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STRUCTURAL PROPERTIES OF HYALURONIC ACID IN MODERATELY CONCENTRATED SOLUTIONS*

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ABSTRACT

The solution properties of hyaluronic acid having various d.p. values have been examined at physiological pH and ionic strength by light-scattering techniques at various concentrations. The angular dependence of Kc/R_θ was linear at low concentrations, whereas substantial curvature was present at small angles on increasing the concentration. This phenomenon was affected by the conditions of centrifugation. Moreover, the function Kc/R_0 exhibited a maximum value at concentrations of polymer inversely related to the molecular weight. The maximum concentration was always higher than the critical concentration. Ageing, molecular-weight distribution, and polymer purity influenced the light-scattering response. A model is proposed in which the polysaccharide chains intertwine at relatively low concentrations of polymer and become entangled at high concentration, eventually leading to a non-homogeneous polymer network.

INTRODUCTION

The structural properties of moderately concentrated solutions of polymers are of importance both in the synthesis and processing of polymers and in phenomena involving biological macromolecules[1–6]. Polysaccharides and their protein conjugates are present in substantial amounts in animal tissues and fluids, and hyaluronic acid (**1**) is found in cartilage, in eye vitreous humour, and in synovial fluid[7]. In contrast to other mucopolysaccharides, hyaluronic acid exhibits molecular weights up to 8×10^6, a highly regular structure, and no sulfur-containing groups. In order to evaluate the behaviour in solution of hyaluronic acid under conditions approaching the physiological, samples with various d.p. have been examined at various concentrations at physiological pH and ionic strength using light-scattering techniques.

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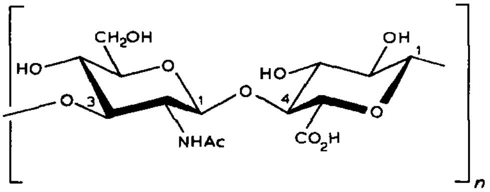

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MATERIALS AND METHODS

Sodium hyaluronate (HA). — Highly purified HA[8], extracted from rooster comb and provided by FIDIA Research Laboratories (Italy), typically contained 98–99% of HA, ~0.2% of other mucopolysaccharides, <0.2% of protein, and <1% of sodium chloride. There were no peaks of absorption above 250 nm. The molar extinction at 258 nm was in the range 20–40 $\text{M}^{-1}.\text{cm}^{-1}$, except for sample 070 which was deliberately less purified so as to contain ~0.8% of protein and 1.2% of polynucleotide contaminants. C.d. measurements showed one absorption minimum at 208 nm. Excluding sample 070 for which lower values were found, the molar ellipticity ranged between −13,500 and −14,000 $\text{deg.cm}^2/\text{dmol}$. Whereas the minimum wavelength corresponded to other literature data[9], the band intensity of our samples was substantially higher, probably due to increased purity of the HA and consequently a higher ordering of the polysaccharide. The intrinsic viscosity and molecular weight of HA samples used are listed in Table I. Molecular weights were evaluated from intrinsic viscosity measurements in 0.15M NaCl (pH 7) at 25.0°, using the relationship[10] *1*.

$$[\eta] = 5.7 \times 10^{-4}\, M_w^{0.75} \qquad (1)$$

The above figures accord with other literature data[11,12] in the same solvent system.

HA solutions. — Mixtures of HA and 0.01M phosphate buffer (pH 7.0) containing 0.15M NaCl of known composition were stored overnight at 4–5° to allow polymer swelling, and then shaken for 48 h. Each solution was diluted as required with buffer and shaken for 6–8 h in the presence of NaN_3 (~0.1 g/L) to prevent bacterial growth. The NaN_3 did not interfere with light-scattering measurements. HA concentrations were checked by the carbazole method[13], using D-glucuronic acid as the standard.

Light-scattering (l.s.). — These measurements were performed at 25° using a SOFICA Model 42000 photometer with cylindrical cells immersed in toluene. Non-polarised light (546 and 436 nm) was used, covering scattering angles ϑ between 30° and 150°. A Rayleigh ratio $R_{90} = 15.8 \times 10^{-6}\ \text{cm}^{-1}$ at 546 nm was used for calibration of the instrument with benzene[14]. Before l.s. measurements, dilute solutions and solvent were vigorously shaken for 1 h with 5:1 $CHCl_3$–isopentyl

TABLE I

CHARACTERISTICS OF INVESTIGATED SAMPLES

Sample	$[\eta]$ *(dL/g)*	M_w[a]
002	11.7	562,000
025	15.2	797,000
034	14.9	776,000
040	19.1	1,080,000
068	4.3	148,000
070	3.1	95,000

[a] Determined from $[\eta]_{0.15M\ NaCl}$ using Eq. *1*.

alcohol[15]. The aqueous phase was separated and centrifuged for 2.5 h at 13,000 r.p.m. in a Beckmann L8-70 centrifuge.

This procedure is not possible for highly viscous systems since chloroform bubbles were observed in the aqueous phase after centrifugation. Satisfactory results were obtained by centrifugation for 2.5 h at 17,000 r.p.m. The data obtained by the two procedures, using relatively dilute solutions of HA of high molecular weight, were almost identical.

L.s. data were treated as previously reported[16]. The dn/dc value was 0.166 $mL.g^{-1}$, in agreement with literature data[17] for HA.

L.s. and dn/dc measurements before and after extensive dialysis of the polymer solution against the solvent showed negligible differences. It is therefore concluded that the effect of non-equilibration chemical potential does not appreciably modify our results.

