

THE MOLECULAR SIZE, SHAPE AND WEIGHT OF MUCOPROTEIN
FROM CARTILAGE

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INTRODUCTION

The chondroitin sulfate-protein complex (CS-P) from cartilage accounts for at least a third of the chondroitin sulfate of cartilage, the remaining two thirds most likely being linked to collagen¹. The chondroitin sulfate and protein contents of CS-P have been estimated to be 60-75% and 25-40%, respectively, for various preparations². Chondroitin sulfate is a linear acidic mucopolysaccharide³, whose repeating unit is a disaccharide consisting of D-glucuronic acid and N-acetyl-2-deoxy-2-amino-D-galactose-6-sulfate. The glucosidic linkage is a β -linkage; the glucuronidic linkage involves C₁ and C₃, the hexosaminidic C₁ and C₄⁴. The molecular weight of chondroitin sulfate is about 50,000⁵. The protein moiety of CS-P shows an amino acid composition different from that of collagen. The nature of the linkages between the protein and polysaccharide is unknown.

In the present work, the aim was to obtain information about the molecular size, shape and weight of CS-P.

EXPERIMENTAL

Preparation of CS-P

All operations were carried out at 2°C. 1 kg of fresh beef nasal septa cartilage, carefully cleaned, was homogenized in a Waring blender, which was operated intermittently to avoid warming, suspended in 5 l of 30% KCl and mechanically shaken for two days. The suspension was squeezed through cheesecloth and centrifuged at 20,000 r.p.m. for 15 min in a Spinco centrifuge. The supernatant showed two components in the analytical ultracentrifuge; the concentration of the faster one was negligible compared to that of the slower component. The supernatant was then dialyzed against running tap water for three days; K acetate (5 g/l) and two volumes of absolute alcohol were successively added. The precipitate that formed was allowed to stand overnight and was then recovered by centrifugation at 10,000 r.p.m., for 15 min; afterwards it was dried with alcohol and ether and suspended in 500 ml of phosphate buffer *M*/15, pH = 7.0. The turbid suspension was centrifuged at 40,000 r.p.m. for 2 h, a very small quantity of precipitate being formed. The clear, viscous supernatant exhibited in the analytical ultracentrifuge only a sedimenting boundary; thymol was added and the solution was stored at 2°C for use in the physical studies, which were, therefore, performed with phosphate buffer *M*/15, pH = 7.0 as the solvent.

Analytical and chromatographic results

Our CS-P solution had a concentration (dry weight at 110°C) of 0.72 g%. The nitrogen content of CS-P determined by the micro-Kjeldahl method, was 5.85 g%. Qualitative data on the amino acid composition of the protein of SC-P were obtained by paper chromatography.

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Samples of CS-P were digested in 6 *N* HCl at 120°C for 18 h, and one- and two-dimensional analyses were carried out; butanol-acetic acid-ethanol-water (4:1:1:2) and phenol saturated with water (in the two-dimensional analyses) were used as solvents. Amino acids were located by developing the chromatogram with ninhydrin, by U.V. absorption and by specific reactions (histidine and proline).

It was possible to identify the following amino acids by their R_F values: aspartic acid, serine, glycine, glutamic acid, threonine, alanine, leucine (+++); ornithine, lysine, valine, phenylalanine (++) ; histidine, tyrosine, proline (+). The evaluation of the intensity of spots, as indicated by (+), (++) , (+++) , is to be considered as only tentative and is based on the intensity of the spot corresponding to alanine. Proline is possibly due to trace contamination with collagen. Further spots pertaining to the carbohydrate moiety of CS-P were not investigated.

Physical methods and results

Viscosities were determined with a Couette apparatus at a velocity gradient $g = 0.246 \text{ sec}^{-1}$, and a temperature $t^\circ = 25^\circ\text{C} \pm 0.05$. The viscosity results are shown in Fig. 1.

