Structural Study of Fractionated Hydroxyethyl-Amylopectin in Aqueous Solution

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SYNOPSIS

Fractionated hydroxyethyl starch was well characterized by coupled LALLS-SEC (low-angle laser light scattering and steric exclusion chromatography), QELLS (quasi-elastic laser light scattering), MALLS (multiangle laser light scattering), and viscosimetry. The scaling laws of the molecular weight dependences of the following dimensions are found to exhibit low exponents consistent with a highly grafted structure:

Intrinsic viscosity	$[\eta_{37} = 0.0023 M^{0.33}$ (dl g^{-1})
Hydrodynamic radius of gyration	$R_H = 0.110 M^{0.37}$ (nm)
Static radius of gyration	$R_z = 0.022 M^{0.48}$ (nm)

Additional X-ray synchrotonic scattering (SAXS) studies permit us to give a preliminary description of the internal structure of the macromolecules.

INTRODUCTION

The various medical uses of hydroxyethylamylopectine HAP particularly as plasma volume expanders have caused considerable interest in its molecular characterization. First, a series of studies on HES fractions were performed using conventional methods such as elastic light scattering, viscosimetry, and osmometry.[1-3] More recently, the development of the low-angle light scattering technique (LALS) coupled with size exclusion chromatography (SEC)[4] has permitted a fast and accurate determination of molecular weight distribution of these polymers.[5,6] Nevertheless, there is a lack of available studies about their conformational properties and structure in aqueous solution. In fact, what is really important to know is the rheological behavior of the solutions, which is related to the hydrodynamic volume and then to the branching and conformation of the macromolecules. It is well known that a description of the polymer branching may be obtained from the comparison of their molecular dimensions with those of their linear homologous.[7,8] Such a comparison has never been made for HAP. Yu et al. have exploited the possibilities of the SEC/LALS technique to determine the Mark Houwink law of amylopectine and draw some conclusions on the branching,[9] but these authors must assume the validity of the universal calibration of SEC.

The present work aims at presenting and discussing results of a full investigation of the conformational properties of well-characterized fractions of HAP. We particularly use small-angle X-rays scattering (SAXS) technique able to give direct informations about polymer branching and local structure as already made by Garg et al.[10] for dextran fractions.

EXPERIMENTAL

Materials

Depolymerized hydroxyethyl starches were obtained from Roquette Frères S. A.: HAP1 and HAP2 with, respectively, a molar substitution of 0.5 0.7 (MS).

HAP1 fractionation was performed on a preparative low-pressure size exclusion chromatography using ACA 22 gel from ICS. Two series of fractions—HAP1 F_1–F_6 and HAP1 F'_2–F'_7—were studied.

Methods

Analytical Size Exclusion Chromatography (SEC)

The SEC device includes a model LCII Shimadzu pump, a Waters injector, and two detectors: a differential refractometer (Waters R410), as concentration detector and a low-angle light scattering apparatus (KMX Chromatix) as mass detector. We used three columns from Shodex TSK SW4000 SW3000 Pw60.

Our data analysis does not take into account the axial diffusion effect as described by Tung.[11] Hamielec and Meyer[12] have shown that the number-average molecular weight (M_n) and consequently the polydispersity index (PI) may be affected by such an effect while the weight average (M_w) is simply obtained by the integration of the KMX scattering peak. One can, however, assume that the resolution power of these columns is high enough to minimize such errors.

Some values given in Table I were obtained without LALS detector, and in these cases we used either a simple molecular weight calibration or a universal calibration, both obtained with polysaccharide standards from Toyo Soda.

The solutions prepared for the SEC were heated to 70°C for 12 h before using. The volume of the injector loop was 200 μL. The aqueous mobile phase was 0.1 Na_2SO_4 (pH 6).

Elastic Laser Light Scattering (LS)

Elastic light scattering measurements were performed at 37 ± 0.1° on two home-built automatized photogoniodiffusiometers[13] equipped with laser sources, $\lambda_1 = 632$ nm and $\lambda_2 = 488$ nm. The angular dependence was measured within the scattering angle range 30° and 150°.

Polymer was dissolved $(2.10^{-4}$–2.10^{-3} g cm$^{-3})$ in buffered solvents (pH 7 phosphate buffer) containing 400 ppm of sodium azide prepared with a prior three times distilled water. The complete solubilization is discussed later on. They were made clear by centrifugation at 15,000 rpm for about 4 h.