Intrinsic viscosity. — Intrinsic viscosity data ($[\eta]$) were obtained by means of Huggins and Kraemer equations[18,19]. Viscosities were determined using a multigradient suspended-level Ubbelohde viscometer at 25.0 ±0.1°, having solvent flow times of ~150 s. Relative viscosities ranged between 1.1 and 1.6. Dilutions were made directly in the viscometer. No shear effect was observed up to molecular weights of 500,000. Higher polymer viscosities were extrapolated[20] to zero flow-velocity gradient.

RESULTS

Dilute solutions. — L.s. measurements of dilute solutions of HA enable a more complete physicochemical characterisation. The diagrams relative to samples 068, 002, and 025 are reported as an example in Fig. 1. The average molecular weights (M_w), second virial coefficients (B), and radii of gyration (R_G) are given in Table II. Comparison with the data in Table I indicates a good agreement between molecular weight data obtained from l.s. and viscosity.

Concentrated solutions. — *(a) Angular dependence of l.s.* The angular

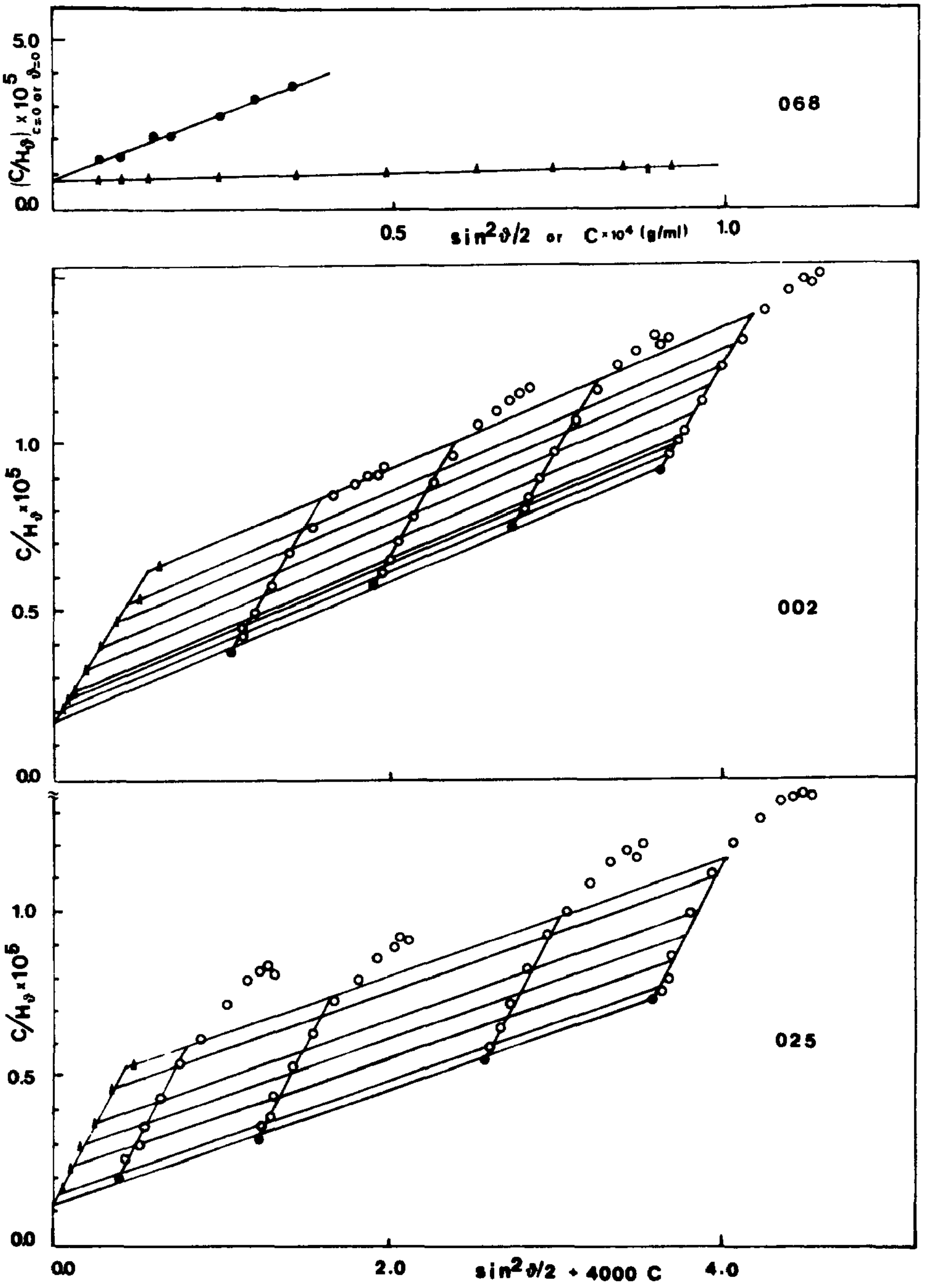

Fig. 1. Zimm plots of samples 068, 002, and 025 (H_ϑ is the instrumental response of scattered light).

TABLE II

DILUTE SOLUTION PARAMETERS OF HA SAMPLES

Sample	M_w[a]	B[a] (*mol.mL.g*$^{-2}$)	R_G (*Å*)[a]
002	605,000	4.0×10^{-3}	1140
025	796,000	3.2×10^{-3}	1270
034	760,000	2.2×10^{-3}	1165
068	135,000	3.8×10^{-3}	470

[a] Estimated error ±10%.

