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.

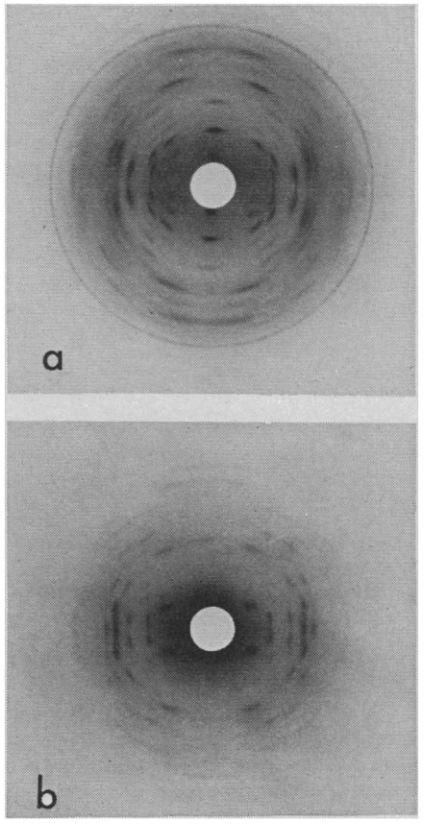


Fig. 1. X-ray diffraction photographs obtained from two new crystalline forms of potassium hyaluronate. Similar patterns were also obtained for sodium hyaluronate. The pattern in (a) is interpreted in terms of a double-strand helix (*3*), and that in (b) in terms of an untwisted version. The fiber axis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

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

certain properties of the proposed model. On annealing under tension, the x-ray pattern changes (apparently without passing through an amorphous phase) to one interpreted by us in terms of a system of antiparallel, single-strand, left-handed, threefold helices (*1*). Finally, the measured density favors two molecular chains per unit cell. Thus, any postulated molecular structure must contain a minimum of two chemical disaccharide repeats per structural repeat and have two chains of opposite polarity.

The x-ray diffraction pattern in Fig. 1b indicates another unique conformational state for potassium hyaluronate, intermediate between the double-strand helix and the single-strand helical structures. The pattern can be indexed on an orthorhombic unit cell with dimensions $a = 10.4 \pm 0.1$ Å, $b = 9.0 \pm 0.1$ Å, and $c$ (fiber axis) $= 37.1 \pm 0.3$ Å. The unit cell volume of 3470 Å$^3$ is intermediate between the double helix structure (3590 Å$^3$) and the threefold, single-strand helix structure (3380 Å$^3$) containing two chains in the unit cell (*2*). The first meridional reflection occurs on the fourth layer plane at a spacing of 9.3 Å, which enables the structural repeat to be equated with the disaccharide repeat (*4*). The higher axial projection of 9.3 Å [compared with an average of (33.0 Å)/4 = 8.25 Å for the double helix] suggests that the double helix is likely to have untwisted into individual regular fourfold helices. We cannot be absolutely certain that the two chains have completely untwisted, since heavily exposed x-ray photographs give a hint of a weak meridional reflection on the second layer plane. However, the pattern is readily reproducible and represents a definite conformational state. On slight annealing, this conformation relaxes into threefold, single-strand helices, which are capable of forming a well-organized system of interchain linkages (*2*).

Specimens of hyaluronate suitable for x-ray diffraction were prepared by allowing a viscous solution (2 percent if the molecular weight is 1 million) to dry down on a clean glass slide. Good results were obtained with a film thickness of about 200 $\mu$m. The film was cut into long strips 1 to 2 mm wide, peeled off, and folded concertina fashion five to six times to produce a thick specimen of the same width as the original. The ends were stuck to a piece of paper with Durafix. After the adhesive dried, the paper was cut in two, and the separation between the two parts during stretching gave a reliable, yet simple, measure of the degree of stretch. The specimens were stretched in sealed glass containers under constant load of 5 to 15 g and at a relative humidity (maintained by saturated salt solutions) in the range 66 to 98 percent. The appropriate humidity was that which gave a fairly rapid extension without breakage. As crystallization proceeded both relative humidity and load could be increased. The use of elevated temperatures to promote crystallization was also introduced (*5*).