The data were analyzed by computer using classical Zimm procedure and a linear polynomial fit to the classical equation:

$$\frac{Kc}{I} = \frac{1}{M_w P(\theta)} + 2A_2 c \qquad (1)$$

where

$$P(\theta) = 1 + \frac{q^2}{3} R_G^2 + Bq^4 \qquad (2)$$

I = scattered intensity excess of the solution and solvent intensity

c = polymer concentration in g cm^{-3}

M_w = weight-average molecular weight of the solute

q = scattering vector $q = [4\pi n \sin(\theta/2)]/\lambda$

A_2 = second virial coefficient

R_G^2 = Z-average radius of gyration

θ = scattering angle

K = optical constant proportional to the square refractive index increment $(dn/dc)^2$

Table 1

Sample	M_w (LS)	M_w (SEC/LALS)	PI	M_n (osmometry)
HAP1	200,000	194,000		—
HAP2	556,000	521,000	6.2	—
HAP1 F_1	930,000	—	1.3[a]	
F_2	428,000	526,000	1.23	
F_3	304,000	280,000	1.47	
F_4	209,000	183,000	1.30	
F_5	103,000	89,000	1.2	102,000
F_6	66,000	—	1.63[a]	48,600
HAP1 F'_1	1,630,000		—	
F'_2	860,000		—	
F'_3	390,000		1.43[b]	
F'_4	263,000		1.32[b]	
F'_5	157,000		1.48[b]	
F'_6	64,000		1.93[b]	

[a] SEC with universal calibration (polysaccharide standards) pH 7.
[b] SEC with universal calibration ph 6.

The data analysis is often made by neglecting the third term of $P(\theta)$, which is justified when the angular dependences are linear.

Quasi-Elastic Laser Light Scattering (QELS)

The translational diffusional coefficient D were measured by QELS experiment using a home-built device[14] operating with a coherent laser light source from Spectra Physics (488 nm). The scattering amplitude, which is time dependent, may be described by the time autocorrelation function $G(\tau)$ measured by photon counting. Further data analysis provide information on the dynamical properties of the chains, the z-average diffusion coefficient D_Z and the hydrodynamical radius of giration R_H. By assuming quasi-spherical particles we obtain the following usual exponential function:

$$G(\tau) = K_1 \exp(\tau/\tau_c) + K_2 \qquad (3)$$

where K_1 and K_2 are constant and τ_c is related to D by

$$\tau_c = (2Dq^2)^{-1} \qquad (4)$$

and R_H is given by the well-known Einstein relation:

$$D = \frac{kT}{6\pi\eta R_H} \qquad (5)$$

where η is the solvent viscosity, k the Boltzmann constant, and T the temperature in Kelvin.

The experiments were carried out on the solutions prepared for elastic light scattering. Preliminary investigations have shown that D does not depend on concentration and scattering angle. So we have used only one concentration at one scattering angle (between 45° and 75° according to the scattering intensity due to the remaining dusts).

Refractometry

The dn/dc values have been determined with two different devices. The first one is a classical Brice–Phoenix apparatus operating with red light ($\lambda = 632$ nm) at polymer concentrations $> 5.10^{-3}$ g cm^{-3}. The second one is a home-built refractometer based on the same principle with a higher sensitivity photoelectric detection. This second apparatus operates at a lower concentration and allows dn/dc measurements on solutions prepared for light scattering experiments ($10^{-4} < c < 10^{-3}$ g cm^{-3}).

In a second set of experiments, we have calculated the dn/dc values from the integration of the peak given by a differential refractometer at the output of the SEC columns working with a $0.1N$ Na_2SO_4 water as eluent.

Osmometry

The number-average molecular weight have been determined by a Mechrolab Model 502 device operating in $0.1N$ NaCl at 25°C.

Viscosimetry

An automatic viscosimeter of the Gramain-Libeyre type[15] regulated at ±0.1°C and equipped with a 0.43-mm diameter capillary was used. In this type of viscosimeter the shear rate is around 1000 s^{-1} for dilute aqueous solution. We have checked by using a low shear rheometer from Contraves that the HAP solutions are Newtonian within the investigated range of concentration.

Intrinsic viscosity is calculated from the extrapolation of the reduced viscosity at $c = 0$ according to

$$\eta_{red} = [\eta] + K'[\eta]^2 c \qquad (6)$$

where K' is the Huggins constant.

Small-Angle X-Ray Scattering

SAXS measurement was done in the LURE laboratory (Laboratoire Universitaire du Rayonnement Electromagnétique). The X-ray wavelength was selected with a curved mirror at 1.08 Å. At least 5000 pulses were counted at each of the 256 angles with a linear detector. The wave number q range was between 0.3 and 10^{-2} Å^{-1}.

In the Guinier domain the radius of gyration of the polymer is obtained with the same expression as the one used for small-angle light scattering [relation (1)].

