Reports

Hyaluronic Acid: A Novel, Double Helical Molecule

Abstract. *Films prepared from a deformable gel (or putty) of hyaluronic acid show high crystallinity and orientation in their x-ray diffraction patterns. We have derived a probable structure for the molecules in these films. This is a double helix in which two identical, left-handed strands are antiparallel to one another. Each strand has four disaccharide residues per pitch length. Although the putty is prepared at* pH *2.5, at which dilute solutions of hyaluronic have exaggerated rheological properties, the double helical form can also exist at physiological* pH *and therefore may be a biologically important form.*

Hyaluronic acid is a glycosaminoglycan that is present in some bacterial capsules and in the intercellular matrix of the connective tissue of most vertebrates. The molecule is a linear polydisaccharide of the form -G-N-, in which G is glucuronic acid and N is *N*-acetylglucosamine. The linkages G-N and N-G are $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 4$, respectively. A pure form, with a protein content less than 0.5 percent and with a molecular weight of 1 to 3 million, can be conveniently prepared from various tissues (umbilical cord, rooster comb, vitreous humour, skin, and so forth) or from synovial fluid. The long polymeric chain has been characterized as a "random coil with some stiffness" when studied in low concentration (less than 0.1 percent) in dilute salt solution (0.1 to 0.2*N* NaCl, *p*H 6 to 7) (*1*). In natural fluids and in solutions of purified sodium hyaluronate of concentrations greater than 0.1 percent, the biopolymer has pronounced viscoelastic characteristics (*2*). When dilute (less than 1 percent) aqueous solutions of sodium hyaluronate of high molecular weight (1 to 2 million) are acidified to *p*H 2.5 in the presence of monovalent salts (less than 0.1*N*), a "viscoelastic putty" forms (*3*). When the concentration of sodium hyaluronate is 1 percent or more, the putty forms a solid that is quite different rheologically from gels of agar or gelatin. The viscoelastic characteristics of the putty are highly dependent on strain frequency—the putty flows slowly under stress, and cut surfaces heal when pressed together (*3*).

Seeking an explanation of the peculiar rheological properties of sodium hyaluronate of high molecular weight in neutral solution or in the "viscoelastic putty," we investigated oriented films prepared from putty made from hyaluronic acid of human umbilical cord. Films suitable for x-ray diffraction analysis were prepared by a technique similar to that used by Atkins *et al.* (*4*). The putty, warmed to 60°C, was squashed to a film by clamping between two glass plates and then dried at 22°C. The film was removed from the glass plates and further dried at this temperature. The dried film was placed at 80 percent relative humidity in a chamber and then stretched under constant load (10 g).