As soon as hyaluronates could be induced to form semicrystalline films and fibers (*6*), it was obvious that a series of different conformational states existed. Furthermore, when samples were annealed a transition from one structural state to another took place; this could be monitored by x-ray diffraction techniques. We realized that the biopolymer would respond to environmental changes and wished to pursue this further by monitoring molecular conformation as a function of a series of independent variables such as relative humidity, *p*H, temperature, time, and applied stress. The question arose as to whether the first encountered crystalline form (*3*) was in reality the first, or had a number of states with higher free energy been missed?

We would now formulate the relation between the molecular structures as shown in Fig. 2. They are all interrelated; by adjusting the independent variables mentioned above, all the forms can be obtained from solution. In addition, once a particular form is obtained, it may be converted, by annealing, through a sequence of conformational states, each with lower free energy. By substantially increasing the humidity the conversion can be reversed, but at present with less control. The double helix is not a very stable structure, because it can easily be con-

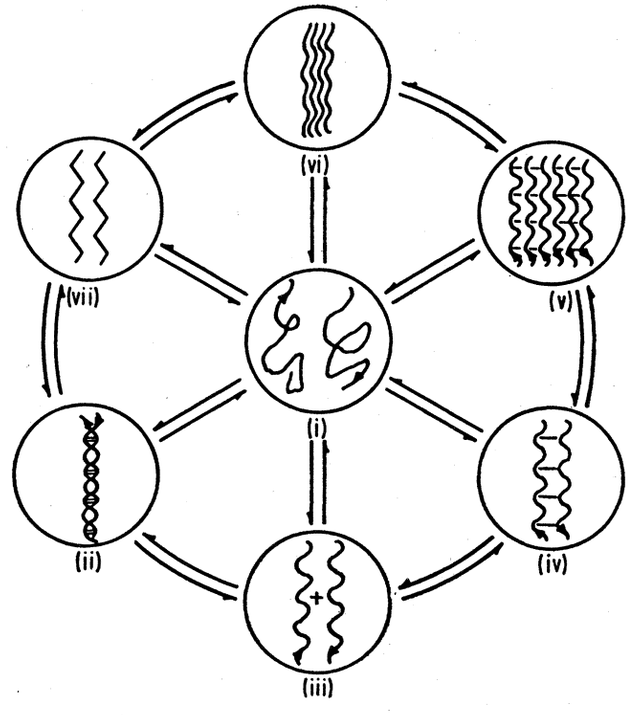


Fig. 2. Scheme for the interrelation of the hyaluronate conformations. The different conformations of both sodium and potassium hyaluronate are described as follows: (i) the molecular chains in dilute solution; (ii) the double helix (Fig. 1a); (iii) the two antiparallel untwisted helices (Fig. 1b); (iv) the relaxed threefold single helices with two antiparallel chains per unit cell, each chain having three near neighbors (*2*); (v) the same helices packed closer together in a six-chain unit cell in two groups of three of similar polarity, each chain having four near neighbors (*2*); (vi) an even more compact state, each chain having five near neighbors; and (vii) a free acid form, obtained by lowering the *p*H to less than 2.5, with molecular chains as fully extended twofold helices (*2*). Each individual state can be obtained by crystallization from dilute solution and reversed by dissolution. As anticlockwise path from (ii) to (vi) is obtained by annealing under tension at elevated temperatures. The return path is accomplished by increase in humidity and is less controllable. State (vii) is obtained by acidifying the univalent salts.

verted to the next conformational state, presumably of lower free energy. There is a plausible explanation for this transition. The chains in the double helix are not regular helices; therefore, alternate disaccharide repeats have different molecular environments. Thus, it may be argued that if a particular disaccharide is forming a maximum number of interchain linkages with the intertwining chain, it is unlikely that the adjacent dissaccharide (in its own chain) will also equally form an efficient interchain bonding arrangement. It is expected that in the structure as a whole, only about half the number of energetically favorable interchain linkages are utilized. Thus, on annealing the chains untwist to form single-strand regular helices capable of forming structures with lower free energy.

Hyaluronic acid is a good example of a material for which study of a variety of conformations, and their interrelation, is needed for better understanding of molecular behavior. Particular aspects of this behavior may then be related to the physical properties and biological functions of the substances.

