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Aggregates in acidic solutions of chitosans detected by static laser light scattering

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Chitosans having degrees of *N*-acetylation, F_A, ranging from 0 to 0·6, were randomly degraded to different molecular weights and studied by multi angle static laser light scattering (LLS). Under the given experimental conditions, negative second virial coefficients of the solutions, A_2', revealed the presence of concentration dependent aggregates.

Attempts to remove the aggregates, or to influence the aggregation behavior, were made by ultracentrifugation and extensive filtering of the solutions. Modification of the solvent conditions such as pH, ionic strength and temperature were carried out, and chitosan solutions were digested with an acidic proteinase. Non-degraded samples and chitosans prepared by both heterogeneous and homogeneous *N*-deacetylation of chitin were also studied. In all cases, the negative A_2' remained. However, it was observed that ultracentrifugation and filtering of the solutions decreased the measured molecular weights and radii of gyration, indicating that some of the material of high molecular weight and size could be removed by ultracentrifugation and filtration.

The chemical nature of the physical basis of the molecular association was not revealed. Nevertheless, by the use of gel permeation chromatography coupled to an on-line low angle laser light scattering instrument and a differential refractive index concentration detector (HPSEC-LALLS-RI), a bimodal molecular weight distribution was observed in which about 5% of the sample had a very high molecular weight. These results coupled with the positive virial coefficients obtained earlier from osmotic pressure measurements suggest that a small fraction of the chitosan is aggregated to high molecular weight material, probably following a closed association model. Electron microscopy revealed the presence of some supramolecular structures. The positive second virial coefficients obtained earlier from osmometry are in harmony with these findings.

The results demonstrate the occurrence of reversible aggregation in chitosan solutions. Static laser light scattering therefore cannot readily be used to determine molecular weights and sizes of chitosans under these conditions.

It was not possible to correlate the extent of aggregation with the chemical composition of the chitosans.

INTRODUCTION

Chitosan is a linear polysaccharide consisting of 2-amino-2-deoxy-β-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc). It is made by alkaline *N*-deacetylation of chitin, which is a structural polysaccharide in the exoskeleton of many arthropods. The potential uses of chitosan derive from its unique chemistry: it is a polycation in neutral and acidic solutions, and it is biodegradable by lysozyme, chitin-, and chitosanases, both in solution and in the solid state. The solution properties of chitosan are governed by the relative content of the two monomers as designated by the fraction of *N*-acetylated units, F_A, and by the distribution of the monomers along the chain, the molecular weight, the pH, and the ionic strength of the solution.

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A study of the conformation and solution behavior of chitosans having different values of F_A based on measurements of number average molecular weights, M_n, and intrinsic viscosities, $[\eta]$, has been reported (Anthonsen *et al.*, 1993). In that study it was found that chitosans having F_A values ranging from 0 to 0·6 exhibited conformation and solution behavior similar to such relatively stiff polyelectrolytes as e.g. alginate, carboxymethylcellulose and other wormlike coils. Moreover, the second virial coefficients, A_2, as determined from osmotic pressure measurements were found to be positive and in the region of 10^{-3} ml mol g^{-2}, as is expected for polyelectrolytes in thermodynamically good solvents. Also, the chain stiffness of the chitosans was found to be comparable to that of alginate, cellulose, and cellulose derivatives as might be expected from the similarity of the structure of the polymeric backbone of chitosan with these substances.

Many polysaccharides extracted from plant and animal materials have been shown to be prone to associate or aggregate under various solvent conditions. These are often charged polymers, e.g. carrageenans (Vanneste, 1993; Smidsrød & Grasdalen, 1984), pectins (Davis *et al.*, 1980; Fishman *et al.*, 1986; Jordan & Brant, 1989; Kravtchenko *et al.*, 1992), alginates (Smidsrød & Haug, 1968; Mackie *et al.*, 1980), hyaluronic acid (Terbojevich *et al.*, 1986), but not necessarily so, e.g. (1 → 3), (1 → 4)-β-D-glucans (Vårum *et al.*, 1992), amyloses (Burchard & Cowie, 1972) and cellulose acetate (Tanner & Berry, 1974; Burchard, 1993). The physical basis for the aggregation is generally poorly understood. Aggregation imposes difficulties for molecular weight determinations of polysaccharides. Sometimes the aggregates can seemingly be removed completely by combined filtration and ultracentrifugation as for alginates (Smidsrød & Huag, 1968) and pectins (Berth, 1988, 1992), whereas in other instances the aggregates can only be partially removed. Complete removal of aggregates implies that they are 'stable', i.e. that they are not in rapid equilibrium with smaller polymeric species and that the proportion of aggregated material is therefore not sensitive to overall polymer concentration. Aggregates that can only be partially removed are either too small to escape the effects of filtration or sedimentation, or they arise from a rapid equilibrium with the lower molecular weight constituents. Macromolecular association phenomena have recently been reviewed by Burchard (1993).

The use of light scattering to study macromolecular aggregation has been described by Elias (1972). The concentration dependence at fixed scattering angles reveals different types of association phenomena. An association is said to be 'closed' when the size of the largest aggregates is determined by the mechanism of association and independent of the total polymer concentration. When a closed association is also 'cooperative' in the sense that only those aggregates in a particular narrow range of sizes are stable, a bimodal molecular weight distribution (MWD), consisting essentially only of unimers and multimers of well defined size, will result. This circumstance can lead to large differences between superficially similar physical properties measured by light scattering and osmotic pressure, because these techniques yield different averages over the very broadly dispersed molecular weight distribution.

