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The Collagen and Ground Substance of Human Intervertebral Disc at Different Ages

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The collagen and ground substance of human intervertebral disc has been studied by means of hydroxyproline, hexosamine and nitrogen analyses, as well as extraction experiments. It was found that an increase in a non-soluble fraction containing collagen and components of the ground substance takes place with rising age.

As demonstrated in Schmorl's laboratory by Püschel [1], and verified by several investigators [2–6], the dry substance of the intervertebral disc increases with rising age, and there is a corresponding decrease in its water content. This applies especially to its central part, the *nucleus pulposus (N.P.)*, but also to the peripheral *annulus fibrosus (A.F.)*. A parallel change takes place in the properties of the tissue, which is colourless and has good turgor in the young individual, whereas it is brownishly discolored and almost disintegrating in the highest age groups, as already noted by Luschka [7]. Since postnatal intervertebral disc (from about 1 year of age) is a kind of fibrocartilage containing large quantities of collagen and ester-sulphated mucopolysaccharides [5,8,9], it is highly probable that these changes depend on a change in the composition of the intercellular substances. Sylvén *et al.*[5,8] suggested that an increase in the collagen content might be the responsible factor, and similar explanations have appeared in the case of other connective tissues [10–12]. It nevertheless seems unlikely that this is the whole truth, since there are reasons to believe that changes in the composition and extractability of the mucopolysaccharides [13], as well as the deposition of yellow-brown lipofuscin pigment [6,14], must also be taken into account.

The reasons underlying the changes in the properties of the *N.P.* and *A.F.* with age cannot be known until all the intercellular components have been appropriately characterized, and determined quantitatively. The present investigation was intended as a step in this direction, including a chemical study of the collagen. The components of the "ground substance", *i.e.* the rest of the intercellular substances, are as yet only partly characterized, and at this stage the discussion must be confined to results of hexosamine and nitrogen analyses, and extraction experiments.

EXPERIMENTAL

Preparation of the tissue. Lumbar and lower thoracic parts of the vertebral column were taken from human autopsy cases, and the intervertebral discs were cut out, after freeing them from muscle and loose connective tissue. The *N.P.*, consisting of the most central part, about 2—3 cm^2, was dissected out. The surrounding *A.F.* was also dissected out, after removal of a small peripheral margin of the disc. The intermediate zone between the *N.P.* and *A.F.* was discarded. For the tissue analyses, these specimens were freeze-dried and then minced.

Collagen extraction experiment. A 0.5 g *N.P.* specimen was taken from a 26 year old subject and extracted, without previous drying, for 24 h at room temperature with 10 ml of 0.15 M ammonium carbonate. In this step, the tissue was finely divided with a glass rod. The pH was 9.0 at the beginning of extraction, and 8.6 at the end (glass electrode). The residue was removed by centrifugation at 2 000 *g* for 1 h, washed once with distilled water, and centrifuged down again. Extraction was repeated with 20 ml of 0.2 M citrate buffer, pH 3.8, as described above. At the end of this extraction, the pH was 3.9. The residue was removed and washed as before. The extracts, together with the respective washing water and the residues, were dried in a desiccator, taken up in strong hydrochloric acid and transferred quantitatively to the hydrolysis tubes. After desiccation, hydrolysis and the hydroxyproline analyses were performed essentially as described below.

Preparation of N.P. collagen. About 5 g of pooled *N.P.* from subjects 20—60 years old, that had been stored for several weeks at −15°C, was thawed, minced by hand, and extracted overnight at 0°C with 500 ml of 10 % calcium chloride. The residue obtained on centrifugation was extracted twice more in the same way with 250 ml of 10 % calcium chloride. The residue was then washed with water, freeze-dried, washed with acetone, and ground in a mortar. The yield was 1.3 g of calcium chloride-treated collagen.

83 mg of this collagen preparation was extracted for 2 days at room temperature with 20 ml of 2 % sodium hydroxide. The residue obtained on centrifugation was washed with distilled water, dried by several washings with acetone, and minced in a mortar. 50 mg of sodium hydroxide-treated collagen were obtained.

Hydroxyproline analysis. After hydrolysis of the tissue, the hydroxyproline was oxidized to 4-hydroxypyrrole, and the colour developed with Ehrlich's reagent [15] was read in a photometer according to Neuman and Logan [16], including some important modifications [17,18]. About 8 mg of the dried *N.P.* was hydrolyzed in sealed tubes with 2 ml of 6 M hydrochloric acid for 24 h at 100°C, or for 22 h at 110°C, both conditions giving the same result. The acid was completely removed *in vacuo* in a desiccator over potassium hydroxide. The hydrolysate was dissolved to 25 ml in distilled water, and 2 ml pipetted off into a 2 × 20 cm Pyrex tube. Standards were prepared containing 0, 10, 20 and 30 μg of hydroxyproline in 2 ml of distilled water. To unknowns and standards, about 20 tubes in each experiment, were added 2 ml of equal amounts of 0.01 M copper (II) sulphate and 2.5 M sodium hydroxide mixed immediately before use, and 1 ml of 6 % hydrogen peroxide. The contents were gently mixed, and left standing for 5 min. In order to remove excess peroxide, each tube was vigorously stirred in a mechanical shaker for 5 min, after which the contents were mixed and left standing for 5 min with 0.1 ml of a 0.01 M iron (II) sulphate solution, containing 0.5 % (v/v) sulphuric acid. 6 ml of a solution containing 1 part of 4 M sulphuric acid and 2 parts of 2 % *p*-dimethylaminobenzaldehyde in propanol, mixed immediately before use, was added. After mixing, heating at 70°C for 20 min and cooling with tap water, the red colour was read at 555 mμ in 1 cm absorption cells.