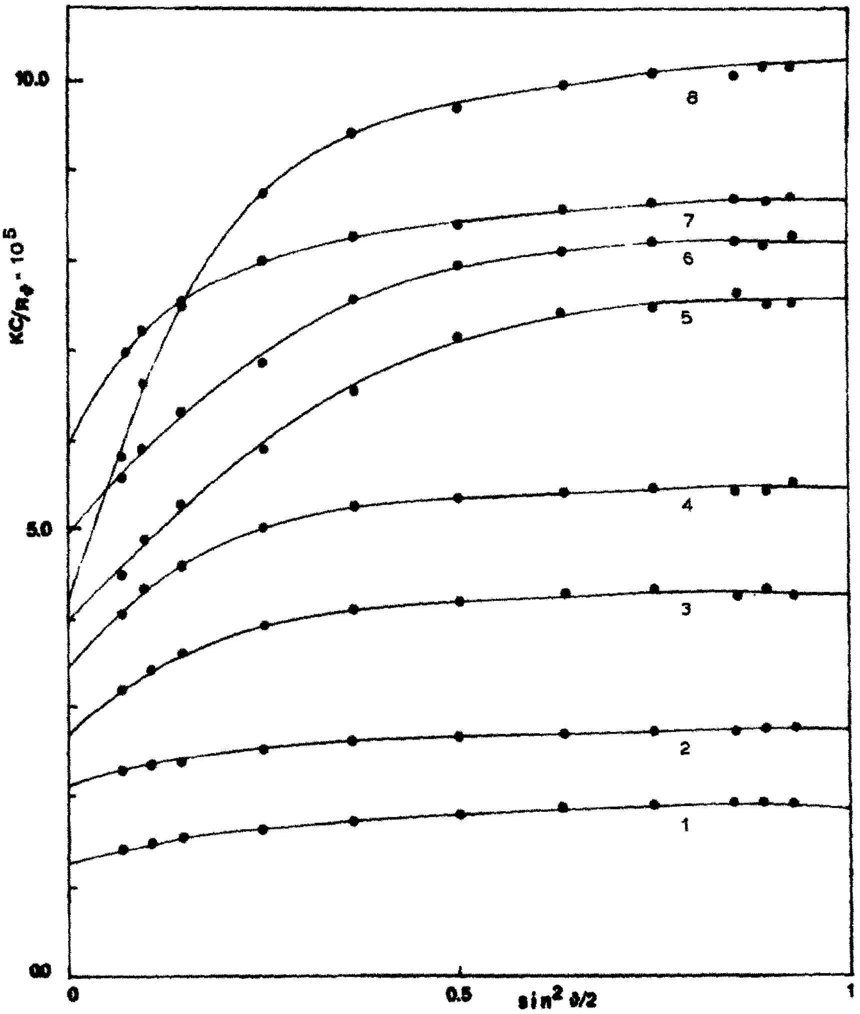

Fig. 2. Angular dependence of Kc/R_θ for sample 068. Concentration (g/mL × 10^3): 1, 1.0; 2, 1.5; 3, 3.0; 4, 4.0; 5, 5.0; 6, 6.5; 7, 7.8; 8, 9.7.

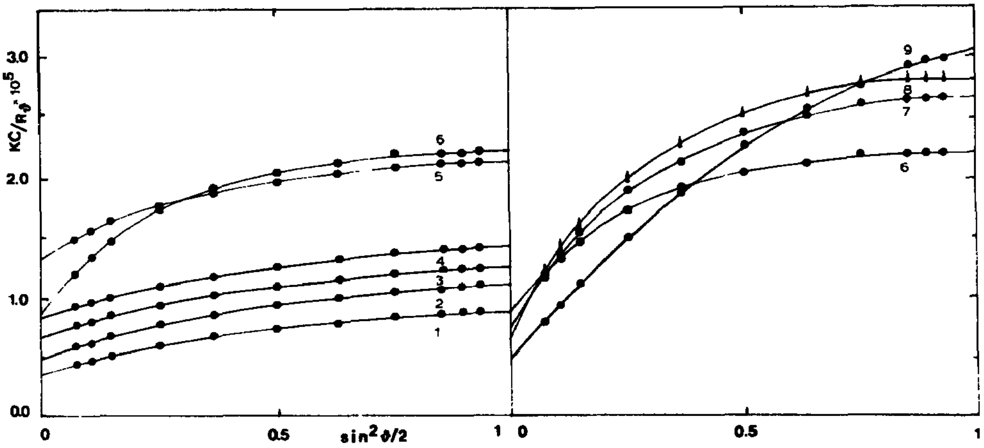

Fig. 3. Angular dependence of Kc/R_ϑ for sample 002. Concentration (g/mL × 10^3): 1, 0.3; 2, 0.5; 3, 0.7; 4, 0.9; 5, 1.7; 6, 2.0; 7, 3.0; 8, 5.0; 9, 6.0.

