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INFRARED DICHROISM OF SODIUM HYALURONATE

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SUMMARY

The infrared dichroism of sodium hyaluronate isolated from umbilical cords was investigated. Films of hyaluronate swollen in 82% ethanol were elongated and the infrared absorption spectra of the dried films were taken at different elongations with the electrical vector of polarized infrared radiation parallel and perpendicular to the direction of elongation. The dichroic ratios were calculated as a function of elongation. The orientations of different atomic groups in the polymer molecule with elongation were interpreted as indicating a rather stiff molecular configuration of the chains in the gel form. It is proposed that the elongation is mainly due to the turning and orientation of the molecular aggregates rather than to uncoiling of a random isotropic chain configuration.

INTRODUCTION

Hyaluronic acid exists in many animal tissues in the form of a gel mostly associated with proteins. Many of these tissues: skin, umbilical cords, vitreous humor, etc., exhibit a high degree of elasticity which is largely or partly due to the presence of hyaluronate in the tissue[1-4]. Physical chemical measurements on hyaluronates in dilute aqueous solutions were reported by light scattering[5,6] streaming birefringence measurements[7-9] and by viscometry[10-12]. The overall indication is that hyaluronic acid of high molecular weight has the shape of a stiff random coil in such solutions. On the other hand, BETTELHEIM[13] reported that sodium hyaluronate of low molecular weight (70000) can form crystallites in the solid state and the chains in the crystalline region are extremely stiff. Electron microscopic data on dehydrated sodium hyaluronate gels[14] confirmed the rigidity and anisotropy of the molecules and their aggregates. The shape of a single molecule in an infinitely dilute solution or, for that matter, the shape of the polymer molecule under the influence of crystalline aggregation might not represent the physiological shape in the gel state. In any case, if hyaluronic acid contributes to the elasticity of the tissues, a quasi-rubber-like behavior[15,16] is expected from the molecules, that is, the polymer chains should orient with their long geometrical axis in the direction of stress. A previous report[17] from our laboratory using strain-birefringence measurements indicated that swollen hyaluronate films exhibit rubber-like behavior up to 30% elongation. Above this elongation the intermolecular hydrogen bonding greatly hinders any further orientation. Since in birefringence measurements one deals with the vector sum of bond polarizabilities parallel and

perpendicular to the direction of stress rather than the orientation of the individual molecule, it was found necessary to follow the molecular orientation during elastic elongation with infrared-dichroism measurement. The result of this investigation is reported here.

MATERIALS AND METHODS

Preparation and analysis of sodium hyaluronate

Sodium hyaluronate was isolated from umbilical cords by a combination of two methods. In the first part JEANLOZ AND FORCHIELLI's method[18] was used in purifying the hyaluronate from proteins and the method of SCHILLER *et al.*[19] was applied to separate the hyaluronate from sulfated mucopolysaccharides. Acid hydrolysis with 4 N HCl and subsequent paper chromatography showed only D-glucuronic acid and D-glucosamine spots. No amino acids were present in the hydrolysate. ANDERSON's micro-method for sulfur determination[20] gave negative results. DISCHE's method for the determination of uronic acid content[21] gave 53 % uronic acid as anhydro-sodium glucuronate. A modification of the Elson–Morgan method[22] gave 41 % *N*-acetyl-glucosamine. The preparation contained 3.2 % nitrogen as determined by micro-Kjeldahl. The weight-average molecular weight was $1.2 \cdot 10^6$ as determined by light-scattering measurements[17]. The sodium hyaluronate preparation was amorphous as viewed by X-rays.

Preparation of films

Thin films for infrared-dichroism studies were prepared by solvent casting onto a ferrotype plate and dried in a vacuum at room temperature. Film strips were prepared approx. 1 × 4 cm. These strips were marked at 0.5-cm intervals with inked lines and were then placed in the jaws of a lathe-type stretching device. The stretching device and film were placed in a tray containing 82 % ethanol and the films were slowly elongated. The amount of elongation was determined by measuring with a caliper the average distance between the inked lines upon stretching.