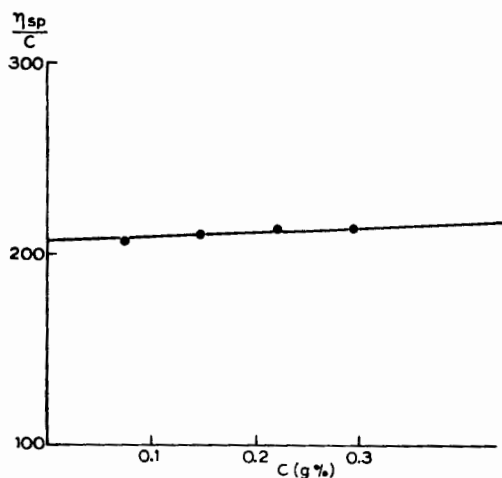


Fig. 1. Reduced viscosity plotted against concentration.

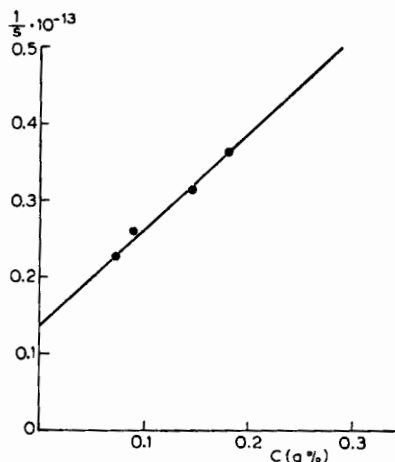


Fig. 2. The inverse of the sedimentation coefficient plotted against concentration.

The intrinsic viscosity was found to be $[\eta] = 207 \text{ cgs units}$.

A model E Spinco ultracentrifuge operating at 60,000 r.p.m. was used in the sedimentation analysis; sedimentation coefficients corrected to standard conditions were computed in the usual way. The sedimentation constant at infinite dilution, s_0 , was obtained by linear extrapolation of the plot of $1/s$ against c to $c = 0$ (Fig. 2). Only results for concentrations below 0.2% were used. The sedimentation boundary showed considerable spreading and the peak was very asymmetric. s_0 was found to be $6.85 \cdot 10^{-13} \text{ cgs u}$.

The flow birefringence of CS-P was measured at 25°C at velocity gradients ranging from 0 to 390 sec^{-1} . For a 0.72% solution of CS-P and a velocity gradient $g = 390 \text{ sec}^{-1}$, the steaming birefringence was $\Delta n = 4.5 \cdot 10^{-8}$. Therefore, the intrinsic streaming birefringence, defined as $[\Delta n] = \lim_{\substack{g \rightarrow 0 \\ c \rightarrow 0}} \Delta n / gc\eta_0$, was $[\Delta n] \ll 10^{-6}$.

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In order to correct an error in the labelling of Figs. 3 and 4, the kc/R values should be multiplied by 1.55. The actual points in the figures are correctly placed relative to this new scale. The values of molecular weight, size and shape reported in the paper are correctly calculated.

The refractive index increment was measured at $\lambda = 5460 \text{ \AA}$ with a Rayleigh interferometer; the specific refractive index was $dn/dc = 0.168 \text{ per g/ml}$.

Angular light scattering was measured at wavelength $\lambda \approx 5460 \text{ \AA}$ with the apparatus of WIPPLER AND SCHEIBLING⁶. Solutions of CS-P with concentrations ranging from 2.86 to $14.30 \cdot 10^{-5} \text{ g/ml}$ were used in these measurements. Scattering data were extrapolated to zero angle and zero concentration by the method of ZIMM⁷. The angular scattering envelope obtained is shown in Fig. 3. The anisotropy was negligible (which is important for the following discussion⁸). From the light-scattering measurements it was possible to obtain the following data: the weight average molecular weight, $M_w = 1.98 \cdot 10^6$; the dissymmetry, $I_{45^\circ}/I_{135^\circ} = 2.18$; the Z-average radius of gyration, $R_z = 1180 \text{ \AA}$.