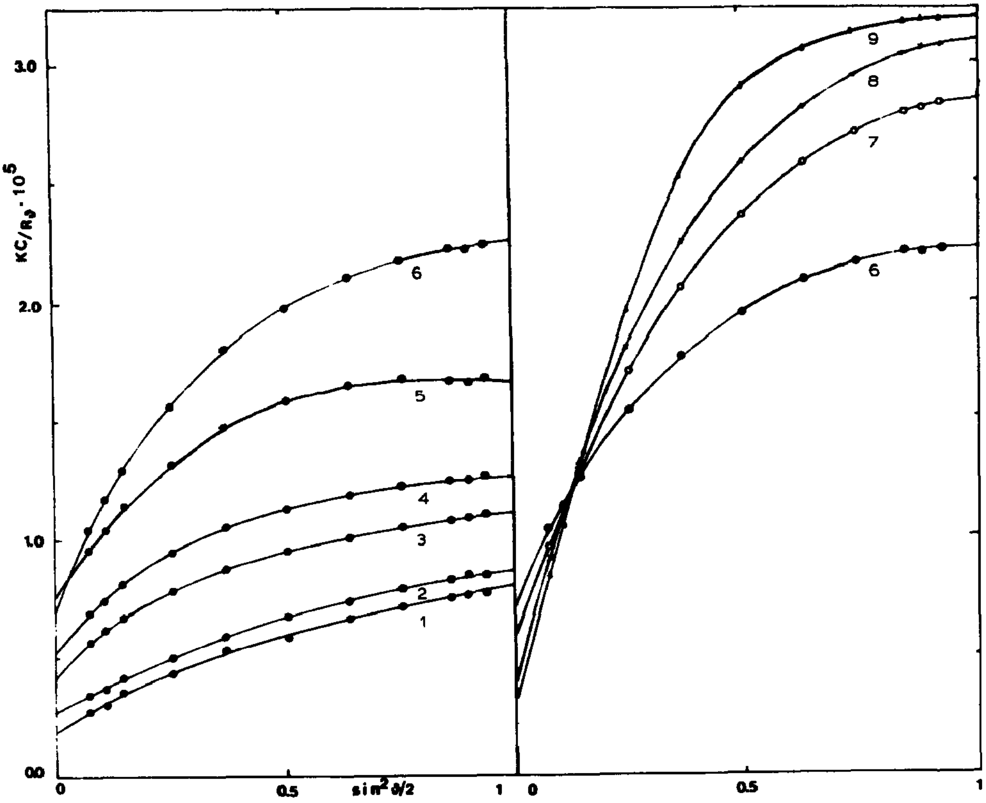

Fig. 4. Angular dependence of Kc/R_ϑ for sample 025. Concentration (g/mL × 10^3): 1, 0.1; 2, 0.3; 3, 0.6; 4, 0.9; 5, 1.5; 6, 2.5; 7, 3.5; 8, 4.5; 9, 6.0.

TABLE III

EFFECT OF CONCENTRATION ON Kc/R_0 FOR SAMPLES 068, 002, AND 025

068		*002*		*025*	
$c \times 10^3$ (g/mL)	*$(Kc/R_0) \times 10^6$*	*$c \times 10^3$ (g/mL)*	*$(Kc/R_0) \times 10^6$*	*$c \times 10^3$ (g/mL)*	*$(Kc/R_0) \times 10^6$*
0.6	9.5	0.3	3.0	0.1	1.7
1.0	11.6	0.5	4.7	0.3	3.0
1.5	19.7	0.7	6.4	0.6	4.4
1.8	15.5	0.9	8.0	0.9	5.6
2.5	17.9	1.0	6.7	1.5	7.4
3.0	24.0	1.7	13.0	2.5	6.0
3.6	27.1	2.0	9.3	3.5	4.8
4.0	34.3	3.0	7.6	4.5	3.5
5.0	39.2	4.0	5.6	6.0	2.9
6.5	48.1	5.0	5.7		
7.8	57.4	6.0	4.6		
9.7	47.0	7.0	4.2		

dependence of Kc/R_ϑ[†] for samples 068 (0.65–9.7×10^{-3} g/mL), 002 (0.3–7×10^{-3} g/mL), and 025 (0.1–6×10^{-3} g/mL) are shown in Figs. 2–4. The limiting values obtained by graphic extrapolation are reported in Table III. Qualitatively parallel and linear plots were found for all three samples at low concentration. The corresponding Kc/R_0[†] values increased on increasing the concentration, according to positive B values generally found in good solvents. Above a certain concentration, however, curved and non-parallel patterns were observed, especially at angles <90°. The Kc/R_0 intercepts decreased slightly and then dramatically on increasing concentration, eventually reaching values comparable to those found for dilute solutions.

(*b*) *Concentration dependence of l.s.* L.s. diagrams of Kc/R_0 *vs.* c are reported in Fig. 5. The intercept with the ordinate axis is $1/M_w$. The initial slope is a function of the second virial coefficient B, whereas the following part is related to the concentration dependence of the Rayleigh factor in moderately concentrated solutions[21]. In our systems, except for the HA at lowest M_w, a maximum value was found (corresponding to c_{max}), which was shifted to lower c on increasing M_w. At high c, Kc/R_0 tended to constant values, irrespective of M_w. The ranges of c_{max} are reported in Table IV together with the critical concentration (c^*) for comparison; c^* is defined as the concentration at which the hydrodynamic volume of polymer chains equals the solution volume and can be obtained both from l.s. and viscosity data ($c^*_{R_G}$, $c^*_{[\eta]}$). Considering a monodisperse polymer in a random coil conformation, the volume V_n, occupied by a single chain, is given by equation 2[22].

[†] K is an optical constant, c the concentration (w/v), R and R_0 are the Rayleigh ratios, at angles ϑ and 0, respectively.