Multi angle static laser light scattering (LLS) gives information about the weight average molecular weight. M_w, the root-mean-square z-average radius of gyration, R_g, and the light scattering second osmotic virial coefficient, A_2', of macromolecules in dilute solution. On the other hand, the osmotic pressure of a macromolecular solution is a colligative property and is hence a function of the number of molecules in the solution, yields information about the number average molecular weight, M_n, and a different average of the osmotic virial coefficient, A_2. This implies differences in the information obtained from these two techniques, especially if the samples have a bimodal MWD in which a very small fraction of the sample (impurity) has a very high molecular weight. Similar situations have been illustrated for pectins (Berth, 1988) and for the (1 → 3), 1 → 4)-β-D-glucans (Vårum *et al.*, 1992). Vårum *et al.* (1992) showed than an aggregation model for the β-D-glucans, possessing a small fraction of polymer that followed a cooperative closed association model, could mimic the positive and negative second virial coefficients obtained from osmotic pressure and light scattering measurements, respectively.

There have been several reports of the use of static laser light scattering to characterize chitosans. Several authors have reported 'anomalies' in the light scattering behavior of chitosans (Domard & Rinaudo, 1983; Terbojevich *et al.*, 1989, 1992), but there have also been reports of the use of light scattering to determine molecular weights of chitosan (Muzzarelli *et al.*, 1987; Terbojevich *et al.*, 1991; Wang *et al.*, 1991) apparently uncomplicated by aggregation effects. Rha (1984) used the results from sedimentation data of Berkovich *et al.* (1980) and concluded that the chitosan investigated had a more compact conformation than a random coil. This conclusion may perhaps be explained by the presence of aggregates. Aggregates have also been detected in chitosan by other techniques such as small angle X-ray scattering (Matsumoto *et al.*, 1991*a*), rheological properties (Matsumoto & Zenkoh, 1989) and LALLS (Matsumoto *et al.*, 1991*b*), and by GPC-LALLS-RI (Domard & Rinaudo, 1986). No correlations have been made between the occurrence of aggregates and the intrinsic properties of the chitosans, the solvents, or the preparation of the chitosans in the cited reports. This suggests that there are several parameters influencing the possible aggregation behavior of chitosan solutions.

Aggregates in polysaccharide solutions may be highly significant, e.g. for practical reasons such as filtration

on the laboratory or industrial scale or because aggregates may affect a biological property such as the immune response. Consequently, it is important to report on observations of chitosan–solvent systems in which aggregation behavior and molecular heterogeneity have been detected. Here we report the observation of aggregates in acidic solutions of chitosans having different F_A values and also different molecular weights. The results are in apparent contradiction to a previous study (Anthonsen *et al.*, 1993) based on osmotic pressure measurements of the same chitosan-fractions. The different signs of the second osmotic and light scattering virial coefficients A_2 and A_2' may be explained, however, by the presence of small fraction of chitosan associating in a concentration dependent way into supramolecular structures. The study also illustrates the importance of providing careful reports of the procedures used for preparing polysaccharide solutions for light scattering study, especially with regard to the clarification procedure.

EXPERIMENTAL

Materials

The chitosan sample with F_A values of 0·6 and 0·15 were kindly provided by Pronova Biopolymers (Drammen, Norway). Homogeneous *N*-deacetylation was used to prepare the chitosan with $F_A = 0·6$ (Vårum *et al.*, 1991*a*). The chitosan with $F_A = 0$ was made in our laboratory by heterogeneous *N*-deacetylation as described by Vårum *et al.* (1991*a*), except that the time and temperature used were 4 h and 95°C, respectively. The sample with $F_A = 0$ was made from a starting material with $F_A = 0·15$. All F_A values were determined by ^{1}H- and ^{13}C-NMR spectroscopy; the same techniques disclosed that the distribution of *N*-deacetylated residues in the chain was random (Vårum *et al.*, 1991*a*, *b*). Proteinase from *Rhizopus*-species (P5027) was supplied by Sigma Chemical Company (St Louis, MO, USA). Measurements of protein were performed with the Bio-Rad assay (Bio-Rad Laboratories, NY, USA), using bovine γ-globulin as a standard. Chitosan did not interact with the assay reagents, i.e., the regression line for the standard curve (γ-globulin) was unaffected by the presence of chitosan in the γ-globulin solutions.

Preparation of chitosan fractions

The chitosan fractions were degraded to fractions having different mean molecular weights and approximately the same type of molecular weight distributions and transformed to the hydrochloride salt as described previously (Anthonsen *et al.*, 1993). Chitosans not degraded with nitrous acid were also converted to the hydrochloride salt.

Viscometry and osmotic pressure

The viscosity and osmotic pressure measurements were performed as described previously (Anthonsen *et al.*, 1993).

Laser light scattering

The multi angle static laser light scattering measurements were made with a Brookhaven Instruments (Holtsville, NY) Model BI-200SM Spectrometer/Goniometer and a Model BI-2030 correlator giving usable data in the angular range of 30° to 120°. The sample was irradiated with vertically polarized light provided by a Lexel Model 85 argon ion laser tuned to the 488 nm line. The solutions were filtered through Millipore-HA filters (0·45 μm) or Millipore-GS filters (0·22 μm) directly into cylindrical borosilicate measuring cells containing ca. 5 ml (Fisher Scientific, Pittsburgh, PA). The instrument was calibrated with pure benzene, and alignment of the laser was checked daily (Bishay, 1989). Details of the experimental procedure (Urbani & Brant, 1989) and the processing of data (Haache *et al.*, 1987) are presented elsewhere.