After stretching, the films were clamped in a jig and kept under absolute alcohol prior to infrared measurements. Samples were elongated to 5, 10, 20, and 40 % of the original length. To obtain the thickness of the films, an American Optical Spencer Metalstar compound microscope with a tungsten light source and green filter was used.

Dichroism measurement

The instrument used was a Beckman IR-7 double-beam infrared spectrophotometer. Two identical silver chloride polarizers were placed in the path of the sample and reference beams. The elongated film was mounted in the jig in the path of the sample beam so that the direction of the elongation was vertical. A spectrum was then obtained with the electrical vector of the polarized infrared radiation perpendicular to the direction of stretch. The polarizers were then rotated 90° and a spectrum was obtained with the electric vector parallel to the direction of stretch.

EXPERIMENTAL RESULTS

When a substance absorbs one of the components of polarized radiation more strongly than the other the substance is said to exhibit dichroism. Absorption by molecules

of polarized radiation at characteristic wavelength depends on changes in dipole moments due to particular intramolecular vibrations. The absorption at a characteristic peak is proportional to the interaction between the dipole change vector M and the electric vector $\bar{P}$ of the vibration. For those bands which depend on the direction of $\bar{P}$, the absorption is maximum when $\bar{P}$ and $\bar{M}$ are parallel and zero when $\bar{P}$ and $\bar{M}$ are perpendicular. Thus, with a knowledge of the groups which a polymer contains and of the characteristic wavelengths of vibration associated with those groups, important deductions can be made for those substances which exhibit dichroism under infrared radiation. For example, when a group exhibits dichroism with respect to one orientation of the electric vector, one may conclude that there

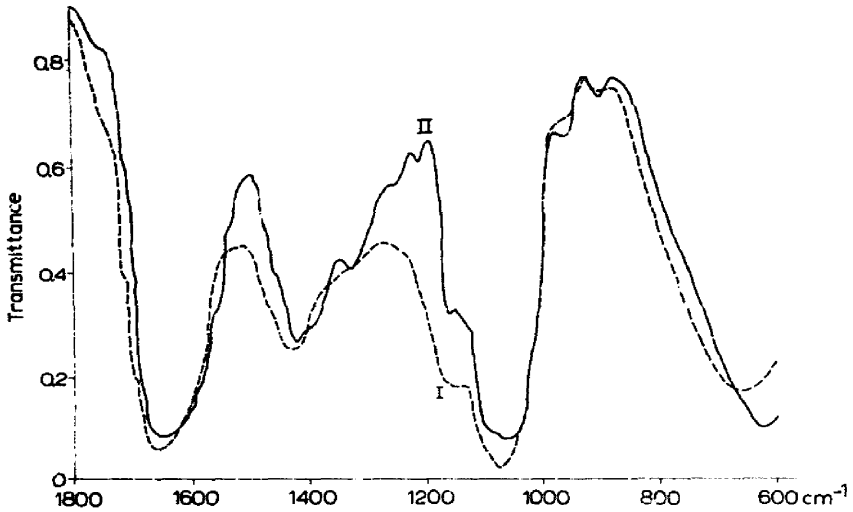

Fig. 1a. Infrared absorption spectra of sodium hyaluronate films, 40% elongation, from 600–1800 cm^{-1}. Electrical vector of polarized infrared radiation parallel (———) and perpendicular (– – – –) to the direction of elongation.

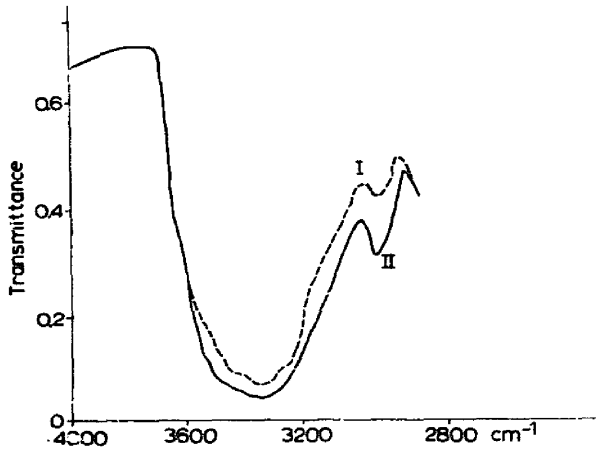

Fig. 1b. Infrared absorption spectra of sodium hyaluronate films, 40% elongation, from 2800–4000 cm^{-1}. Electrical vector of polarized infrared radiation parallel (———) and perpendicular (– – – –) to the direction of elongation.

is orientation in the molecular aggregate. Further analysis of the dichroism thus exhibited with respect to the mode of vibration associated with the particular band assigned to a group can furnish information about the position of that group with respect to the rest of the molecule.