TABLE IV[a]

VALUES OF c_{max} AND c^* FOR SAMPLES 068, 002, AND 025

Sample	*$c_{max} \times 10^3$ (g/mL)*	*$c^*_{[\eta]} \times 10^3$ (g/mL)*	*$c^*_{R_G} \times 10^3$ (g/mL)*	*$c^* \times 10^3$ (g/mL)*
068	8	2.45	1.70	2.2
002	1.5–2.5	0.90	0.50	0.7
025	1–2	0.69	0.50	0.6

[a] See text for definition of c_{max} and c^*.

$$V_n = 4/3\ \pi\ \xi^3 R_G^3, \tag{2}$$

where ξ is a numerical factor between 0.5–0.7 and R_G is the radius of gyration.

At c^*, the solution volume equals the hydrodynamic volume of the chains ($V = nV_n$, if n is the total number of chains). Thus,

$$c^* = \frac{M}{NV_n} = \frac{3M}{4\pi N\ \xi^3 R_G^3}, \tag{3}$$

where M is the molecular weight, and N is Avogadro's number. Therefore, given R_G and M, c^* can be immediately evaluated. If equation *4*[23] is considered further,

$$[\eta] = \phi\ 6^{3/2}\ R_G^3/M, \tag{4}$$

where ϕ has a constant value of 2.6×10^{23}, it is also possible to correlate c^* and $[\eta]$ as in equation *5*.

$$c^* = \frac{3\phi 6^{3/2}}{4\pi N[\eta]} \simeq \frac{1.5}{[\eta]} \tag{5}$$

Comparing the theoretical and experimental data, equation *5* was slightly modified by Simha and Zakin[24] in the form

$$c^* = 1.08/[\eta]. \tag{6}$$

Considering the shape of the Kc/R_0 *vs.* c plot, by analogy with the other samples, compound 068 will probably also exhibit a c_{max} exceeding 8×10^{-3} g/mL. Unfortunately, this concentration range could not be examined due to the very high viscosity of the solutions.

Table IV shows that the c_{max} values are considerably higher than the c^* values for all compounds.

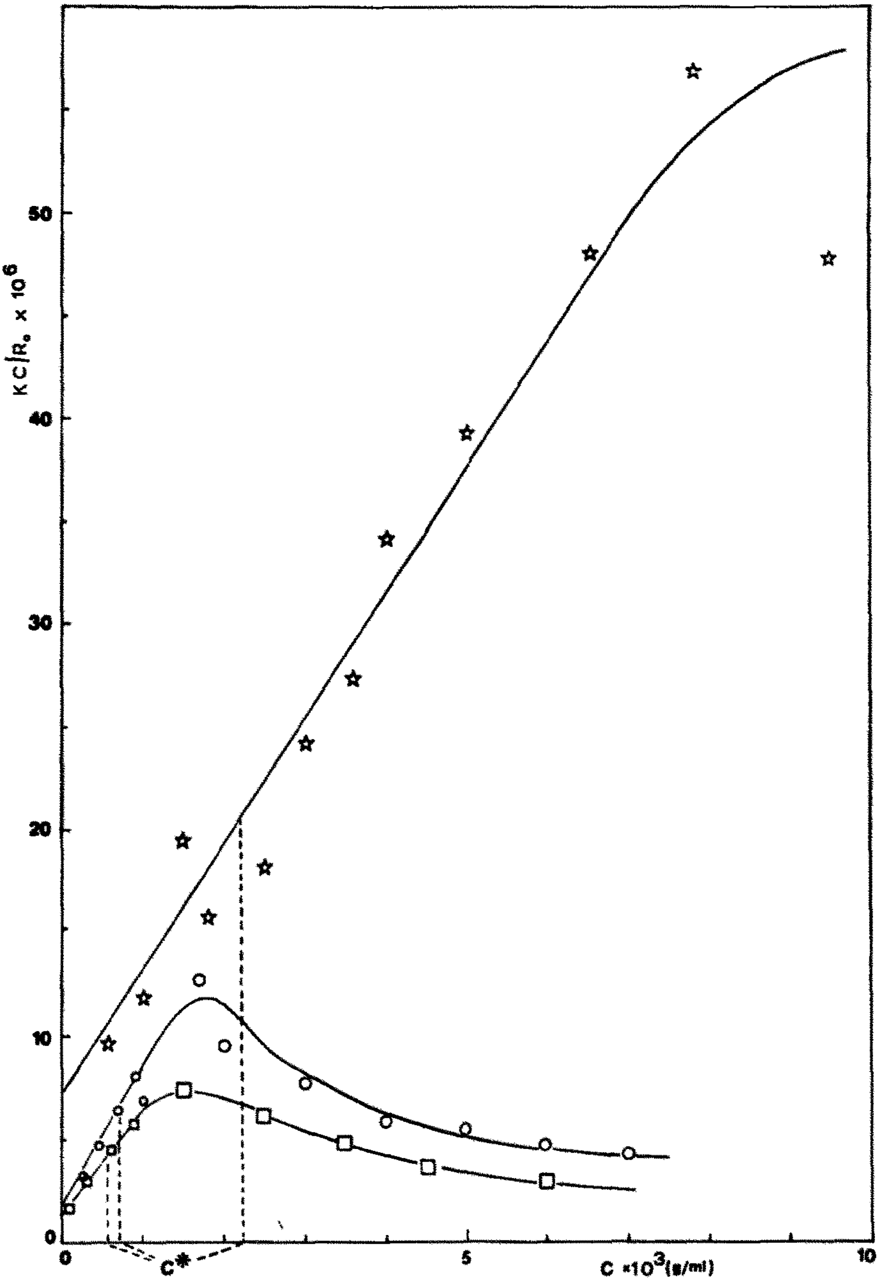

Fig. 5. Concentration dependence of Kc/R_0 for samples 068 (☆), 002 (○), and 025 (□).

