THE MAMMALIAN INTERVERTEBRAL DISK:  
FIBROUS STRUCTURES OF THE WHALE  
NUCLEUS PULPOSUS

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SUMMARY
Striated fibres showing a beaded appearance in the electron microscope were prepared from the insoluble collagen residue of foetal nucleus pulposus. The assembling and stability of the beaded fibres are controlled to a significant degree by electrostatic forces. In acid solutions (pH 2.0) the organization of the structures is preserved. Analytical data on the preparations show the presence of proteoglycans containing chondroitin sulphate and keratan sulphate, as well as collagen.

INTRODUCTION
Study of the ultrastructure of human and rabbit nucleus pulposus has revealed a sparse cell population with a large number of necrotic cells (Smith & Serafini-Fracassini, 1968; Cornah, Meachim & Parry, 1970). The extensive cellular necrosis seen in this tissue is considered a normal rather than a pathological phenomenon (Meachim & Cornah, 1970). Banded structures with a periodicity which differs from that of a native collagen fibre have been shown to be present in the extracellular matrix of the nucleus pulposus (Smith & Serafini-Fracassini, 1968; Cornah et al. 1970). The prevalence of these structures around viable and degenerating cells suggests that their formation could have been initiated by an abnormal biological situation, with alteration in the constituents of the tissue. Experimental difficulties in their isolation have been pointed out. Briefly, aqueous extraction procedures seem to dissociate the components of the structure (Smith & Serafini-Fracassini, 1968).

The extracellular structure of the nucleus pulposus can be regarded as a network of non-oriented collagen fibrils and banded collagen fibres embedded in a proteoglycan gel matrix. Most of the proteoglycan can be separated from the nucleus pulposus by aqueous extractions (Ludowieg et al. 1972). The use of proteases or extensive and elaborate extractions with several solvents are needed in order to remove the remaining proteoglycan from collagen. In the course of the fractionation process, fractions containing collagen and proteoglycan, which in the electron microscope showed a beaded appearance, were isolated from whale foetal nucleus pulposus by using pronase. Because of their heterogeneity it was difficult to recognize and be certain of the fibrous forms observed. Samples were therefore treated with buffer solutions of different pH and subjected to enzymic digestions in an attempt to obtain a better view of the components and to identify them.
METHODS AND MATERIALS

Nucleus pulposus of foetuses (6–8 months) of finback whales was used. Colorimetric methods for the analysis of carbohydrate and collagen have been described before (Ludowieg et al. 1972). Glycosaminoglycans were identified by electrophoresis in cellulose acetate strips after step-wise enzymic-alkali hydrolysis of the sample (Anderson, Hoffman & Meyer, 1965). With the same preparative method, chondroitin sulphate and keratan sulphate isolated from the nucleus pulposus were used as standards.

Preparation of CP-2C (nodal fibres)

Freeze-dried foetal nucleus pulposus (1 g) (see the flow-sheet outlining its preparation, Fig. 1) was gently extracted by means of magnetic stirring with water (60 ml) for 2 periods of 14 h each at 5 °C. The thick opalescent mixture was centrifuged at 37000 g for 1 h and the residue (RA) washed twice with water. Fraction RA was suspended in 0·1 M calcium acetate, pH 7·2–7·6 (12 ml) and gently stirred with 2 mg of filtered pronase (grade B, Cal Biochem) for
Fibrous structures of nucleus pulposus

20 h at 5 °C. The reaction mixture was centrifuged at 34 000 g for 0-5 h, the residue (RB) was separated, and the supernatant dialysed (5 °C) against 0-05 % acetic acid (1 day) and 0-05 % acetic acid (2 days). Upon dialysis, the clear supernatant rapidly became turbid, and after several hours a precipitate formed which was harvested by centrifugation at 34 000 g for 0-5 h. This material (CP-2C) was washed twice with cold 0-05 % acetic acid (yield 20 mg).