According to theory,[16,17] the scattering curve of an elongated particle follows the relation

$$I = 2\pi I_q/\lambda q \qquad (7)$$

where I_q is the cross-section factor. The radius of gyration of the cross section R_q may be obtained by the slope of $\ln(I^*q)$ as a function of q^2 according to

$$\ln(I^*q) = \ln(I^*q)_0 - R_q^2 q^2/2 \qquad (8)$$

The persistence length a^* may be calculated according to the theory of Kratky and Porod[18] using the relation

$$a^* = 2.3/q^* \qquad (9)$$

where q^* is the transition point between the $1/q^2$ and $1/q$ behavior of I.

EXPERIMENTAL

Preliminary remark: We must point out that great care has to be given to the solution make up. Indeed in a first series of experiments, we have homogenized the HAP solutions by simple gentle stirring for at least 24 h at room temperature. This method does not always lead to well-dispersed solutions and the presence of aggregates is shown by the high Huggins constant ($K' > 1$) in the viscosity measurements and a downward curvature of the Kc/I plot versus q^2. Under such conditions, the values of intrinsic viscosity [η], R_g and M_w at a given temperature and pH and their variations with these parameters are unreproducible. In fact, such effects seem to be more or less evident according to the sample and its degree of purification. For all the results discussed in this study, the solutions were heated at 70°C for 12 h and stirred at room temperature for some hours.

Refraction Index Increment

The value of dn/dc may play an important role in the determination of M_w by light scattering. The dn/dc of a given polymer is expected to vary with refractive index of the solvent, the wavelength, and

temperature.[19] The Greenwood et al.[20] review of dn/dc values for various polysaccharide systems in aqueous solution shows that the variation with λ, salinity, and temperature are very low and may be negligible. dn/dc may also depend on the purity of the sample and the solution homogeneity. We have measured with both refractometers dn/dc values for HAP1 and HAP2 samples solubilized at room temperatures and at 70°C are, respectively, within the range 0.145–0.155 cm^3 g^{-1} and 0.138–0.144 cm^3 g^{-1}. Even for the well-solubilized solutions, such values are significantly higher than that obtained by Sommermeyer et al.[5] (0.135 cm^3 g^{-1}) and Lederer et al.[6] (0.1325 cm^3 g^{-1}). One must point out that the latter authors have worked on polymers available in physiological saline state that have been deionized through a mixed-bed ion exchange column. With a dialysis purification of sample HAP1 we obtained a dn/dc value of 0.134 cm^3 g^{-1} in much better agreement with literature data. The same value was obtained with the highly purified HAP F' fractions.

Molecular Weight and Polydispersity Index

Figure 1 shows a comparison of the chromatogramms of fractions F_1–F_6 with that of the unfractionnated HAP1. A Zimm plot specimen is represented in Figure 2 for HAP2.

Table I summarizes the different values of HAP
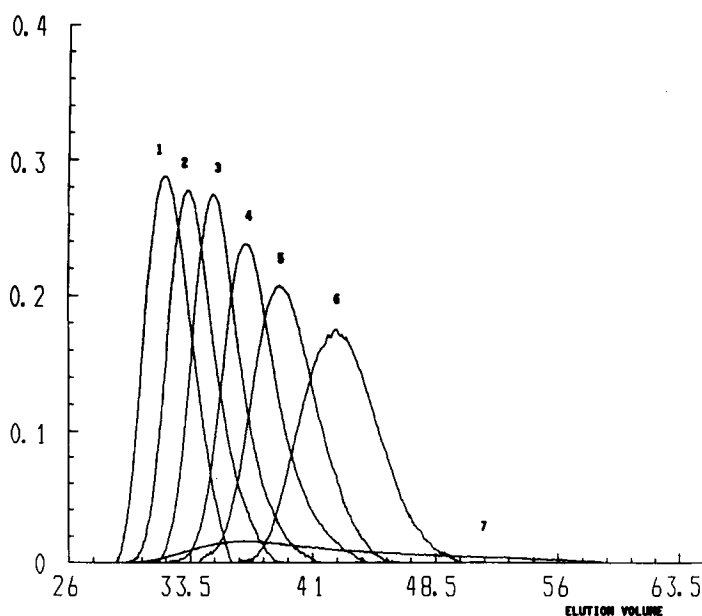
Figure 1 Normalized chromatographs in aqueous solutions of fractions F_1 to F_6 (1 to 6) and of unfractionated HAP1 (7).
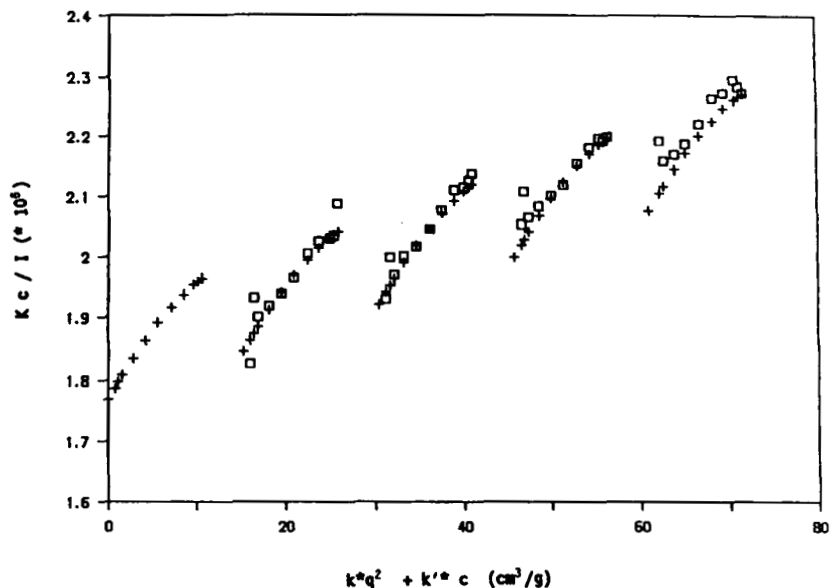
Figure 2 Example of Zimm plot (HAP2).