Dichroic measurements have been used with considerable success in the elucidation of polymer structures, particularly in the case of synthetic polymers, as well as in the case of polysaccharides[23,24]. In Fig. 1 the infrared absorption spectrum of an oriented sodium hyaluronate film is given at 40 % elongation. Parallel polarization means that the electric vector of the infrared radiation is parallel to the direction of the elongation of the film.

The assignments of the various bands were made in comparison with spectra of similar compounds reported in the literature. These assignments and the polarization of the bands are given in Table I.

TABLE I

ASSIGNMENT AND POLARIZATION OF INFRARED ABSORPTION BANDS

The polarizations given in the table represent that orientation of the electric vector which was absorbed more strongly at the highest elongation.

Frequency (cm^{-1})	*Polarization*	*Interpretation*
905	Perpendicular	Ring stretching[26]
960	Perpendicular	C–O stretching[26]
1055	Perpendicular	C–O–H stretching and bending[25]
1090	Parallel	C–O stretching[23]
1155	Parallel	Assymmetric bridge oxygen stretching[23]
1230	Parallel	Acetyl group[26]
1330	Perpendicular	Amide III[26]
1395	Perpendicular	CH bending and symmetric CH_3 deformation[24]
1420	Parallel	Ionized carboxyl[26]
1560	Perpendicular	Amide II band, ionized carboxyl[26]
1625	Perpendicular	CO of *N*-acetylamine group[25]
1655	Parallel	Amide I band[24]
2900	Perpendicular	CH stretching[24]
2940	Perpendicular	CH_3 stretching[24]
3300	Perpendicular	NH stretching[24]
3480	Perpendicular	OH stretching[24]

The absorption spectra of the parallel and perpendicular polarized radiation of the hyaluronate films at different elongation were replotted as absorbance.

$$\text{Absorbance} = \log \frac{1}{\text{transmittance}}$$

The assigned peaks then were resolved by assuming Gaussian distribution curves in each case. The resolved peaks were then plotted as absorbance index "*a*" *versus* frequency in wave numbers where the absorbance index was defined as $a = 2.303\, d \times \log (1/\text{transmittance})$, d being the thickness of the film. Such a plot is given in Fig. 2. The area under the curves was determined by a polar planimeter and the perpendicular dichroic ratio was calculated from the relationship

$$D = a_{\perp}/a_{\parallel}$$

Perpendicular dichroic ratios were calculated for five absorption bands (at 1090, 1155, 1420, 1655 and 2940 cm^{-1}), the resolutions of which could be accomplished with the least error.

Although for a random orientation of polymer chains one would expect a dichroic ratio of one, not all of our absorption bands yielded this ratio at zero elongation. Small differences in the transmittance of parallel and perpendicular polarized infrared radiation were observed in some of the bands indicating a slight degree of preferential