Considering a narrow distribution of molecular weight, the term Kc/R_0 is related to osmotic pressure π by equation 7[21].

$$Kc/R_0 = (1/RT)\,(d\pi/dc) \tag{7}$$

Thus, the reduced osmotic pressure $\pi M/cRT$ can be evaluated from l.s. measurements. Logarithmic plots of $\pi M/cRT$ *vs.* c/c^* are reported in Fig. 6. For HA, the reduced osmotic pressure is not a monotonic function of c/c^*, as a drop in this function occurs at c/c^* values of ~3.

(*c*) *Centrifugation effects.* L.s. measurements on polysaccharide solutions may be dramatically affected by the presence of polymeric microgel or aggregates[25]. In order to monitor the onset of this phenomenon, both the time and speed of centrifugation were increased, so as to allow sedimentation of polymer particles with very high molecular weight. Experiments up to 25,000*g* (18,000 r.p.m.) for 2–4 h did not show any appreciable modification of the l.s. response. Under more drastic conditions (30,000*g*, 19,500 r.p.m. for 8 h), dilute solutions still gave the same results, whereas the angular dependence of c/H_ϑ, proportional to Kc/R_ϑ, appears to be sensibly affected at high c values. The behaviour of sample 002 is reported as an example in Fig. 7.

Quantitative determinations show that the polymer concentration remained unchanged throughout the centrifugation experiments. Thus, sedimentation of negligible amounts of HA occurred under the conditions described above.

(*d*) *Ageing effects.* In order to evaluate the effects of prolonged storage of concentrated HA solutions, sample 034, at a concentration of 12×10^{-3} g/mL, was sealed under nitrogen and stored at 17–24° for 24 months.

A comparison between the results obtained immediately or two years after solubilisation is presented in Fig. 8. The drop in c/H_0[†] occurred for the aged solution at remarkably lower concentration values, whereas the curves referring to $c<c^*$ were practically superimposable. Thus, the values of M_w and B were not affected by a long storage. Prolonged centrifugation (30,000*g*, 8 h) led to an increase in c/H_0 values to the levels found for the freshly prepared solution.

DISCUSSION

Very little has been reported in the literature on l.s. studies of concentrated solutions of polysaccharides. In the field of uncharged polymers Noda *et al.*[21] recently examined poly-α-methylstyrene in toluene, a good solvent, with M_w values between 200,000 and 7,000,000 and in the concentration range 0.3–19 × 10^{-3} g/mL. Kc/R_ϑ *vs.* $\sin^2 \vartheta/2$ plots were regular and parallel. The Kc/R_0 value, easily extrapolated to $\vartheta = 0$, always increased on increasing concentration. At high c, the

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[†] H_0 is the limiting value of H_ϑ at $\vartheta = 0$.

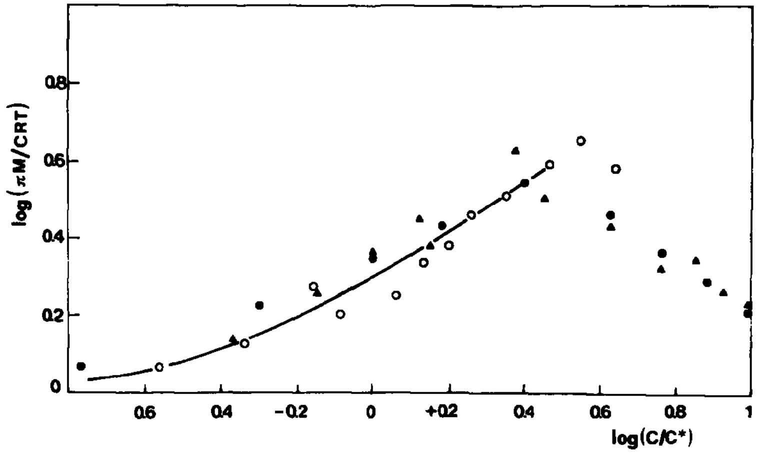

Fig. 6. Logarithmic plots of $\pi M/cRT$ *vs.* c/c^* of samples 068 (○), 002 (▲), and 025 (●).

diffusion values became independent on M_w. Other investigations carried out by Dautzenberg[26-28], using mainly polystyrene but also cellulose nitrate and carbanylate, showed qualitatively similar results. At high concentration, however, curved Kc/R_ϑ diagrams were observed at small angles, especially for the polysaccharide derivatives. In any event, the Kc/R_0 ratio and the reduced osmotic pressure increased monotonically with c.