From this stretched film we obtained diffraction photographs that

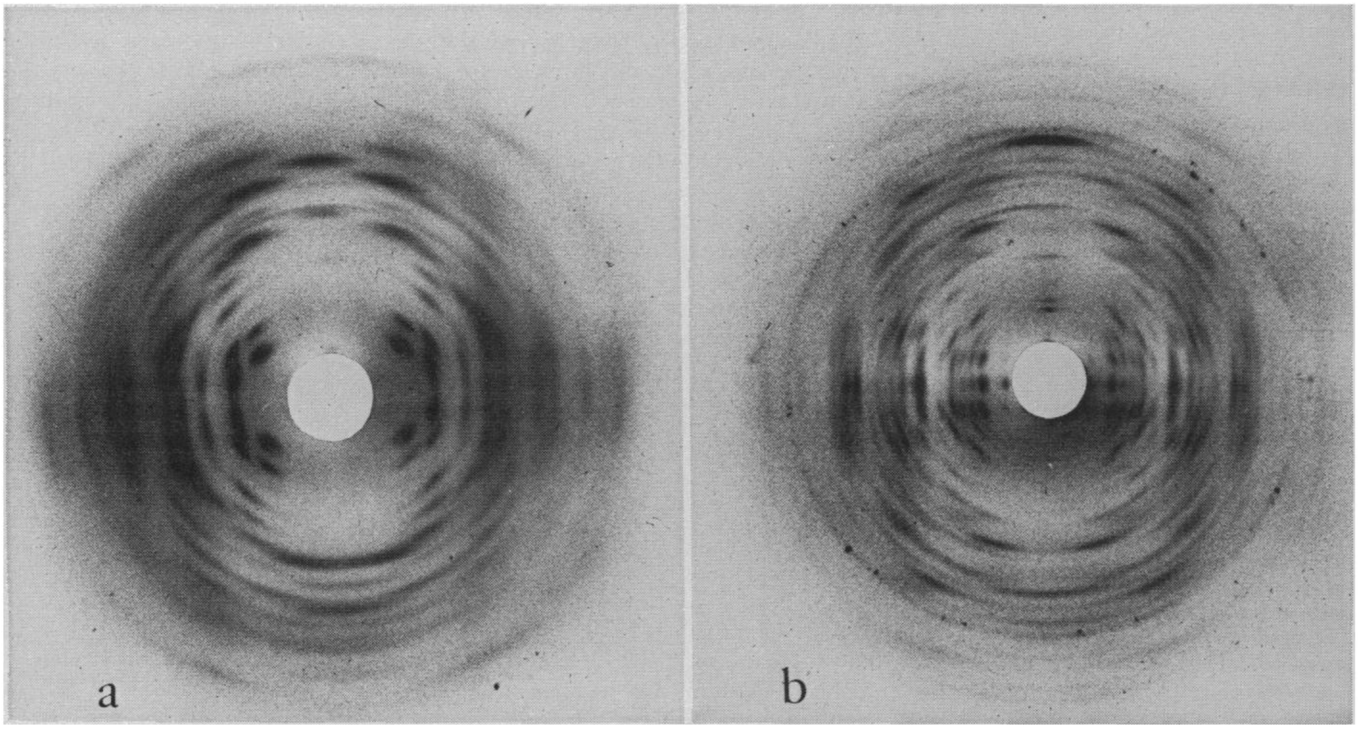

Fig. 1. X-ray diffraction photographs from films of hyaluronate. (a) The new, double helical form shows the meridional reflections only on layer lines with *l* even. This pattern may be indexed on the basis of an orthorhombic unit cell 11.4 by 9.8 by 33.7 Å. (b) This pattern is from a mixed phase containing the orthorhombic form and a hexagonal form previously reported (*4*).

show high crystallinity and high orientation (Fig. 1a). We indexed these on the basis of an orthorhombic lattice with dimensions (and estimated standard deviations) $a = 11.4 \pm 0.1$ Å, $b = 9.8 \pm 0.1$ Å, and c (fiber axis) $= 33.7 \pm 0.1$ Å. When the specimen is suitably tilted, the diffraction photographs also show meridional reflections on all even layer lines up to 14. This is quite different from any of the structures reported previously (*4*) for either sodium hyaluronate (two hexagonal forms, both with $c = 28.5$ Å) or for hyaluronic acid (with $c = 19.6$ Å), in which the chains have threefold and twofold screw symmetry, respectively. However, the new structure can also be obtained from alkali metal salts of hyaluronic acid of relatively low molecular weight (*5*).

Preliminary models for later x-ray refinement were set up by means of a computer search for hyaluronate backbone conformations that match the axial periodicity of 33.7 Å. For this purpose, we assigned fixed values (*6*) to the bond lengths and bond angles of the covalent structure and varied the conformation angles step by step through the ranges that were stereochemically reasonable (*7, 8*). The search indicated that only left-handed, fourfold helices of the type shown in Fig. 2 would lead to the required pitch length. The fixed geometrical features were from a careful survey of the best available data from relevant single-crystal structures (*6*), but searches also were made by using slightly different assumptions (*7, 9*). These searches confirmed our preference for left-handed helices.

The density measured by flotation was 1.45 g/ml, which corresponds to the presence of eight disaccharide residues in the unit cell. This indicates that two chains pass through the unit cell (each having four disaccharide residues in the *c*-axis repeat distance). However, the meridional reflections that are present when $l = 4n - 2$ show that the chains cannot have regular fourfold helical symmetry but strictly only twofold screw symmetry. Since there are eight disaccharide residues in each unit cell, and the dimensions of the base of the unit cell preclude the presence of four molecules at the cell corners and another at the center, we assumed that one strand drapes around the other to form a double helical structure.