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## Strengite Dissolution in Flooded Soils and Sediments

Abstract. *Strengite ($FePO_4 \cdot 2H_2O$) undergoes partial dissolution under the reducing conditions existing in a flooded soil. The greatest release of phosphate and iron occurs under conditions of low oxidation-reduction potential in combination with low* p*H.*

A major source of phosphorus for lowland rice and swamp and marsh plants is phosphate compounds which are ordinarily insoluble but which are partially dissolved under the anaerobic conditions that exist as a result of oxygen depletion in flooded soils and sediments. The release of phosphorus from the sediments of freshwater lakes and streams is also enhanced by reducing conditions. Although the increased solubility and availability of phosphorus to plants under these conditions is well documented (*1*, *2*), there is still lack of agreement about the cause. Among the major mechanisms proposed to account for the increase in phosphate solubility are: (i) the reduction of highly insoluble ferric phosphate to the more soluble noncrystalline ferrous form (*1*, *3*); (ii) the displacement of phosphate from insoluble ferric phosphate and aluminum phosphate by organic anions produced in an anaerobic flooded soil (*4*); and (iii) the hydrolysis of ferric phosphate as a result of the almost invariable increase in soil *p*H which results when an acid soil is flooded (*5*).

The experiment reported here was designed to enable one to differentiate between the effects of reduction processes and *p*H changes in a waterlogged soil on the release of phosphorus from strengite ($FePO_4 \cdot 2H_2O$), a ferric phosphate mineral commonly occurring in soils. Suspensions (25 g of soil and 80 g of water) of a rice soil (Crowley silt loam from the coastal prairie of Louisiana) were incubated at oxidation-reduction (redox) potentials of +300, +100, −100, and −250 mv. These redox potentials were controlled to within ± 5 mv by means of an apparatus described previously (*6*). Microbial metabolism within the soil medium results in a release of electrons and hence in a lowering of the redox potential. The decrease in the redox potential can be controlled accurately by the automatic introduction of oxygen into the suspension. Native soil organic matter acts as an energy source for the microorganisms, although in this case we found it necessary to add glucose as an additional energy source to sustain redox potentials of −250 mv. At each redox potential the suspension was maintained at a *p*H of 5.0, 6.0, 7.0, or 8.0 ± 0.02 by the manual addition of either 0.5*N* HCl or 0.5*N* NaOH.

The strengite was synthesized according to the method of Cate *et al.* (*7*) and contained 31.2 percent iron and 17.4 percent phosphorus (by weight). The compound was uniformly labeled with $^{59}Fe$ to give an initial activity of 0.08 μc/mg. X-ray diffraction patterns of our compound and a sample of the preparation of Cate *et al.* showed the same crystalline structure (*8*).

After the soil suspensions had been maintained at the various redox potential–*p*H combinations for 3 days, 0.0478 g of finely powdered labeled strengite was added to each of the suspensions and incubation was continued for an additional 7 to 10 days. Samples of the suspensions were removed without contact with air, and iron ($Fe^{2+}$ and $Fe^{3+}$) and orthophosphate ($H_2PO_4^-$ and $HPO_4^{2-}$) were extracted from the soil with 1*N* ammonium acetate solution adjusted to the same *p*H as that of the suspension. Extraction was carried out in a nitrogen-filled glove bag to prevent atmospheric oxidation. We analyzed the iron by means of the α,α′-dipyridyl method (*9*) and phosphate by means of the chlorostannous–reduced molybdophosphoric blue color method (*10*). We determined the activity of $^{59}Fe$ in the extract by using a deep-well gamma counter.

The results show that both *p*H and redox potential had marked effects on the dissolution of strengite in flooded soil. Using as independent solubility indicators either the amount of labeled iron released from the added strengite (Fig. 1A) or the total amount of extracted phosphate (Fig. 1B), we found that the amount of strengite dissolved ranged from no detectable amount at the highest *p*H in combination with the highest redox potential (*p*H 8 and +300 mv) to a marked amount at the lowest *p*H in combination with the lowest redox potential (*p*H 5 and −250 mv). At all *p*H levels decreases in the redox potential increased the amounts of iron and phosphate dissolved. The amounts dissolved were greater, however, at the lowest *p*H value.

More than one half (59 percent) of the $^{59}Fe$ in the added strengite was released under the most acid condition in combination with the most reduced condition (*p*H 5 and −250 mv). At *p*H 6, a more realistic value for a flooded soil, 21.5 percent of the total $^{59}Fe$ was extracted at a redox potential of −250 mv.

The effect of *p*H at constant redox