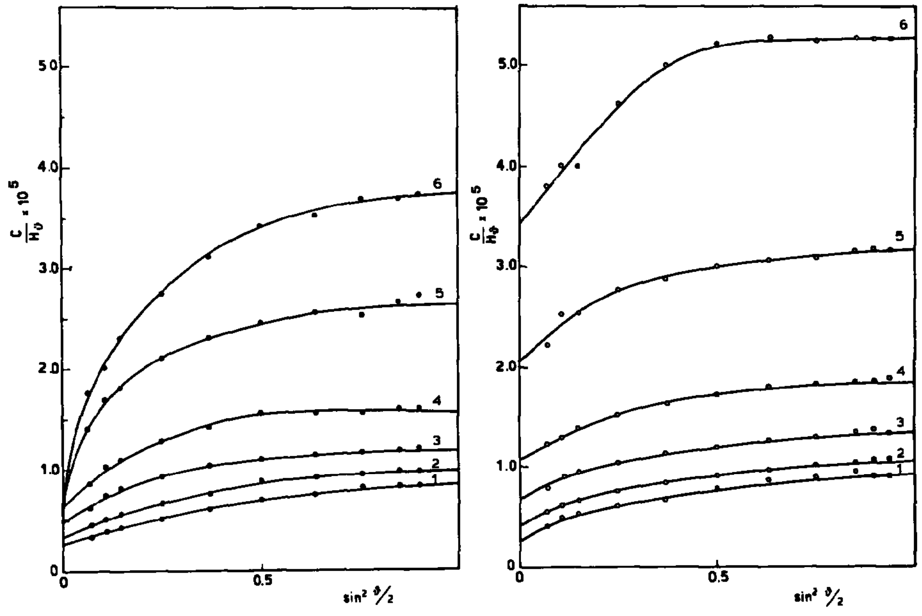

Fig. 7. Angular dependence of c/H_ϑ for sample 002 after centrifugation: (●) 2.5 h at 17,000 r.p.m., (○) 8 h at 19,500 r.p.m. Concentration (g/mL × 10^3): 1, 0.16; 2, 0.50; 3, 0.66; 4, 1.16; 5, 2.50; 6, 4.50.

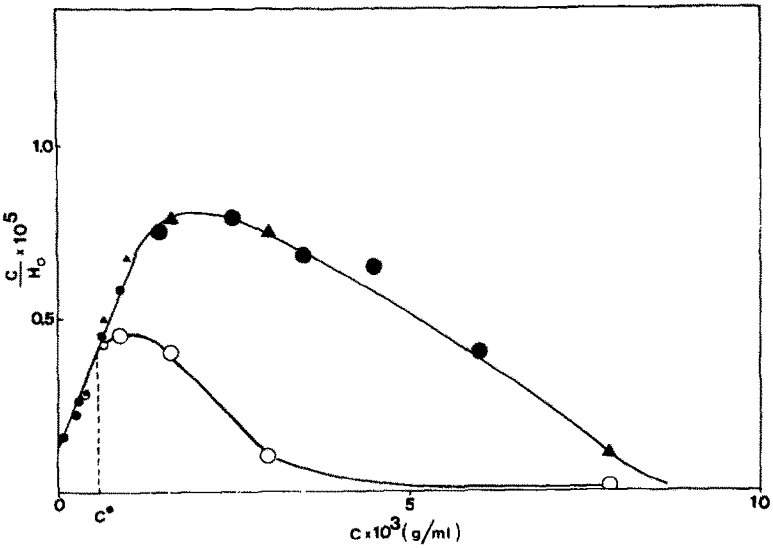

Fig. 8. Concentration dependence of c/H_0 for sample 034: freshly prepared (●) an aged (○) solutions after centrifugation for 2.5 h at 17,000 r.p.m., and an aged solution (▲) after centrifugation for 8 h at 19,500 r.p.m.

For HA, a comparable trend was found up to c_{max}. In agreement with the cited literature, it is concluded that, for our system, entanglement of polymer chains occurred, rather than reduction in coil dimensions, on increasing c. Under these conditions, each chain interacts with solvent molecules belonging to the hydrodynamic volume of other chains. This phenomenon is probably related to the characteristics of a linear semi-rigid polymer in a good solvent as presented by HA in neutral aqueous solution, and leads finally to a polymer network independent of the molecular weight of single units.

At concentrations exceeding c_{max}, a drop in Kc/R_0 and reduced osmotic pressure occurred as shown in Figs. 5 and 6. To explain this aspect, which has not been observed in other polymer systems thus far investigated, the onset of concentration-dependent, specific, chain–chain association phenomena is proposed, which intensify entanglement coupling (hyperentanglement). L.s. is especially suited to study multimerisation processes. Apparent parameters such as $(M_w)_{app}$ or $(R_G)_{app}$ can be calculated from experimental data at finite concentration[29]:

$$Kc/R_0 = (1/M_w)_{app} = 1/M_w + 2Bc + \cdots\cdot$$

On the reasonable assumption of a constant dn/dc value, the plot Kc/R_0 *vs.* c is a direct measure of the dependence of $(1/M_w)_{app}$ on c. Before c_{max} is reached, $(M_w)_{app}$ decreases, due to non-ideality deviations, and, after c_{max}, $(M_w)_{app}$ increases dramatically. Thus, irrespective of high B values, HA chains associate at moderately high concentrations. The process is a function of molecular weight as c_{max} increases on decreasing M_w (see Fig. 5).

Substantial curvature of the scattering envelope at low angles and the fact that the aggregates sediment under appropriate conditions without appreciably modifying the content of polymer in solution indicate that the self-association process causes formation of hyperentangled particles having quite large dimensions, but involves only a very small fraction of dissolved chains. The known tendency of polysaccharides to associate and the comparable angular dependence of l.s. found for systems in which microgels are present support our interpretation[30,31]. For HA, the onset of hyperentanglement takes place well after reaching c^*, as indicated from the data in Table IV.

Data reproducibility, quite satisfactory at $c<c^*$ under all circumstances, appeared to be more critical at $c>c^*$. Small changes in the preparation of the solutions (concentration of mother solution, shaking and dilution conditions) and in centrifugation may introduce relatively important modifications in the angular dependence of scattering and in the extrapolated value of Kc/R_0. Non-homogeneity of the polymer network, along with non-equilibrium phenomena[26–28], could account for the observed limitations in reproducibility. Measurements on aged solutions clearly confirmed the importance of kinetic factors in the hyperentanglement process. The phenomenon was much more pronounced after prolonged storage of the solution. Additional evidence was obtained after sedimentation of the hyperentangled polymer chains by centrifugation at 30,000*g*. As expected in the absence of high aggregates, at $c>c_{max}$ the Kc/R_0 value of the centrifuged material was initially well above the original one. On storage of the solution, this value began to drop, indicating reaggregation of HA. Also, this finding ruled out the possibility of incomplete solubilisation of the polymer in the mother solution as the cause of low Kc/R_0 values.