Multi angle static laser light scattering was also carried out with a DAWN model F photometer (Wyatt Techonologies, Santa Barbara, CA) in the microbatch mode using the K5 flow cell and a He–Ne linearly polarized laser with wavelength 632·8 nm (Jackson *et al.*, 1989). The scattering angles measured ranged from 54° to 150°. The instrument was calibrated with toluene and the detectors were normalized with allow molecular weight dextran sample. The software used was DAWNF-87 and AURORA (Wyatt Technologies).

The low angle laser light scattering (LALLS) instrument used was a KMX-6 instrument (Chromatix, Sunnyvale, CA) with a light source of 633·5 nm and operating at a scattering angle of 6°.

The light scattering experiments were usually done at room temperature, but experiments were also performed on the Brookhaven instrument at 60°C. This was accomplished by circulating a thermostated water–ethylene glycol mixture through the coils of the refractive index matching vat containing toluene. The temperature was always measured in the vat.

The highest chitosan hydrochloride concentrations used for the different experiments ranged from 0·160 to 0·04 g/100 ml (Brookhaven), from 0·120 to 0·03 g/100 ml (Wyatt) and from 0·120 to 0·08 g/100 ml (LALLS). The solvent used was usually 0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl. Every concentration in each experiment was filtered within 30–60 min before measuring light scattering.

Clarification of light scattering solutions by ultracentrifugation, when carried out, was done with Beckman Model L preparative ultracentrifuge using rotot Model 30 operating at 100 000 × *g* for 4–5 h. After

centrifugation, the polymer solution was diluted to different chitosan hydrochloride concentrations and filtered through Millipore-HA filters (0·45 μm).

Electron microscopy

Samples were prepared for transmission electron microscopy (TEM) by high resolution metal shadowing. Chitosan samples were mixed with glycerol, sprayed onto a freshly cleaved mica surface, vacuum dried and rotary shadowed with Pt/C at 6° in a Balzer 400 T freeze etching unit. The effect of a number of preparation techniques on chitosan structures has been studied in great detail and will be discussed in a separate paper (Hermansson *et al.*, in preparation). Samples mixed with glycerol and vacuum dried revealed the same supramolecular structures as rapidly frozen and freeze etched sample. The chitosan hydrochloride concentrations used were 0·03, 0·01 and 0·003 g/100 ml. The solvent used was 0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl.

RESULTS AND DISCUSSION

Representative light scattering results (Brookhaven) plotted according to the method of Zimm (Zimm, 1948*a*, *b*) are given in Fig. 1. The plots illustrate typical results obtained for chitosans with $F_A = 0$, 0·15 and 0·6 and different molecular weights (as obtained from osmometry). Molecular weights measured by osmotic pressure (M_n) and by light scattering (M_w) are given in Table 1 along with other properties measured by light scattering. The samples listed in Table 1 represent extremes of F_A for water-soluble chitosans and also cover a relatively large range of molecular weight. The angular dependence of the scattering is essentially linear in the angular range 30°–120°, whereas the concentration dependence of the scattering shows significant positive curvature with an initial slope that is negative. The shape of the Zimm plots in Fig. 1, which results in negative second virial coefficients, A_2', is typical of concentration dependent aggregation behavior of a of a macromolecular solution (Elias, 1972). A solution

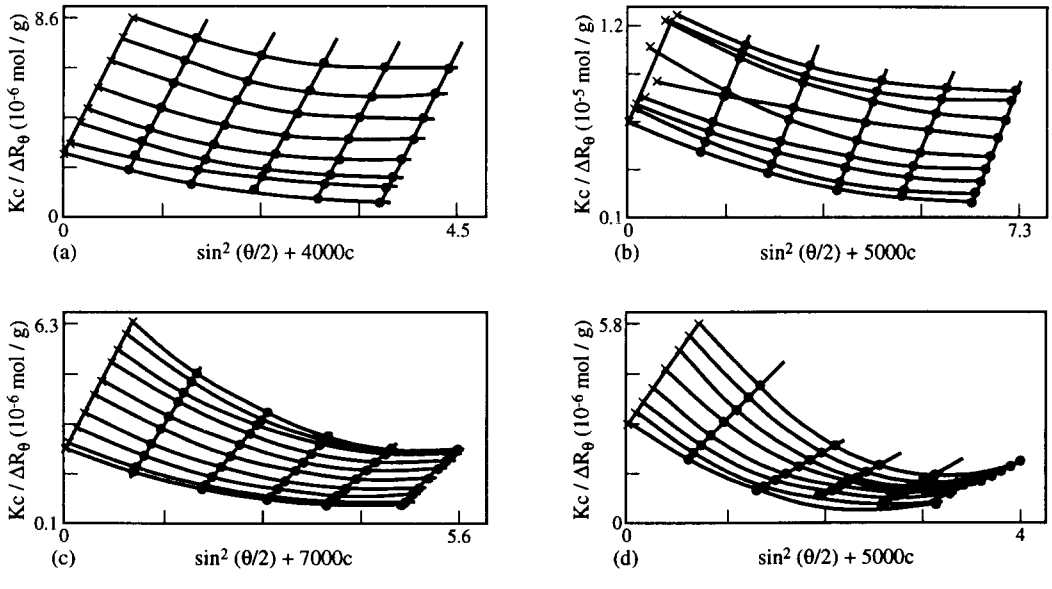

Fig. 1. Zimm plots of chitosan fractions. Concentrations are plotted as g/ml. (a) $F_A = 0$, $[\eta]_{0\cdot1} = 665$ ml/g, (b) $F_A = 0$, $[\eta]_{0\cdot1} = 379$ ml/g, (c) $F_A = 0{\cdot}6$, $[\eta]_{0\cdot1} = 813$ ml/g, (d) $F_A = 0$, $[\eta]_{0\cdot1} = 135$ m/gl. Number average molecular weights, osmotic second virial coefficients, intrinsic viscosities and extrapolated values from the Zimm plots are given in Table 1.