samples (M_w, M_n, and polydispersity index PI) by LS, osmometry, and SEC/LALS. These characterizations were made at pH 6 or 7 and 37°C. There is a rather good agreement between M_w values obtained by LS and SEC/LALS. The ratio between the two values is between 1.17 and 0.82. An M_w value of 532, 000 was found for HAP2 in DMSO at 37°C.

The osmometric determinations performed for the lower molecular weight fractions are also consistent with the two other ones. The different frac-

tions have a PI between 1.2 and 1.5 for F_1 series and 1.43 and 1.93 for the second series.

Molecular Dimensions

Variation with Molecular Weight

The molecular weight dependencies of the static R_G and hydrodynamic R_h radius of gyration are shown in Figure 3. Figure 4 gives the variation of intrinsic
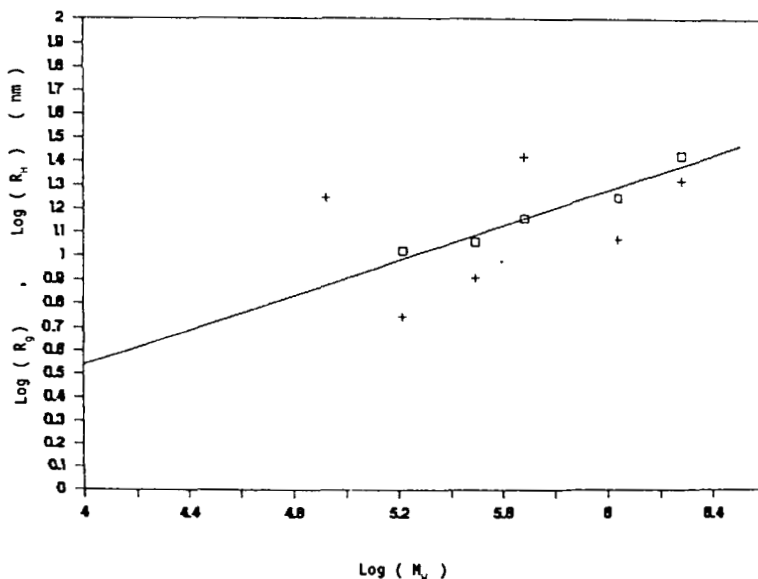

Figure 3 Molecular weight dependences of static (+) and hydrodynamic radii (□) for HAP fractions.
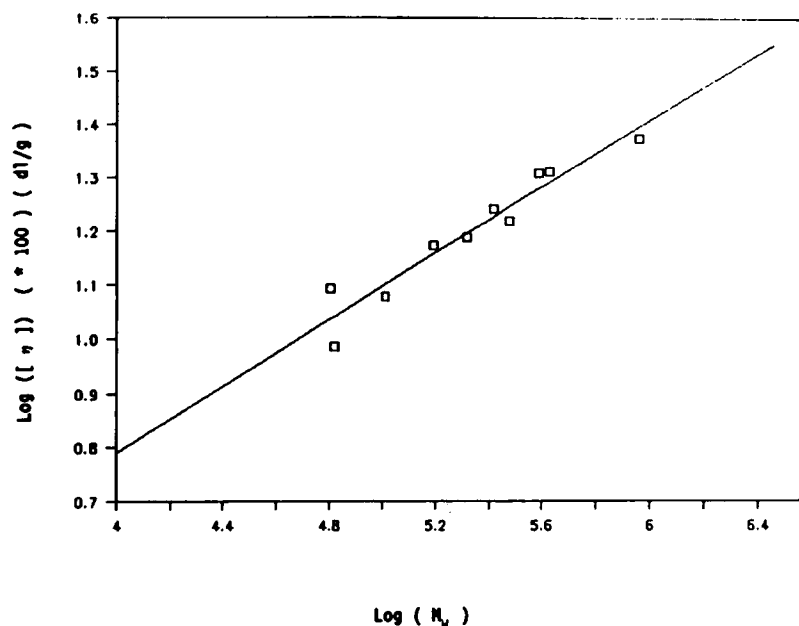
Figure 4   Molecular weight dependence of intrinsic viscosity for HAP fractions.