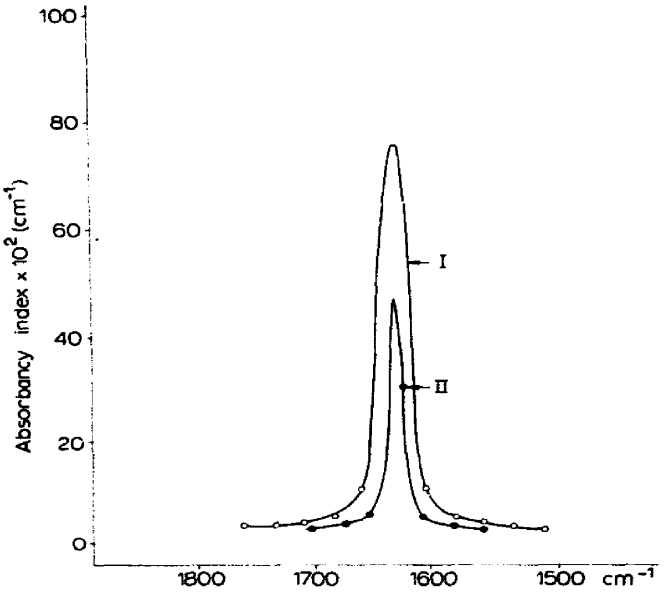

Fig. 2. Resolution of the band at 1625 cm^{-1} at 40% elongation. Parallel and perpendicular polarized absorbance *versus* frequency of radiation.

orientation during the film-casting technique. Since the purpose of our investigation was to relate the average degree of orientation of a specific group of atoms within the polymer to the degree of elongation, the dichroic ratios obtained at the different elongations were normalized so that each band was assigned a dichroic ratio of one at zero elongation. In Fig. 3 these normalized perpendicular dichroic ratios are plotted *versus* elongation. The diagram indicates, therefore, the different degree of orientation of the different atomic groups upon stretching. The greatest orientation and the fastest is observed with the 1153-cm^{-1} band which was assigned to the antisymmetric stretching vibration of the glycosidic oxygen bridge. Since the perpendicular dichroic ratio is decreasing with elongation, the vibrations of this group of atoms orient their dipole change vector in the direction of stretch. The vector sum of the antisymmetric vibration of the C–O–C bridge is along the long geometrical axis of the polymer molecule. This, therefore, substantiates previous observations that long-chain polymers upon stretching orient themselves with their long axis in the direction of stretch.

The same result can be observed with the 2940-cm^{-1} band. Here the perpendicular dichroic ratio increased with elongation. Therefore, the vibrations of the CH_3 groups of the *N*-acetylamine side chain become more and more perpendicular to the direction of stretch.

The other three bands for which the dichroic ratios were measured, namely the vibrations of C–O, COO^- and amide C=O oriented at a lesser degree parallel to the direction of elongation.

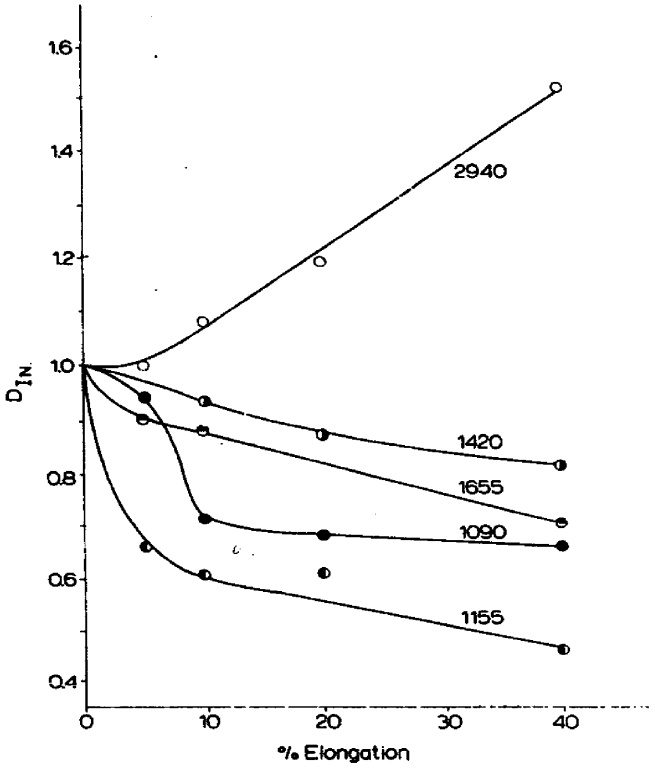

Fig. 3. Normalized perpendicular dichroic ratio of sodium hyaluronate as a function of elongation for different absorption bands.

DISCUSSION

The correct interpretation of the results of this investigation rests largely on the validity of the assignments on the one hand and on the minimization of errors in the resolution of the individual bands when calculating dichroic ratios.