Table 1. Intrinsic viscosities, number average molecular weights, osmotic second virial coefficients and measured properties from light scattering (Zimm plots given in Fig. 1)

F_A	$[\eta]_{0\cdot1}$ (ml/g)	$M_n \times 10^{-5}$ [a] (g/mol)	$M_w \times 10^{-5}$ [b] (g/mol)	A_2 [a] (ml mol g^{-2})	A_2' [b] (ml mol g^{-2})	R_g [b] (nm)
0	665	2·1	3·3	$7{\cdot}7 \times 10^{-3}$	-2×10^{-3}	86
	379	0·64	1·3	$7{\cdot}6 \times 10^{-3}$	-3×10^{-3}	51
0·6	813	1·64	6·0	$1{\cdot}5 \times 10^{-3}$	-3×10^{-3}	67
	135	0·3	4·8	$3{\cdot}5 \times 10^{-3}$	-5×10^{-3}	59

[a] From osmotic pressure measurements (Anthonsen *et al.*, 1993).
[b] From light scattering measurements.

containing stable aggregates, in which the presence, amount and molecular weight of the aggregates are not dependent on the concentration of the macromolecule, would give positive A_2', provided that the solvent was thermodynamically good.

The observed curvature in the concentration dependence of the scattering data (Fig. 1) is frequently rather strong. This introduces problems for the choice of polynomial used to fit the data and carry out the extrapolation to vanishing concentration. In all cases shown in Fig. 1 the concentration dependence (at finite and at zero angle) was fitted with a second degree polynomial. Choosing a linear fit would result in smaller intercepts (larger M_w) and less negative slopes (less negative A_2') for the concentration dependencies at zero angle. Choice of a third degree polynomial fit would produce the opposite effects on M_w and A_2'.

Figure 2 illustrates the important effect of changes in the range of measured concentrations on M_w, R_g and A_2'. Five concentrations (0·055, 0·044, 0·033, 0·022, and 0·011 g/100 ml) are included in Fig. 2(a) from which $M_w = 4{\cdot}6 \times 10^5$ g/mol and $A_2' = -3{\cdot}2 \times 10^{-3}$ ml mol g^{-2} are obtained, respectively, from the intercept and initial slope of the concentration dependence at zero angle, and $R_g = 64$ nm is obtained from the initial slope of the angle dependence at zero concentration. In Fig. 2(b) a sixth (lower) concentration (0·006 g/100 ml) is included. Because the equilibrium aggregation is shifted toward greater dissociation at lower overall polymer concentration, the scattering experiment reports a smaller mean molar mass ($M_w = 3{\cdot}1 \times 10^5$ g/mol), smaller mean molecular dimensions ($R_g = 56$ nm), and a larger negative

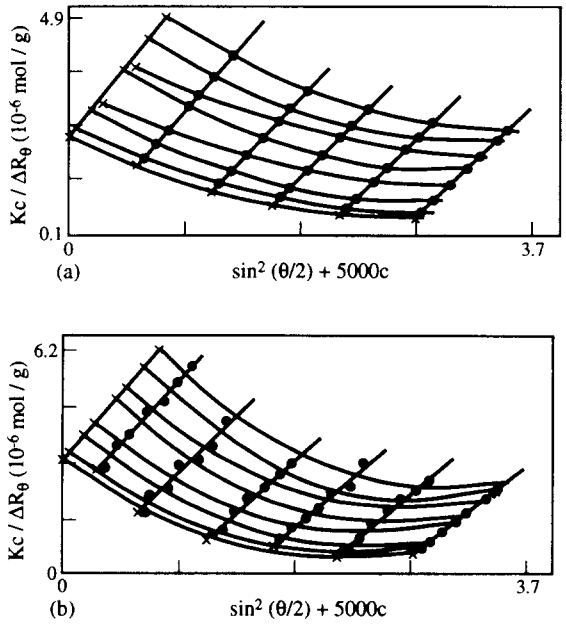

Fig. 2. Zimm plots of chitosan with $F_A = 0{\cdot}6$ and $[\eta]_{0\cdot1} = 282$ ml/g. Concentrations are plotted as g/m. Five (a) and six (b) chitosan hydrochloride concentrations are included (extrapolated values are discussed in the text).

second virial coefficient ($A_2' = -6{\cdot}4 \times 10^{-3}$ ml mol g^{-2}) when the lower concentration is included. The larger negative virial coefficient reflects the strong effect of dilution on the equilibrium association process.