viscosity $[\eta]$. The results for the two series of fractions are in good agreement and lead to the following scaling laws, at 37°C:

$$R_G = 0.022 M_v^{0.48} \ (\text{nm}) \tag{10}$$

$$R_H = 0.11 M_w^{0.37} \ (\text{nm}) \tag{11}$$

$$[\eta] = 2.32^{-3} M_v^{0.33} \ (\text{dl g}^{-1}) \tag{12}$$

While the two later expressions are well defined, the first one must be considered with some suspicion: In fact R_g varies between 7 and 25 nm, where the accuracy is pretty low in LS. Such measurements just confirm the order of magnitude of the radius of gyration.

The exponents of these scaling laws are very low as expected for branched polymers and found by different authors for hydroxyethyl starch or dextrans. For example, Granath obtains Mark Houwink exponents equal to 0.35[2] and 0.37[21] for HAP and branched dextran fractions, respectively. At our knowledge, no scaling law of R_G and R_H have already been published for HAP. The law obtained by Granath for dextrans[21] is close to that given in relation (10)

$$R_G = 0.057 M_w^{0.44} \ (\text{nm})$$

Variation with pH and Temperature

We have not observed variations of $[\eta]$ with pH. $[\eta]$ was found to slightly decrease by heating while the Huggins constant k' slightly increases (see Fig. 5). Nevertheless k' remains lower than 1, which means that the solutions are rather well dispersed. The following emperical laws were found for HAP2:

$$[\eta] = 26.6 - 0.14T \ (\text{cm}^3 \ \text{g}^{-1})$$

$$K' = 0.55 + 0.0008T$$

SAXS Investigations

X-ray scattering study was performed on a buffered solution (pH 7) on HAP1 (concentration $1.9 \ 10^{-2}$ g cm^{-3}). In Figure 7 we give the variation of I^*q^2 versus q. In this Kratky plot three domains can be distinguished:

(i) In the Guinier domain I, for $qR < 3$, a plateau is observed corresponding to the linear variation of I^{-1} versus q^2 as verified by the Zimm representation of Figure 6. The radius of gyration found from this plot is 8.5 nm. This value is farely consistent with the ones calculated from relations (10) and (11) using the M_w value of HAP1: 7.6 and 10 nm, respectively. One could expect a slightly higher value for HAP1 due to its high polydispersity compared to that of fractions since LS and SAXS given the z average radius of gyration.

(ii) In the Debye domain II, $1/I$ is expected to vary as $q^{1/n}$ if n is the exponent of the molecular weight dependence of R_G. Then if n is lower than 0.5 a new plateau shifted toward higher q values must appear in a representation $I^*q^{1/n}$ versus q. This has
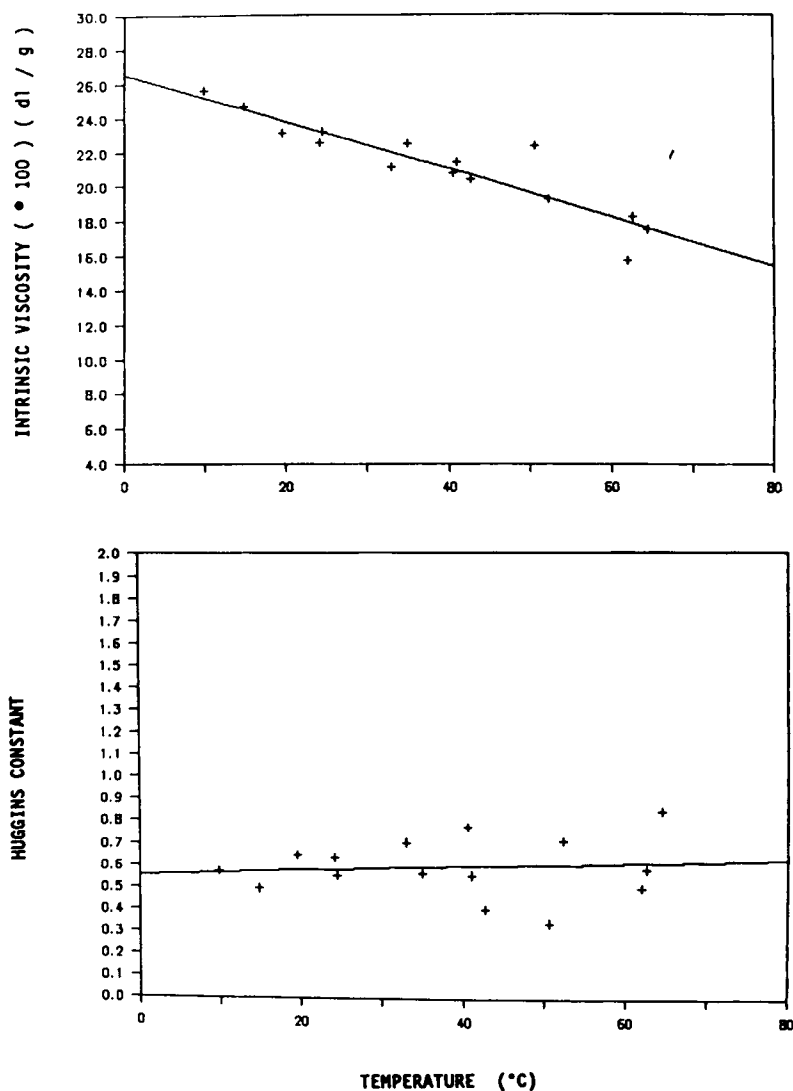
Figure 5   Variation with temperature of the intrinsic viscosity (a) and the Huggins constant (b) for HAP2.