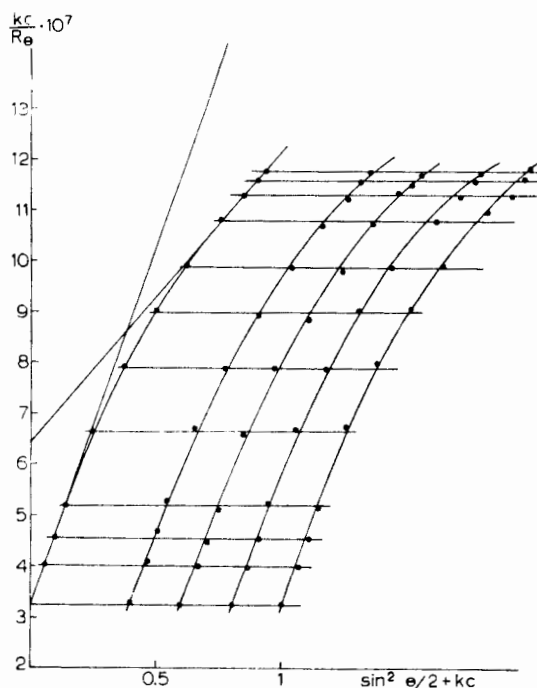


Fig. 3. Zimm plot obtained with CS-P.

DISCUSSION

The double extrapolation of the Zimm plot obtained for our CS-P was recorded in a diagram $P^{-1}(\theta)$ vs. $\mu^2 R_z$ ($P^{-1}(\theta)$ is the reciprocal particle scattering factor⁹; $\mu = \frac{2\pi}{\lambda'} \sin \frac{\theta}{2}$, λ' being the angle between the incident and scattered beams), in order to compare the plot with the curves pertaining to monodisperse systems of spheres, coils and rods, respectively (Fig. 4). It is known (BENOIT¹⁰) that the curves of polydisperse systems are lower than those of the monodisperse ones.

We shall now discuss the three possibilities as to the shape of CS-P macromolecules, namely polydisperse spheres, coils and rods. As is evident from Fig. 4, the sphere model seems to be quite improbable; furthermore, it would require a hydrated specific volume $V' = 4450 \text{ ml/g}$, which appears to be enormously high; the given value of V' is easily computed from R_z ; the hydrated volume should be $V = 14.7 \cdot 10^{-15} \text{ cm}^3$,

and the anhydrous volume (assuming a partial specific volume for CS-P, $\bar{v} = 0.60$ ml/g), $V_a = V/7800$. All these data decidedly rule out the possibility of a system of spheres.

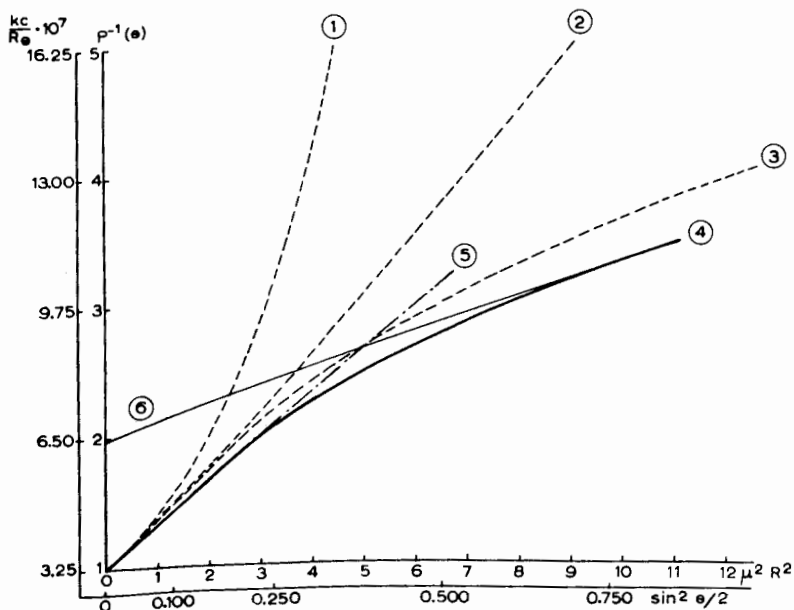


Fig. 4. Curves 1, 2, 3 pertain to monodisperse systems of spheres, coils and rods, respectively. Curve 4 is the Zimm plot extrapolation to $c = 0$, with its tangent at the origin, 5, and the asymptote, 6.