The weight-equivalent colour development of tyrosine was 2 % of that of hydroxyproline, but the absorption spectrum was completely different. The colour of the *N.P.* hydrolysate was found to have the same absorption spectrum as the standard, which implies negligible interference by non-specific chromogens. The hexosamines gave no colour in the reaction, and the colour development of proline was neglected, since it amounted to only some 0.1 % of the hydroxyproline.

Shrinkage-temperature determination. The powdered preparation, immersed in water, was viewed under a cover-glass in the microscope at 75 × magnification during gentle heating, and the temperature for maximal shrinkage recorded as described by Borasky and Nutting [19].

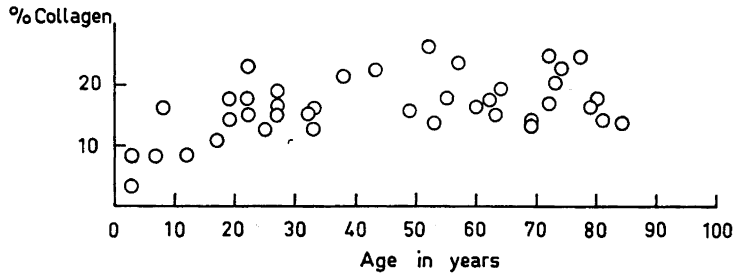


Fig. 1. Collagen content of human freeze-dried *N.P.* at different ages.

Analysis of the ground substance. Hexosamines were determined according to Gardell [20], and nitrogen by a modified Kjeldahl procedure. For further details, reference is made to an earlier paper [13].

RESULTS

Collagen analyses. Fig. 1 is a graphical representation of the results of the hydroxyproline analyses of *N.P.* The hydroxyproline content was multiplied by the factor 7.41, based on the assumption that *N.P.* collagen contains about 13.5 % hydroxyproline, like other mammalian collagens [16,21]. It can be inferred from Fig. 1 that the collagen content of *N.P.* was about 15—20 % of the dry weight, the values increasing slightly with rising age. Since the dry substance increases with age (see Fig. 2), it implies a fairly appreciable increase in collagen per unit volume of native tissue. This is in agreement with the observations of Sylvén *et al.*[5,8]

A.F. contained more collagen, *i.e.* about 50 % of the dry substance. This could be expected from the morphological properties of the tissue [1,9,22].

The extraction experiment showed that the collagen was mainly non-soluble. Thus, only 0.4 mg of collagen was found in the ammonium carbonate extract, and 0.4 mg in the citrate extract, whereas 13.6 mg, *i.e.* 95 %, was present in the residue. These results are in accordance with earlier brief reports [13,23], *i.e.* that only a small percentage of the collagen in *N.P.* can be extracted with physiological saline, slightly acid 10 % calcium chloride or slightly alkaline 30 % potassium chloride.

Attempts to isolate the *N.P.* collagen met with some difficulty, probably because parts of the ground substance were extremely difficult to remove [8,13]. The calcium chloride-treated collagen, obtained in some 95 % yield, contained only 7.4 % hydroxyproline (corresponding to 55 % collagen), and was contaminated by as much as 3 % hexosamine. The sodium hydroxide-treated collagen obtained in some 80 % yield, was practically devoid of hexosamines, and the hydroxyproline content had increased to 10.4 % (corresponding to 77 % collagen).

It proved impossible to see any shrinkage in freeze-dried *N.P.* during heating. In *A.F.*, on the other hand, it was easily visible, and the shrinkage temperature was found to be 60—62°C. In the *N.P.* collagen preparations treated

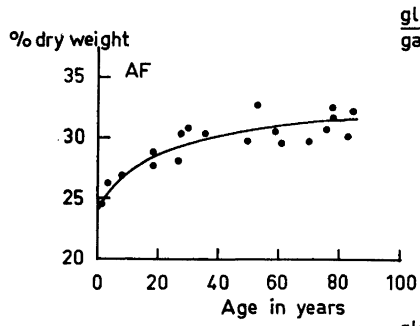


Fig. 2. Dry substance content of human *A.F.* and *N.P.* at different ages. Note the increase in *A.F.* during childhood, and the increase in *N.P.* throughout life. Based on Püschel's data [1].