Digestion with pepsin (2 times crystallized, Worthington) was performed in 0-05 M glycine-hydrochloric acid, pH 2.0. Digestion with bacterial collagenase (special grade CLSPA 8 BA, Worthington) was performed in 0-067 M phosphate buffer in 0-45 % sodium chloride, pH 7.4. Digestion with ribonuclease (once crystallized, Worthington) and deoxyribonuclease (once crystallized, Worthington) was performed in 0-1 M acetate buffer, pH 5.0. Digestion with bovine testicular hyaluronidase (Type VI, Sigma) was performed in 0-1 M acetate buffer containing 0-15 M sodium chloride in 0-005 M sodium pyrophosphate, pH 5.0. Enzyme solutions were made 0-1% in their cold buffer solvents. Before enzymic incubations 1 ml of a freshly prepared complex suspended in cold 0-05 % acetic acid (ca. 0-2%) was centrifuged.

The residue after centrifugation was briefly washed twice with a small amount of cold water and incubated with 0-5 ml of enzyme solution. Digestions were limited to 1-3 h at 37 °C. In controls the enzyme solutions were kept at 100 °C for 2 min before they were added to the substrate. Samples for electron-microscopic examination were taken from these mixtures.

Electron-microscopic examination

Aliquots of suspended or dispersed aqueous preparations were placed directly on carbon-Formvar-coated grids for 1 min, drained, dried, and stained with freshly prepared 2 % aqueous uranyl acetate or mixed with equal portions of fresh 1-2 % aqueous phosphotungstic acid at pH 2-2, 5-0 or 7-2 (pH adjusted with potassium hydroxide) and then dried on grids. All samples were examined and photographed in a Zeiss EM 9A electron microscope at magnifications ranging from 1900 to 40 000 times.

RESULTS

The nodal fibres (CP-2C) showed a regular repeating pattern of an electron-dense band alternating with an electron-lucent filamentous gap (Figs. 2-6). The repeating axial period between nodes had an average measurement of about 0-1 µm. Scattered through the preparation were also collagen segment-long-spacing (SLS) forms which appeared centrally banded and attached to sections of a nodal fibre (Figs. 2-4). In certain instances they appeared to cover a nodal fibre to form part of a banding pattern with a repeating axial period of about 0-1 µm (Figs. 3, 4). Nodal fibrils were also observed which appeared to be derived from a nodal fibre (Figs. 2, 3). The structure of a nodal fibril and of a nodal fibre at high magnification is seen in Figs. 5 and 6. Collagen segment-long-spacing forms in proximity to a nodal fibre are shown at high magnification in Fig. 7. The spherical nodes of a nodal fibril are about 50-60 nm in diameter; dense particles of about 5-10 nm in diameter are seen as part of their structure.

Our measurements can only be considered to be rough estimates of the quantity of structural elements because of uneven dispersion and the tendency of the preparations to clump. Furthermore, the evident role of electrostatic forces in the preparations necessitates caution in the interpretation of results with different methods of staining. Thus, the compactness and organization of a nodal fibre was not clear with phosphotungstic acid at pH 7.0 (negative staining). The same method enhanced, however, the structural details of collagen components. At acid pH (0-05 % acetic acid) the nodal
fibres were clearly seen; at higher pH both nodal fibres and nodal fibrils were fewer and disorganized. Segmented collagen forms, amorphous elements, and fibrous long-spacing (FLS) collagen fibres were seen in increasingly larger numbers. Preparations were soluble at neutral pH, and at pH 7-6 no nodal fibres were seen. The preparation, after being dissolved in an alkaline buffer (pH 7-6), failed to reorganize to produce the original nodal fibre upon dialysis against acetic acid. The composition of the fractions isolated is shown in Table 1. These preparations contained chondroitin sulphate and keratan sulphate.