On the other hand, a polydisperse system of rods, such as was put forward by MATHEWS AND LOZAITYTE², must receive serious consideration. It is, indeed, by no means incompatible with the light scattering envelopes. The length of the rods, as deduced from R_z , would be $L \cong 4000$ Å. For such a system it is possible to obtain approximate values for the number average molecular weight, M_n , and number average length of rods, L_n , by the method of HOLTZER¹¹, which is an application to rod systems of the method originally developed by SADRON AND BENOIT¹² for polydisperse systems of coils. These values are $M_n = 1.3 \cdot 10^6$ and $L_n = 2330$ Å; consequently $M_w/M_n = 1.52$. We can now compute the value of the rotary diffusion constant for such rods. The volume intrinsic viscosity is easily obtained for a suspension of rods by dividing the weight intrinsic viscosity by the partial specific volume (assumed to be $\bar{v} = 0.60$ ml/g); we find $[\eta]_v = 350$ cgs u. By applying SIMHA'S formula as modified by SADRON¹³ for rod-like particles, we obtain an axial ratio $a/b \cong 70$; the diameter of the rods is also $b \cong 60$ Å, and the rotary diffusion constant $D \cong 0.2$ sec⁻¹. Such a system should show a very good orientation already at velocity gradients as low as $g \cong 0.2$ sec⁻¹, because in this case the ratio g/D is equal to unity¹⁴, and the system could hardly fail to exhibit a strong flow birefringence in the gradient range examined. On the contrary, we found a negligible streaming birefringence, a result in agreement with the negative data of WEBBER AND BAYLEY¹⁵ for an analogous product.

All the above considerations show that the rod model is also highly improbably and that the hypothesis of a polydisperse system of random coils appears to be the

most satisfactory. On the assumption that we were dealing with such a system, we were able to obtain other informations about the CS-P macromolecule. Firstly, it may be said that there are indications that there is not branching or at most to only a very slight extent. Indeed, BENOIT¹⁰ showed that branching increases the general upward curvature of curve 2, while our curve 4 exhibits a strong downward curvature (Fig. 4). This latter is substantially due to the wide polydispersity of the system. The possibility of a system of non-gaussian coils, which could also explain such a curvature¹⁶ seems to be ruled out by the high value of the ratio Λ/r , where Λ is the length of the filament (which may be estimated on the basis of an end-to-end arrangement of chondroitin sulfate molecules and polypeptide chains in the macromolecule of CS-P), and r is the mean root-square end-to-end distance of the coil (computable from R_z). Finally, using the method of SADRON AND BENOIT¹², we found an approximate value for M_n (strictly speaking, its upper limit); we obtained $M_n = 480,000$; therefore, $M_w/M_n \cong 4$, a result which confirms the wide polydispersity observed in the sedimentation analysis. With the aim of checking the mutual consistency of our experimental data as well as our conclusions, we applied the following equation proposed by FLORY¹⁷:

$$\Phi^{1/3} \rho^{-1} = \frac{s_0 N [\eta]^{1/3} \eta_0}{M^{2/3} (1 - \bar{v} \rho)}$$

where $\Phi^{1/3} \rho^{-1}$ should be a universal constant independent of solvent, temperature and nature of the polymer. Introducing into the above equation our values s_0 , M_n , $[\eta]$, and the assumed value of \bar{v} , we found $\Phi^{1/3} \rho^{-1} = 2.1 \cdot 10^6$; the good agreement between this value and the theoretical one seems to confirm the consistency of our experimental results and the validity of the proposed model.

CONCLUSION

The results of the present study on CS-P support the view of an end-to-end arrangement of chondroitin sulfate linear chains and polypeptide chains in the macromolecule of CS-P; the same conclusion but on a different basis, was reached by WEBBER AND BAYLEY¹⁵ for a product analogous to our CS-P. Such a structure would account satisfactorily for the stabilizing and cementing function of CS-P on collagen.

It is suggested that such a function of CS-P involves more collagen structural units than chondroitin sulfate does^{18,19}; therefore, the first is extractable by milder methods than the latter.

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SUMMARY

The chondroitin sulfate - protein complex of cartilage was studied by viscosimetry, streaming birefringence, sedimentation and light scattering. It showed a $M_w = 1.98 \cdot 10^6$, a Z-average radius of gyration $R_z = 1180 \text{ \AA}$; the molecular shape is probably linear with a random coil configuration in saline solution.

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