Following the above discussion, one should expect that the reproducibility of light scattering results from solutions exhibiting concentration dependent aggregation behavior was low. This may be seen from Tables 2 and 3 in which are given M_w, A_2' and R_g from extrapolations in the Zimm plots obtained for chitosans having F_A of 0 and 0·6 and different values of $[\eta]_{0\cdot1}$. These results suggest therefore that, especially due to the difficult extrapolation to zero concentration, static light scattering does not provide a reliable method for determining the molecular weight of substances exhibiting such concentration dependent association.

The values of dn/dc used in this study were adopted from the studies of Terbojevich *et al.* (1991, 1992) and Wang *et al.* (1991) displayed in Fig. 3. Their values were reported for chitosans in the free amine form in HAc/NaAc-buffers at wavelengths of 633 and 436 nm, respectively. The values used in this study were corrected to correspond to chitosan hydrochloride concentrations by using:

$$(dn/dc)_{\text{hydrochloride}} = (dn/dc)_{\text{free amine}} \times M_{oF_A(\text{free amine})} \times [M_{oF_A(\text{hydrochloride})}]^{-1},$$

where M_{oF_A} is the average monomer weight corresponding to the F_A of chitosan in either free amine or hydrochloride form. The fact that our light scattering experiments were performed at a different wavelength from those for which dn/dc was reported has been neglected due to the very slight dependence of this parameter upon the wavelength (Johnson & Smith, 1972). Another source of uncertainty is the procedure for determining F_A: in the studies mentioned UV, IR and colloid titration were used to measure F_A, whereas in this study we used ^{1}H NMR. Because determination of the exact M_w is inevitably not the main focus of this study, we are content with the values of dn/dc estimated as described.

From Tables 2 and 3 we note, keeping in mind the relatively large uncertainties in M_w, that the polydispersity index, M_w/M_n, may be larger in the fractions having the lower values of $[\eta]_{0\cdot1}$. The possible increase in M_w/M_n with decreasing $[\eta]_{0\cdot1}$ does not necessarily reflect a greater tendency towards aggregation in the fractions with lower values of $[\eta]_{0\cdot1}$, but might be a result of the filtering procedure. For the samples with larger average particle size some of the larger aggregates may be removed by the 0·45 μm filter, whereas for the samples with smaller average size a larger range of particle sizes may pass through the filter, and the breadth of the distribution will therefore be larger. Moreover, for the samples with $F_A = 0$ and lower $[\eta]_{0\cdot1}$ higher polymer concentrations are usually used in light scattering experiments (Table 2). Thus, for

Table 2. Weight average molecular weights for chitosan hydrochloride, radii of gyration and second virial coefficients as determined from static light scattering (WALLS and MALLS) for chitosan with $F_A = 0$ (0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl)

$[\eta]_{0\cdot1}$ [a] (ml/g)	Conc.[b] (g/100 ml)	$M_n \times 10^{-5}$ [c] (g/mol)	$M_w \times 10^{-5}$ (g/mol)		M_w/M_n [d]	$\langle R_G^2 \rangle_z^{0.5}$ (nm)	A_2 [e] (ml mol g^{-2})	Poresize (μm)[f]
	0·069		1·9[g]	2·0[h]		65	-1×10^{-2}	1 × 0·45
	0·079		3·9	4·2		75	-5×10^{-3}	1 × 0·45
931	0·069	3·1	3·3	3·5	1·5/1·6[i]	81	-5×10^{-3}	1 × 0·45
	0·120[j]		13	14		85	-1×10^{-4}	1 × 0·45
	0·070[j]		6·7	7·2		90	-1×10^{-3}	1 × 0·45
	0·095		3·7	4·0		70	-2×10^{-3}	1 × 0·45
	0·090		3·3	3·5		86	-2×10^{-3}	1 × 0·45
665	0·077[j]	2·1	2·4	2·6	1·4/1·5[i]	65	-6×10^{-3}	1 × 0·45
	0·054[j]		0·75	0·81		45	-2×10^{-2}	1 × 0·45
	0·098		5·5	5·9		114	-2×10^{-3}	1 × 0·45
487	0·084	1·2	3·6	3·9	3·0/3·3[i]	88	-3×10^{-3}	1 × 0·45
	0·100[j]		1·7	1·8		58	-6×10^{-3}	1 × 0·45
	0·130		1·3	1·4		51	-3×10^{-3}	1 × 0·45
379	0·131	0·64	2·2	2·4	2·0/2·2[i]	65	-3×10^{-3}	1 × 0·45
	0·130		0·89	0·95		49	-8×10^{-3}	1 × 0·45
295	0158	0·48	1·4	1·5	4·3/4·6[i]	69	-5×10^{-3}	1 × 0·45
	0·140		2·7	2·9		81	-2×10^{-3}	1 × 0·45

[a] From Anthonsen *et al.*, (1993).
[b] The highest concentration measured, dilutions were made from this concentration.
[c] From osmotic pressure measurements (Anthonsen *et al.*, 1993).
[d] Estimated by deleting the highest and lowest determined M_w when poresize 0·45 μm was used.
[e] Average of values from *g* and *h*.
[f] The poresize of the membrane filters used for filtering the solutions.
[g] Number obtained by using the d*n*/d*c* values from Wang *et al.* (1991).
[h] Number obtained by using the d*n*/d*c* values from Terbojevich *et al.* (1991, 1992).
[i] Numbers obtained by using M_ws from *g* and *h*, respectively.
[j] Results obtained from MALLS experiment.

the samples with smaller average size the association equilibrium is shifted towards aggregation to produce a relatively larger M_w and larger polydispersity. A comparison of quantitative differences in aggregation using HPSEC-LALLS-RI in different samples will be reported elsewhere (Ottøy *et al.*, in preparation).