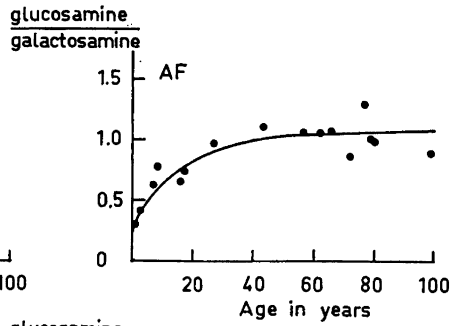
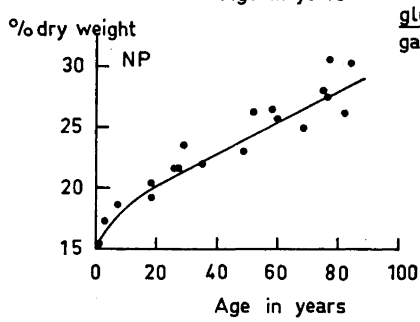
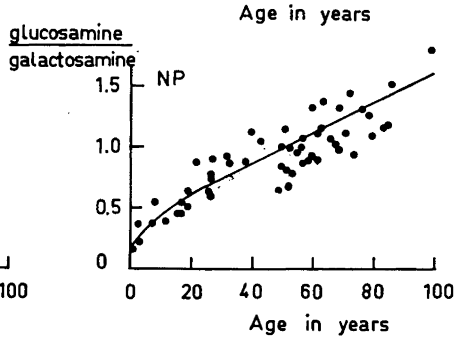


Fig. 3. Ratio of glucosamine/galactosamine of human *A.F.* and *N.P.* at different ages. Note the increase in *A.F.* during childhood, and the increase in *N.P.* throughout life.





with calcium chloride and sodium hydroxide, the shrinkage could be seen with some difficulty. It occurred at the same temperature as in the case of *A.F.*

These experiments substantiate the view that the collagen of the intervertebral disc is identical with collagen of other mammalian connective tissues[21]. This is also in conformity with the results of Sylvén *et al.*[8], who isolated fibrils from *N.P.* with 600—700 Å periodicity, typical of collagen. The difficulties encountered in determining the shrinkage of *N.P.* may possibly depend on the irregular course of the fibrils here [8], in contrast to their very regular pattern in *A.F.*[22]

Analyses of the ground substance. As reported earlier[13], the hexosamine content of *N.P.* is about 14 % in the 10 to 15 year old, and 6 % in the 90 year old. Such a large decrease in hexosamine content must also mean a decrease per unit of native tissue. The *N.P.* of foetuses and newborns has been found to contain very little hexosamine (a few per cent). This is natural, since at this stage the *N.P.* is an epithelial tissue composed of dorsal cord cells [9].

The hexosamine content of *A.F.* is about 3 %, with a tendency to decreasing values with rising age.

The variation in the ratio of glucosamine/galactosamine in *N.P.* and *A.F.* with age is shown graphically in Fig. 3. It is seen that the ratio increases with

rising age in both. As discussed previously [13], this probably represents a change in the mucopolysaccharide-protein pattern from the chondroitin-6-sulphate of the young tissue to the less easily extractable keratosulphate of the old tissue.

The nitrogen content of *N.P.*, after subtraction of the nitrogen ascribable to collagen (18 % of the collagen content [21]) and hexosamine (8 % of the hexosamine content), was found to increase from a few per cent in the child to almost 10 % in the 90 year old. This probably represents an increase in the non-collagenous protein.

DISCUSSION

It seems that an increase in the collagen content and changes in the composition of the ground substance both contribute to the increase in dry substance of *N.P.* and *A.F.* with age. The contribution of the ground substance is indicated by an increase in the non-collagenous protein, and by the fact that there is a parallel between the increase in dry substance and the increase in the ratio of glucosamine/galactosamine in *A.F.* and *N.P.* (*cf.* Figs. 2 and 3). This parallel can be extended to pathological discs, because very brown *N.P.* with a high content of dry substance [1] (*i.e.* discs that have once ruptured [5,6,14,22]) has also proved to have an exceedingly high glucosamine/galactosamine ratio.

Fairly detailed information has recently been given about the way in which the polypeptide structural unit of the collagen is polymerized to fibrils, and about the increase in the number of cross-linkages and in the stability of the collagen with rising age [24,25]. The corresponding process in the ground substance has not yet been as systematically investigated. Certain observations [13,26–28] nevertheless indicate that, here as well, deposition of a non-soluble, highly cross-linked fraction may take place during aging. The following experiment was made to test this hypothesis.

100.0 mg samples of freeze-dried, minced *N.P.* from subjects aged 7, 22, 60 and 81 years were extracted with 10 ml of 10 % calcium chloride for 24 h at room temperature. The residues obtained after centrifugation were washed with 10 % calcium chloride and distilled water, treated with alcohol and ether, and dried. The weight was found to be 11.8, 19.8, 40.7 and 84.4 mg, respectively.

These results support the hypothesis in question, since such a large increase in the non-soluble fraction (to some 80 % of the dry substance) cannot be explained by the fairly inappreciable increase in collagen content with age (to some 20 % of the dry substance).

It seems reasonable to infer that the changes observed in the chemical composition of the intervertebral disc with rising age (increase in dry substance, collagen content, ratio of keratosulphate/chondroitin sulphate and lipofuscin pigment) are partial manifestations of a generalized, successive deposition of non-soluble, intercellular material.

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