Model-building calculations show that only the antiparallel model is completely free from steric compression. Moreover, if the strands are parallel, up- and down-pointing molecules are not superimposable; from the manner of our specimen preparation, we would expect these molecules to occupy lattice positions randomly. This disorder would manifest itself in the diffraction pattern through an increase of continuous intensity between the Bragg reflections (*10*). That no such streaking is observed supports the conclusion that the two strands are probably antiparallel. The preliminary model is being refined by the method of linked-atom least squares (*11*) to optimize the agreement between the observed and calculated intensities, but we do not yet have a model precise enough for the important contacts within the double helices to be identified with certainty.

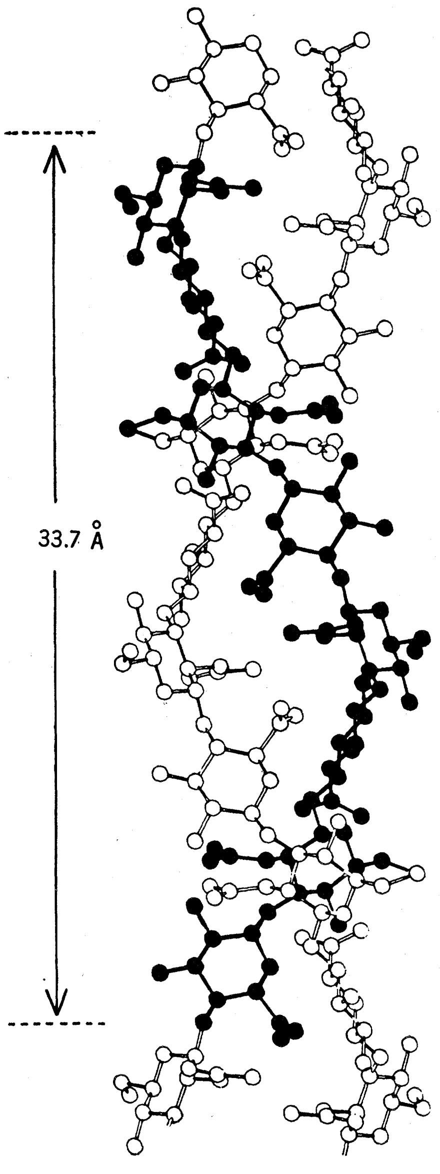

Fig. 2. The antiparallel double helix proposed for hyaluronate. The chains in this model have regular fourfold screw symmetry pending definition of the exact nature of the structural relaxation that leads to the final symmetry.

Hyaluronic acid therefore provides the second example of a polysaccharide double helix, the first being the carrageenan double helix, which is known in two variants (*8*). We can now compare these known types of double helix and consider the physical and biological implications. In the carrageenans the chains are parallel rather than antiparallel and have three rather than four disaccharide residues per turn. Chains can join in double helix formation to form a stable network which, when expanded by osmosis, shows considerable elasticity and capacity to retain its shape even though the system may contain a hundred times as much water as polysaccharide. Such properties are familiar in biochemical laboratories in the agar gels used in bacteriology and in agarose gel filtration media. In the putty from which we prepared our specimens for diffraction analysis, the hyaluronic acid helices appear to give some gel-like properties for short time periods (*3*), but these are modified in a subtle way so that they merge into viscous characteristics over long time periods (*12*). The value to biological systems of such viscoelastic attributes has been pointed out (*2*). The molecular basis of the viscosity could be that the helices, unlike their counterparts in carrageenans, interconvert continually with random coil forms. The more subtle rheological properties of the animal polysaccharides may then derive from a more delicate balance between forces of attraction and repulsion, and may thus reflect the higher level of evolution relative to the algae from which carrageenan and agar are derived. A further difference between the animal and algal systems is that the latter are more extensively helical at neutral *p*H.

Studies of optical rotation and circular dichroism on sodium hyaluronate showed (*13*) that solutions at neutral *p*H, like solutions at *p*H 2.5, have temperature-dependent order-disorder transitions. We also investigated oriented films made from neutral sodium hyaluronate solutions by the abovementioned technique. We could index a diffraction diagram (Fig. 1b) from these as a mixture of hexagonal (*4*) and orthorhombic forms, that is, as a mixture of the form considered to have a single helix conformation (*4*) and the new double helix form. As judged visually from relative intensities, the two forms were present in the ratio 9 : 1. The single helix form is not expected to persist in solution, because there is no mechanism by which it can be stabilized by substantial cooperative effects (*14*). We therefore propose that the polysaccharide in the solution from which this film was prepared was mainly in the random coil form—as indeed is indicated by all the evidence for solutions (*1*)—but with a slight degree of cross-linkage by double helices. Conversion to the putty is then seen as a quantitative change (an increase in helix content) rather than a qualitative change, in agreement with conclusions from the comparison of solution and putty by dynamic viscoelastic measurements (*12*) and by chiroptical measurements (*13*).