Figures 4 and 5 show plots of log $[\eta]_{0\cdot1}$ and log R_g versus log M_w, respectively. The plotted data are taken from the averages of the values in Tables 2 and 3, and only the results that were judged to give the most reliable parameters were included in the linear regression. We also observe that the molecular weight range is rather narrow. With these reservations in mind, the *a*-values in the MHKS-equation are estimated from Fig. 4 to be 0·7 ($F_A = 0$) and 0·6 ($F_A = 0{\cdot}6$), whereas R_g is found to scale with M_w in Fig. 5 with an exponent of 0·3 ($F_A = 0$) and 0·5 ($F_A = 0{\cdot}6$). These results might thus be taken to describe chitosans as having a more compact conformation than that deduced from our earlier osmotic pressure and intrinsic viscosity measurements (Anthonsen *et al.*, 1993). We reject this conclusion and offer the present results to illustrate the difficulties involved in using static light scattering as a method for characterizing chitosans.

Light scattering results from polysaccharide solutions have often been complicated by the presence of aggregates. Ultracentrifugation and filtration of the solutions have been shown in several instances to have a significant effect on the results, e.g. in the case of alginates (Smidsrød & Haug, 1968), cellulose trinitrate (Holt *et al.*, 1976), and pectins (Berth, 1992). In an effort to rid the present samples of aggregated particles two chitosan fractions were ultracentrifuged (100 000 × *g*, 4 h), and on another occasion filtered through three successive Millipore-GS filters (0·22 μm), and analysed by light scattering measurements. Zimm plots with a shape diagnostic for the presence of concentration dependent aggregates remained, but a clear indication of partial removal of aggregates in seen in Table 4. The data in Table 4 illustrate how the results from light scattering analysis are strongly influenced by the filtering procedure used for the scattering solution. It should be kept in mind that $M_w/M_n < 1$ in Table 4 is not realistic. Low M_w values probably result from erroneous extrapolation to vanishing concentration as the aggregation equilibrium is driven towards non-associated molecules.

Figure 6 shows the chromatogram of a chitosan sample from HPSEC-LALLS-RI. A detailed description

Table 3. Weight average molecular weights for chitosan hydrochloride, radii of gyration and second virial coefficients as determined from static light scattering (WALLS and MALLS) for chitosan with $F_A = 0.6$ (0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl)

$[\eta]_{0.1}$ [a] (ml/g)	Conc.[b] (g/100 ml)	$M_n \times 10^{-5}$ [c] (g/mol)	$M_w \times 10^{-5}$ (g/mol)		M_w/M_n [d]	$\langle R_G^2 \rangle_z^{0.5}$ (nm)	A_2 [e] (ml mol g^{-2})	Poresize (μm)[f]
	0·067		6·0	4·4[h]		67	-3×10^{-3}	1 × 0·45
	0·057		9·8	7·3		73	-2×10^{-3}	1 × 0·45
813	0·039	1·64	6·4	4·7	4·0/2·9[i]	84	-5×10^{-3}	1 × 0·45
	0·060[j]		7·0	5·2		82	-1×10^{-3}	1 × 0·45
	0·039[j]		6·3	4·6		85	-5×10^{-3}	1 × 0·45
	0·100		12	8·8		84	-2×10^{-3}	1 × 0·45
	0·100		2·7	2·0		50	-5×10^{-3}	1 × 0·45
	0·044		4·0	2·9		60	-8×10^{-3}	1 × 0·45
	0·036		2·0	1·5		43	-2×10^{-3}	1 × 0·45
644	0·046	1·45	3·6	2·6	3·0/2·2[i]	66	-7×10^{-3}	1 × 0·45
	0·041		2·6	1·9		55	-7×10^{-3}	1 × 0·45
	0·100[j]		10	7·4		93	-1×10^{-3}	1 × 0·45
	0·080[j]		2·9	2·1		56	-7×10^{-3}	1 × 0·45
	0·075		2·6	1·9		46	-8×10^{-3}	1 × 0·45
	0·060		2·9	2·1		62	-7×10^{-3}	1 × 0·45
	0·063		1·9	1·4		41	-1×10^{-2}	1 × 0·45
502	0·064	1·15	4·7	3·5	2·6/1·9[i]	73	-4×10^{-3}	1 × 0·45
	0·064		2·9	2·1		62	-7×10^{-3}	1 × 0·45
	0·071[j]		2·9	2·1		60	-7×10^{-3}	1 × 0·45
	0·065[j]		3·6	2·6		41	-7×10^{-3}	1 × 0·45
	0·100		4·3	3·2		47	-3×10^{-3}	1 × 0·45
	0·067		5·0	3·7		48	-4×10^{-3}	1 × 0·45
	0·055		2·6	1·9		38	-1×10^{-2}	1 × 0·45
	0·055		6·6	4·8		64	-3×10^{-3}	1 × 0·45
282	0·055	0·69	3·4	2·5	7·6/5·6[i]	44	-8×10^{-3}	1 × 0·45
	0·100[j]		9·7	7·2		72	-1×10^{-3}	1 × 0·45
	0·030[j]		0·7	0·5		24	-6×10^{-2}	1 × 0·45
	0·055[j]		14	11		80	-4×10^{-3}	1 × 0·45
	0·073		9·7	10		57	-2×10^{-3}	1 × 0·45
	0·068		5·7	4·2		66	-5×10^{-3}	1 × 0·45
	0·061		4·8	3·6		59	-5×10^{-3}	1 × 0·45
135	0·054[j]	0·3	6·3	4·6	20/15[i]	57	-4×10^{-3}	1 × 0·45
	0·072[j]		0·11	0·08		35	-2×10^{-1}	1 × 0·45
	0·089		0·46	0·34		9	-5×10^{-2}	1 × 0·45

Footnotes *a–j* as in Table 2.