been well observed for $n = 0.4$. Such a result is consistent with the low values of the exponent of the scaling laws (10) to (12) (see Fig. 6).

(iii) The transition point between the $1/q^{1/n}$ and $1/q$ dependence of I is approximate for $q = 0.11$ Å^{-1}, which should correspond to a persistence length of $a = 2.1$ nm. From the linear plot of $\ln(I^*q)$ versus q^2, one obtains a value of the radius of gyration of the cross section $R_Q = 0.47$ nm (see Fig. 8).

These results are roughly consistent with those obtained by Garg et al.[10] for branched dextrans, but a quantitative comparison is difficult since these authors have studied fractions, and they point out a variation of a^* and R_q with molecular weight: 2.03 $< a^* < 2.75$ nm and $0.6 < R_q < 0.2$ nm for 250,000 $> M_w > 11,000$.
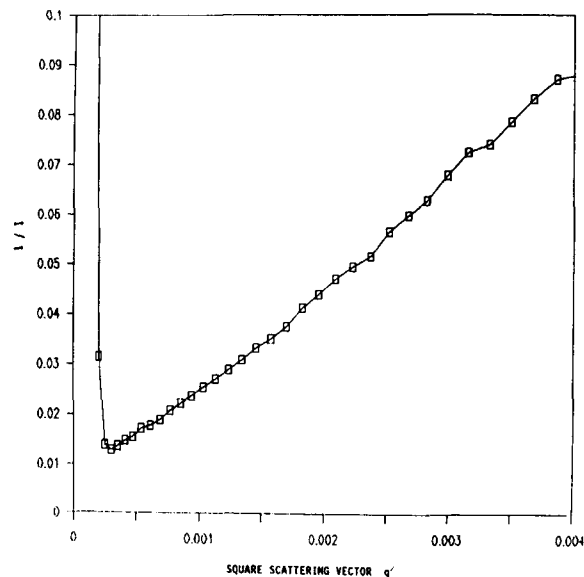
DISCUSSION

Self-Consistency of the Results

The relation between the scaling laws of the dependences of $[\eta]$, R_g, and R_H with M was the object of much debate. From the Kirkwood theory in the limit of free draining, Flory and Fox have proposed the well-known relation:[22]

$$[\eta] = \frac{\Phi_0 6^{3/2} R_G^3}{M} \tag{13}$$

If R_G varies as M^n, the exponent of the Mark Houwink law must be

$$a = 3n - 1 \tag{14}$$

Figure 6 X-ray scattering experiments for HAP1: Kratky plot.

In such an expression no distinction is explicitly made between static and hydrodynamic radii and either one can be introduced.