In a viscometric study of the transition from dilute to concentrated solution regimens for several polysaccharides, Morris and co-workers[32] could not find the specific interactions described here for HA at neutral pH. This discrepancy could be attributed to different polymer samples, with non-reproducibility of the hyperentanglement phenomena. As indicated by the analytical and c.d. data, the samples used in the present work were of higher purity and ordering in comparison with the literature materials. Accordingly, self-aggregation is more likely to occur. This fact was confirmed by a less pronounced curvature in the angular dependence of c/H_θ for HA samples with broader molecular weight distribution (mixtures of samples 040 and 068) or containing higher levels of impurities (sample 070) (Fig. 9).

The different experimental techniques used in the literature and here could also account for the discrepancies in the results. Indeed, whereas l.s. measurements are greatly affected by the presence of almost negligible amounts of very high M_w polymer, this is not so for the flow time in a capillary. Only when massive amounts of polysaccharide are involved in self-aggregation can the process be revealed by viscosity measurements, as confirmed by the hydrodynamic data reported[32] for HA and guar at low pH.

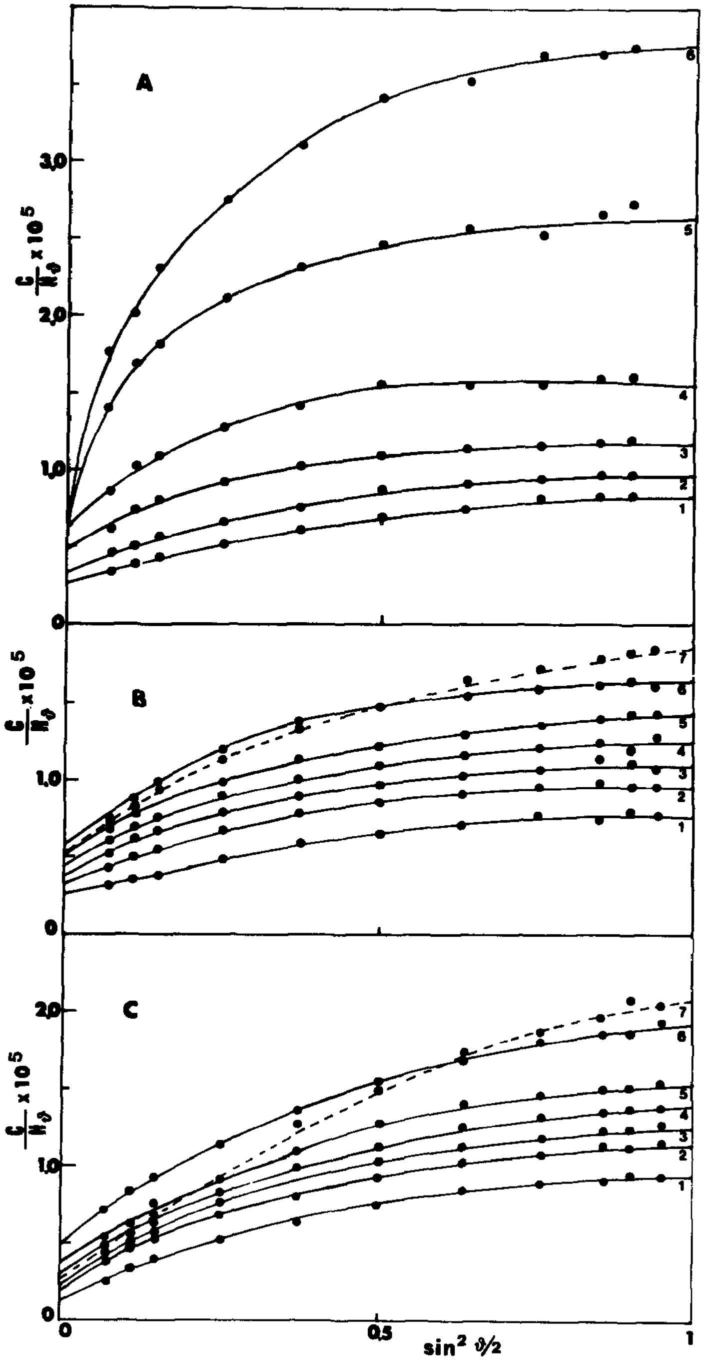

Fig. 9. Angular dependence of c/H_ϑ for *A*, sample 002; *B*, 1:1 mixture of samples 040 and 068; and *C*, 1:1 mixture of samples 040 and 070. Concentration (g/mL × 10^3): *A*: 1, 0.16; 2, 0.50; 3, 0.66; 4, 1.16; 5, 2.50; 6, 4.50; *B*: 1, 0.19; 2, 0.44; 3, 0.65; 4, 0.87; 5, 1.15; 6, 2.09; 7, 4.88; *C*: 1, 0.23; 2, 0.46; 3, 0.69; 4, 0.96; 5, 1.39; 6, 2.77; 7, 6.24. Dashed lines for concentration 7 of panels *B* and *C* are drawn for clarity only.

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