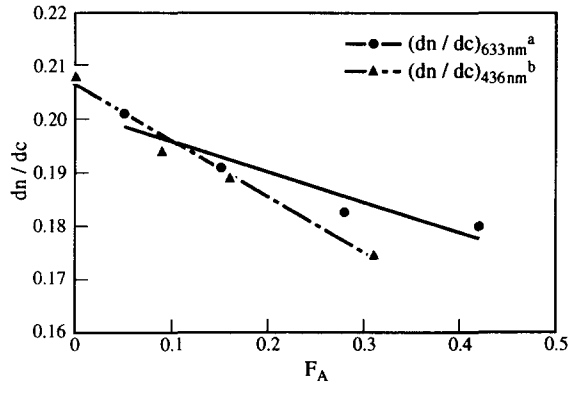

Fig. 3. Plots of dn/dc-values from (a) Terbojevich *et al.* (1991, 1992) at 633 nm and (b) from Wang *et al.* (1991) at 436 nm. The regression lines are (a) $(dn/dc)_{633} = 0.201 - 0.056 \times F_A$ ($R = 0.96$) and (b) $(dn/dc)_{436} = 0.206 - 0.103 \times F_A$ ($R = 0.99$).

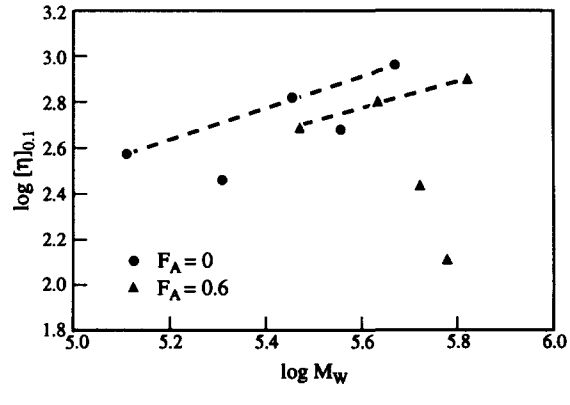

Fig. 4. Log $[\eta]_{0.1}$ versus log M_w for chitosans with $F_A = 0$ and 0·6. The M_w values used for the plots were taken from averaged values of M_w obtained by using dn/dc values from Wang *et al.* (1991) in Tables 1 and 2.

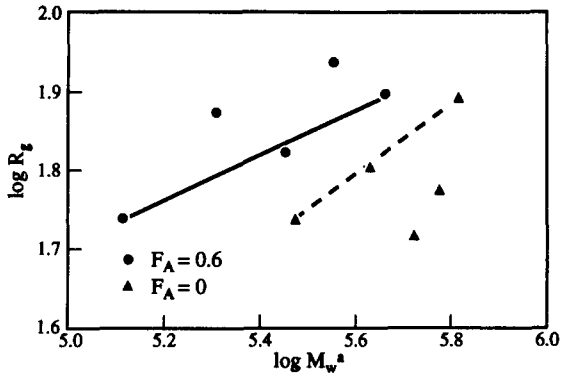

Fig. 5. Log R_g versus log M_w for chitosans with $F_A = 0$ and 0·6. The M_w values used for the plots were taken from averaged values of M_w obtained by using dn/dc values from Wang *et al.* (1991) in Tables 1 and 2. The R_g values are averaged from the values listed in Tables 1 and 2.

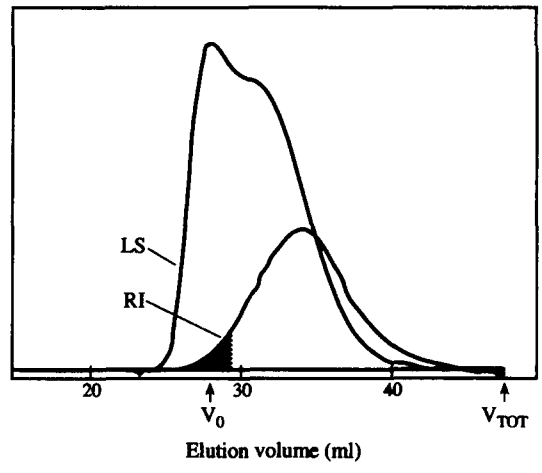

Fig. 6. HPSEC-LALLS-RI chromatogram of chitosan having $F_A = 0{\cdot}6$ and $[\eta]_{0\cdot1} = 644$ ml g^{-1}. The void volume (V_0) of the system was determined using a high molecular weight sample of xylinan (Stokke *et al.*, 1987). The total volume (V_t) was taken as the volume corresponding to the elution volume of glucose. Details will be given elsewhere (Ottøy *et al.*, in preparation.