Despite its wide use, relation (13) is seldom verified even for flexible polymers. The theoretical foundations of eq. (13) have been extensively discussed by Yamakawa[23] and more recently Weill et
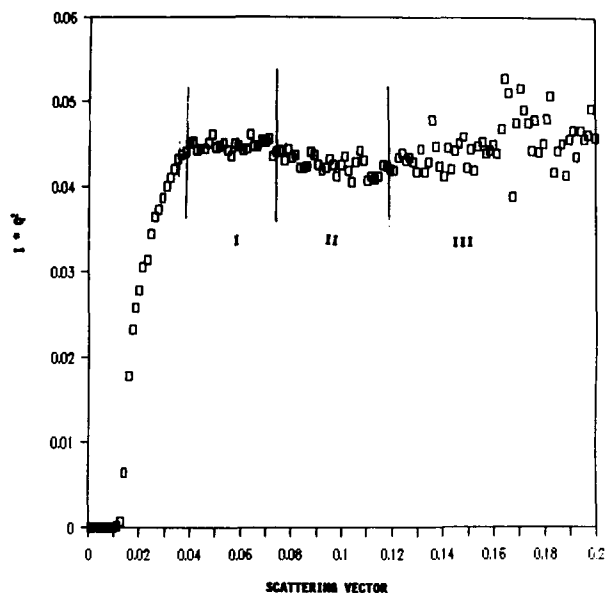
Figure 7 X-ray scattering experiments for HAP1: Zimm representation.
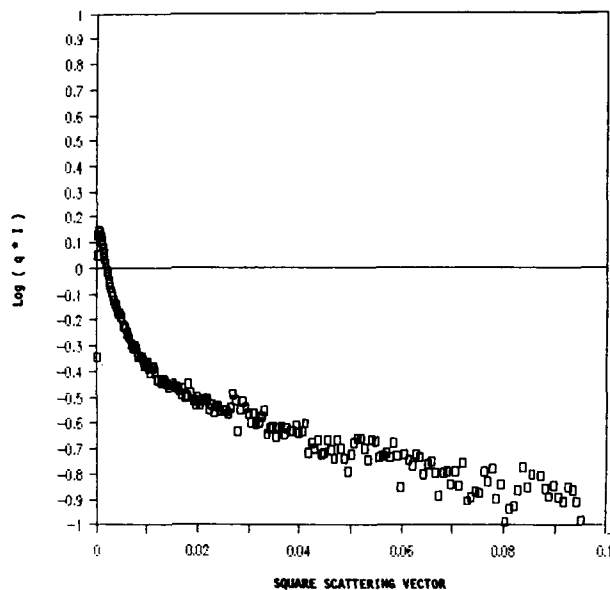
Figure 8 X-ray scattering experiments for HAP1: variation of $\log(qI) = f(q^2)$.

al.[24] have proposed to substitute $R_H^* R_g^2$ for R_G^3 in the Fox–Flory relation from hydrodynamic arguments:

$$[\eta] = \frac{\Phi_0 6^{3/2} R_G^2 R_H}{M} \qquad (16)$$

Such a suggestion was successfully checked on various systems.

In Figure 9 the experimental variation of $[\eta] = f(M_w)$ is compared with the law deduced from relation (11) $[R_H = f(M_w)]$ and relation (13). (We consider that the R_G values are too scattered to lead to a significant comparison). We obtain

$$[\eta] = 4.1 M_w^{0.11} \qquad (17)$$

There is a very high discrepancy and particularly the calculated exponent is much lower than the experimental one.

Now, one must remember that such relations are only applicable to monodisperse samples. Our SEC experiments have shown that PI is not equal for the different fractions and is generally > 1.2. In fact, the measured value of $[\eta]$ corresponds to a so-called viscosimetric average molecular weight (M_v) given by

$$M_v = \left(\frac{\sum c_i M_i^{a+1}}{\sum c_i M_i} \right)^{1/a} \qquad (18)$$
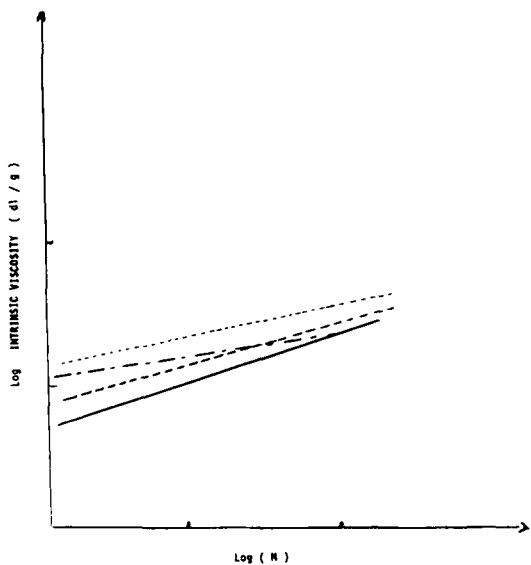
Figure 9 Molecular weight dependence laws of the intrinsic viscosity for HAP fractions, experimental law $[\eta]$ $= f(M_w)$ [eq. (12) (———) corrected experimental law (eq. (23) (---)], calculated law [eq. (17) (— – —), calculated law (eq. (25) (......)].

and the R_G and R_H values correspond to the z average molecular weight M_Z

$$M_Z = \frac{\sum c_i M_i^2}{\sum c_i M_i} \qquad (19)$$

where c_i is the concentration of the molecules of molecular weight M_i.

