated animals), and between the increase in molecular weight and the water content (oestradiol treated animals). Furthermore it was observed, that when the water had been drawn from the connective tissue (the dehydration experiments), the molecular weight was found to be smaller, this also indicating that the waterbinding mechanism is altered (secondarily?). Finally the relation between the decrease in the molecular weight and the decrease in the water content of the connective tissue during growth also supports the hypothesis here forwarded. It must, however, be added, that the drop in the molecular weight of the isolated mucopolysaccharides during growth might partly be caused by alterations in the ratio between the two types of acid mucopolysaccharides in the connective tissue. As is known chondroitin sulphuric acids have smaller molecular weights than hyaluronic acid and do not have nearly as strong hygroscopic properties as this substance (Jensen [7]).

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