The presence of certain amounts of double helical cross-links in neutral sodium hyaluronate solution could explain the unusual rheological qualities of this biopolymer, which are manifest in its rapid transformation from liquid to solid character with increasing stress frequency. The significant lengthening of the molecular relaxation time, calculated from viscoelastic measurement in "viscoelastic putty" at *p*H 2.5, could also be explained by the increased number of double helical cross-links formed as the ionization of carboxyl groups is suppressed. This double helical structure might also be important in the explanation of certain biological activities of sodium hyaluronate of high molecular weight, such as the inhibition of the migration of cells of the lymphomyeloid system (*15*) and the inhibition of the modulation and proliferation of lymphocytes stimulated by mutagens (*16*).

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Hyaluronates: Relation between Molecular Conformations

Abstract. *The discovery that both potassium and sodium salts of hyaluronic acid can exist in a double-strand helical conformation that will convert to the already known single-strand helical structures illustrates the remarkable conformational versatility of this biopolymer. X-ray diffraction was used to monitor variations in molecular conformation as a function of several independent, controllable variables, such as relative humidity, temperature, and applied tension. A scheme is presented for the interrelation of a range of hyaluronate conformations.*

Studies on the molecular architecture of the connective tissue hyaluronates have revealed (*1, 2*) a number of different, yet related and interconvertible, molecular structures for this versatile biological polymer. We now add two distinct forms to this catalog of conformations. The initially bewildering array of molecular structures is now understandable in terms of the general thermodynamic behavior expected of a regular, repeating, unbranched polymer, and this in turn leads to a better understanding of the molecular interactions and their relation to the properties of the polymeric material.

We obtained an x-ray diffraction pattern (Fig. 1a) from a new crystalline form of potassium hyaluronate that has an orthorhombic unit cell with $a = 11.0 \pm 0.1$ Å, $b = 9.9 \pm 0.1$ Å, and c (fiber axis) $= 33.0 \pm 0.3$ Å. These dimensions are similar (but not identical) to those from the double helical structure described in the preceding report (*3*). Moreover, the x-ray intensities are so similar that we have no doubt that the structure proposed by Dea *et al.* (*3*) is in most essentials that needed to interpret our new pattern. That we obtained this form in quite different circumstances argues for the general occurrence of this structure. In addition, we have independent experimental evidence that supports and reinforces

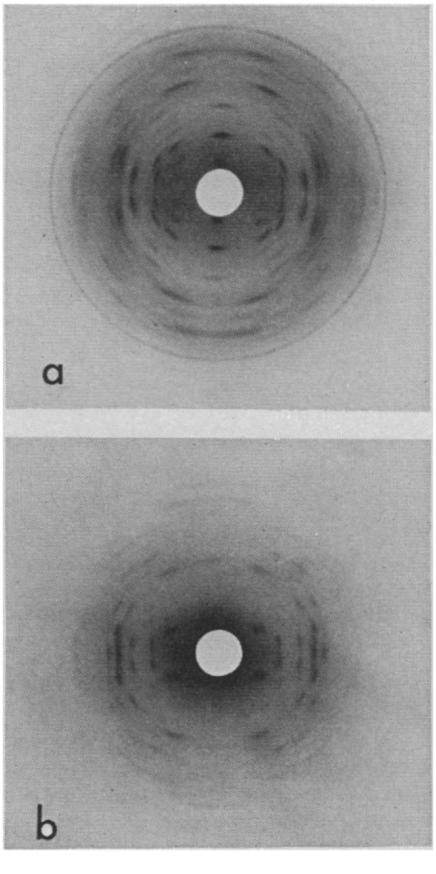

Fig. 1. X-ray diffraction photographs obtained from two new crystalline forms of potassium hyaluronate. Similar patterns were also obtained for sodium hyaluronate. The pattern in (a) is interpreted in terms of a double-strand helix (*3*), and that in (b) in terms of an untwisted version. The fiber axis is approximately vertical in each case.