The chromatograms of Figure 1 are symmetric and can be described by a Gaussian distribution in ln M of the Wesslau type:[25]

$$P(\ln M) = k \exp[-(\tfrac{1}{2}\gamma^2)\ln^2 M/M_0] \qquad (20)$$

where $\gamma^2 = \ln M/M_0$ and M_0 is the molecular weight at the top of the chromatogram. From expressions (18), (19), and (20), it is easy to calculate

$$M_v = M_0 \exp(\alpha\gamma^2/2) \qquad (21)$$

and

$$M_Z = M_0 \exp(3\gamma^2/2) \qquad (22)$$

By successive iterations one obtains a new value for a and finally relations (11) and (12) become

$$[\eta] = 5.52\ 10^{-3} M^{0.28}\ (\mathrm{dl/g}) \qquad (23)$$

$$R_H = 0.091 M^{0.4}\ (\mathrm{nm}) \qquad (24)$$

Figure 9 shows that the Mark Houwink law calculated from relation (24) and (13):

$$[\eta] = 2.34\ 10^{-2} M^{0.2}\ (\mathrm{dl/g}) \qquad (25)$$

is in much better agreement with the new experimental one (23). If now we consider as exact the Weill et al. law,[16] one can try to deduce the molecular weight dependence of R_g starting from the two experimental laws of $[\eta]$ and R_H. The law obtained in this way:

$$R_g = 0.044 M^{0.43}\ (\mathrm{nm}) \qquad (26)$$

fits at least three experimental points. Then by taking into account the polydispersity of the fractions and using a relations of the Flory type improved by Weill et al.,[24] one obtains a satisfactory self-consistency of the different results at least for the exponents of the different laws. In the framework of the Weill et al. theory, the finding of a static exponent lower than the dynamic one is not surprising.

Branching Parameters

According to Zimm and Stockmayer,[7] the branching parameter is defined as the ratio of the average square radius of gyration of branched (R_{gb}^2) and linear (R_{gl}^2) polymer of the same molecular weight:

$$g_M = R_{gb}^2 / R_{gl} \qquad (27)$$

The determination of g_M is usually made from the intrinsic viscosity data

$$([\eta]_b/[\eta]_l) = g_M^e \qquad (28)$$

where the subscripts l and b also refer to linear and branched polymer, respectively.

The value of the exponent e depends on the different theories. In the case of the Fox–Flory theory, it would be $\tfrac{3}{2}$, but for Zimm and Kilb[26] an exponent of 1.0 for free draining behavior and 0.5 for a nonfree draining behavior.

As pointed out by Yu et al.[9] another branching parameter may be defined: the ratio of molecular weight of the linear and branched polymer of same intrinsic viscosity:

$$(M_l/M_b) = g_v \qquad (29)$$

In fact, for hydroxyethyl starch it is difficult to find the laws of radius of gyration and intrinsic viscosity for the homologous linear polymer. To our knowl-

edge no study of hydroxyethylated amylose has already been performed. We will assume that the chemical modifications do not change the local chain conformation and compare the results for HES with those obtained by Burchard[27] for linear amylose, by classical measurements. From the viscosity data, the Mark Houwink laws of amylose are

$$[\eta] = 1.32 \ 10^{-4} M^{0.68} \ (\text{in water}) \tag{30}$$

$$[\eta] = 3.65 \ 10^{-5} M^{0.85} \ (\text{in 0.5 NaOH}) \tag{31}$$

The parameter g_M obtained from the relations (28), (30), (31), and (23) decreases by increasing M according to the following law:

$$g_M = 502 M^{-0.266} \ (\text{in water})$$

The same type of results was also found by Yu et al.[9]; this means that the branching effects disappear for the low molecular weight. A more complete study of such effects by X-ray scattering is in progress.

Characteristics of the Branches

Our preliminary X-ray studies permit us to give approximative values of the persistence length (2.1 nm) and of the radius of gyration of the cross section (0.47 nm), which correspond to average values on all the different branches present in the polymer.

CONCLUSION

In this study, by applying different techniques to modified starchs, we have been able to obtain scaling laws for the molecular weight dependencies of the intrinsic viscosity, the static radius of gyration, and the dynamic radius of gyration by taking into account the polydispersity of the fractions. We show that the branching parameter is a decreasing function of molecular weight. These findings must find their applications in the characterization of such branched polymers by SEC if the apparatus are not coupled to at least three detectors.

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