With respect to the assignment of the bands an empirical approach was followed here, that is, our absorption spectra were compared with those of similar compounds reported in the literature. For these assignments theoretical justifications are also cited in the literature.

A more detailed assignment of the infrared absorption bands of crystalline sodium hyaluronate is in progress now in which the molecular configuration in the unit cell of crystallites is correlated with the infrared frequencies. However, the molecular configuration of an amorphous chain is not necessarily elucidated by the crystalline structure and, therefore, for our present purpose the empirical assignments are sufficient.

A more difficult problem arises with respect to the resolution of the individual bands. Mathematically, even with the assumptions of (a) Gaussian distribution of

each band around its maximum and (b) additivity of absorbance indices, the spectrum represents the sum of terms $A_e^{-\beta(y-y_0)^2}$, where y's are the ordinate of the absorbance curves. In the literature a simplification is often made by assuming that the absorption curve is the sum of $A_e^{-\beta y^2}$ terms rather than the previous formula. Although this makes the calculations possible by not necessitating a separate determination of y_0 and y in addition to the A and β, such a simplification introduces errors which are comparable to those of a visual resolution by trial and error method (10–15 %). Therefore, we decided to use the visual resolution with the understanding of the limitations inherent in this procedure.

For this reason dichroic ratios were calculated only for those bands which lent themselves readily to visual resolution.

The most important among these is the band corresponding to the C–O–C bridge antisymmetric vibration. Since this vector is along the long axis of the polymer this dichroic ratio also indicates the orientation of the polymer chains in the direction of stretch. It is interesting to note that this group orients very rapidly on elongation and upon further stretching it orients to a lesser degree. If the hyaluronate chains have a random-coil configuration in the gel one would expect that upon elongation uncoiling would occur which would result at low elongations in a rather small amount of orientation and the strain optical coefficient should increase at higher elongation when more or less parallel alignment of the chains occurs. This is the type of behaviour one observes in rubber-like materials. The indication of the dichroic-ratio change of the C–O–C band with elongation is, therefore, that we are dealing here with fairly stiff molecules or molecular aggregates which orient themselves by turning in the direction of stretch rather than uncoiling from a random-chain configuration. The presence of anisotropic molecular aggregates was proven by the Wiener-type curves of the form birefringence[17]. The higher elongation would be caused rather by chain slippage than orientation.

The dichroism of the CH_3 vibration at 2940 cm^{-1} is in agreement with this conclusion, although the primary data indicates the CH_3 group orients very little at low elongation perpendicular to the direction of stretch and the optical-strain coefficient increases only at high elongation. If the hyaluronate chains form stiff anisotropic aggregates, and if these aggregates turn upon stretching, one would expect that the side chains entangled in intermolecular hydrogen bonding would be somewhat slow to follow the main chain orientation. This would mean that part of the elasticity at low elongation is provided by the distortion of the side-chain orientation and only upon chain slippage would a belated orientation of the side chains follow.

The dichroism of the 1090 cm^{-1} band indicates a parallel orientation similar if somewhat less than that of the C–O–C bridge. LIANG AND MARCHESSAULT[23] attribute this band in polysaccharides to the ν_{at} antisymmetric and in-phase stretching mode of the C–O in the pyranose ring. Parallel polarization of this band was also observed in cellulose and chitin. The dichroic ratio of this band as a function of elongation reinforces the argument that the main chain orients first in the direction of stretch.

The other two bands resolved, namely the amide I band at 1655 cm^{-1} and the symmetrical stretching mode of the ionized carboxyl group at 1420 cm^{-1}, have only a slight parallel dichroism, their dipole change vector pointing at an angle to the direction of stretch rather than in the direction of stretch. A similar dichroic curve was obtained for the band at 1625 cm^{-1}.

The above interpretation of the infrared dichroism of sodium hyaluronate chains in the gel form is in agreement with the birefringence measurements[17]. One can conclude, therefore, that sodium hyaluronate in the gel form has a rather stiff molecular configuration and its elasticity is provided more by chain orientation, bond distortion and intermolecular hydrogen bonding resisting chain slippage than by the uncoiling of the molecule.

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