Table 1. Composition analysis, %

<table>
<thead>
<tr>
<th></th>
<th>Hydroxyproline</th>
<th>Galactose</th>
<th>Uronic acid lactone</th>
<th>Hexosamine</th>
<th>Ratio of galactosamine to total hexosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>8.6</td>
<td>4.7</td>
<td>2.8</td>
<td>4.1</td>
<td>0.71</td>
</tr>
<tr>
<td>RB</td>
<td>9.0</td>
<td>3.2</td>
<td>1.8</td>
<td>2.6</td>
<td>0.77</td>
</tr>
<tr>
<td>CP-2C</td>
<td>3.8</td>
<td>6.5</td>
<td>5.2</td>
<td>7.5</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Testicular hyaluronidase appeared to produce no profound changes in the structure of at least those nodal fibres which were seen under the conditions of incubation (pH 5.0), while pepsin disorganized the structure. Collagenous structures were digested by bacterial collagenase at pH 7.4. Incubations with ribonuclease and deoxyribonuclease produced no apparent changes.

Discussion

The insoluble collagenous residue (RA) of the nucleus pulposus is solubilized to a certain extent by pronase. After dialysis of the soluble extract against dilute acid the nodal fibres were observed. The method was particularly satisfactory with foetal material. The disorganization of nodal fibres by changes in pH shows that these structures are stabilized primarily by electrostatic forces. Similar forces have been shown to play a major role in the interaction of collagen and proteoglycans (Mathews, 1965; Mathews & Decker, 1968; Podrazky et al. 1971).

The precipitation of collagen and long-spacing fibrils by proteoglycans has been shown to be dependent on the ionic strength, temperature and pH of solutions used to facilitate their interaction (Schmitt, Gross & Hightberger, 1953; Gross, 1956; Wood, 1960; Gross, Hightberger & Schmitt, 1952; Keech, 1961; Fitton Jackson & Randall, 1953). The non-specific character of non-collagenous components in the formation of long-spacing fibrils has been emphasized in earlier experiments by Gross et al. 1952. Some of the fibrillar components of the nucleus show morphological correspondence with fibrillar structures of cartilage. Fitton Jackson (1964) isolated from chicken cartilage fractions which showed in the electron microscope a number of filaments with particulate subunits. It was suggested that these were proteoglycan in nature. Since proteoglycans reveal a beaded filamentous structure in the electron
Fibrous structures of nucleus pulposus (Rosenberg, Hellmann & Kleinschmidt, 1970), it is possible that a beading process takes place in the course of preparing the nodal fibres from interactions between proteoglycans with themselves or with a glycoprotein (or protein).

It is interesting that Schmitt et al. (1955) showed the involvement of beaded fibrils in the formation of FLS fibres. In some of their preparations certain elements described as 'spindles' were observed. It was suggested that the spindles represent tactoidal aggregates of tropocollagen particles anchored at their ends by glycoprotein, from which, upon further aggregation, FLS fibres could be formed. A variety of FLS fibres, some of which are clearly associated with non-collagenous components, have been described (Schmitt et al. 1955; Kühn & Zimmer, 1961). We suggest that although the nodal fibres appear to be one of the variants of such structures (FLS fibres), they may, in fact, be proteoglycan complexes intimately associated with collagen structures.

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REFERENCES


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Figs. 2–6. Nodal fibres and fibrils (CP-2C) at pH 2.6, banded patterns; nl, nodal fibril; nr, nodal fibre; s, segment-long-spacing form of collagen. Band period of about 0.1 μm. Fig. 2, ×8360; Fig. 3, ×18960; Fig. 4, ×23400. All stained with uranyl acetate. Fig. 5, a nodal fibril stained with phosphotungstic acid, ×93600. Fig. 6 is a nodal fibre stained with uranyl acetate, ×127908.
Fig. 7. Dimeric segment collagen forms attached to sections of a nodal fibre. Dimers are about 490 nm in length, with a dense centrally located band of about 90–100 nm in axial extent. Uranyl acetate, × 93600.