of the HPSEC-LALLS-RI study of chitosans will be given elsewhere (Ottøy *et al.*, in preparation), and only some important preliminary findings will be discussed here. The chromatogram shows a bimodal distribution of the scattered light, whereas the distribution of material as measured by the RI detector is unimodal. Since the first peak in the chromatogram in Fig. 6 is eluted in the void volume (V_0) of the column it must consist of material with very high molecular weight. Moreover, the signal from the RI detector reveals that the material with very high molecular weight contributes little to the total mass. An exact determination of the relative amount of this material is difficult, but by cutting out the high molecular weight tail of the distribution as indicated in Fig. 6, a weight fraction of about 4% is obtained. The bimodal distribution in Fig. 6 suggests either a cooperative, closed association where all of the unimers are subject to aggregation, or if only a portion of the unimers have the capacity to aggregate, the bimodal distribution pattern is also consistent with an open association of the aggregating species. For pectins (Jordan & Brant, 1989) and (1 → 3), (1 → 4)-β-D-glucans (Vårum *et al.*, 1992) it has been proposed that only a fraction of the polymeric material has a tendency to undergo aggregation from unimers to multimers and that the aggregates are formed according to a closed association scheme. Vanneste (1993) found that both a closed and an open association model could mimic the static light scattering data on ι-carrageenans.

Neither the molecular weight and shape of the chitosan aggregates, nor the dissociation constants, nor the total amount of material that can undergo association can be determined with confidence from the static light scattering data presented. Nevertheless, with reference to the results from viscometry and osmometry, where positive second virial coefficients and conformational data in the range expected for β-(1 → 4)-linked polysaccharides were obtained, it must be concluded that the aggregates, even at the high concentrations necessary for osmotic pressure measurements, contribute only a small fraction of the total material. This is also confirmed from the HPSEC-LALLS-RI experiment (Fig. 6).

Table 4. Weight average molecular weights, radii of gyration and second virial coefficients as determined from static light scattering (WALLS and MALLS) for chitosan with $F_A = 0{\cdot}6$ after centrifugation and filtering (0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl)

$[\eta]_{0\cdot1}$[a] (ml/g)	Conc.[b] (g/100 ml)	$M_n \times 10^{-5}$[c] (g/mol)	$M_w \times 10^{-5}$ (g/mol)		M_w/M_n[d]	$\langle R_G^2 \rangle_z^{0.5}$ (nm)	A_2[e] (ml mol g^{-2})	Poresize (μm)[f]
644	[j]		4·3[g]*	3·2[h]*	3.0/2.2[i]	64*	-5×10^{-3}*	1 × 0·45
	0·072	1·45	2·0	1·5	1·4/1·0	40	-8×10^{-3}	Ultracentr.
	0·078		1·2	0·86	0·8/0·6	30	-1×10^{-2}	3 × 0·22
282	[j]		5·2	3·9	7·0/5·2	52	-1×10^{-2}	1 × 0·45
	0·098	0·69	1·0	0·76	1·4/1·1	28	-1×10^{-2}	Ultracentr.
	0·098		0·67	0·49	1·0/0·7	30	-1×10^{-2}	3 × 0·22

Footnotes *a–i* as in Table 2.
[j] Average of concentrations in Table 3.
* Average of values (after deleting highest and lowest values) in Table 3.

The chitosan hydrochloride concentration range used in each experiment when measuring osmotic pressure was in the range 0·1–0·6 g/100 ml, whereas the series of concentrations in light scattering measurements ranged from about 0·01–0·16 g/100 ml. In order to investigate the significance of this concentration difference, a light scattering experiment using concentrations in the range of 0·05–0·61 g/100 ml was performed. From the resulting Zimm plot in Fig. 7 it is seen that as the concentration is increased, the lines for the concentration dependence at different angles flatten out, leading, after extrapolation to zero angle, to a change in A_2' to zero or perhaps to positive values at still higher concentrations. This behavior is consistent with completion of a concentration-driven aggregation process as the concentration gets large enough, and consequently also consistent with the notion that only a certain fraction of the chitosan can exist in the aggregated form. When all the possible aggregates are formed, increasing the concentration further results in positive slopes of the concentration dependence representing a weighted average of the concentration dependence of the multimers and the unimers. Such an aggregated system of multimers and unimers is, as discussed above, expected to have positive osmotic second virial coefficients in thermodynamically good solvents. We note finally that A_2' (from light scattering) is not expected to be identical in value with A_2 (from osmotic pressure), even in the same concentration range, because these two observables correspond to different averages over the molecular weight distribution (Elias *et al.*, 1973).

The electron micrographs in Fig. 8 of chitosan with $F_A = 0{\cdot}01$ and $[\eta]_{0{\cdot}1} = 830$ ml/g reveal the presence of aggregates which are in the size range between 200 and 1000 nm. Figure 8 shows that the aggregates decrease in size with decreasing chitosan concentration (0·03, 0·01 and 0·003 g/100 ml in Fig. 8(a, b and c) respectively). Strand-like aggregates can be seen along with larger assemblies apparently based on the same structural motif. At higher magnification than in Fig. 8 (50 000×) a more detailed aggregate structure can be identified. However, the micrographs in Fig. 8 cannot be used to estimate the quantitative relationship between the aggregate structure and the molecular structure of the chitosan. A similar concentration dependence as in Fig. 8(a–c) was also observed for chitosan with $F_A = 0{\cdot}15$ and $[\eta]_{0{\cdot}1} = 1100$ ml/g. Details about characteristics of the aggregate structure of chitosan and the influence of such factors as F_A and ionic strength will be discussed in a separate paper (Hermansson *et al.*, in preparation).

In an attempt to influence the aggregation behavior of the chitosans, some modifications of the solvent conditions were tried. These included measurements at pH 1 to increase possible electrostatic repulsions

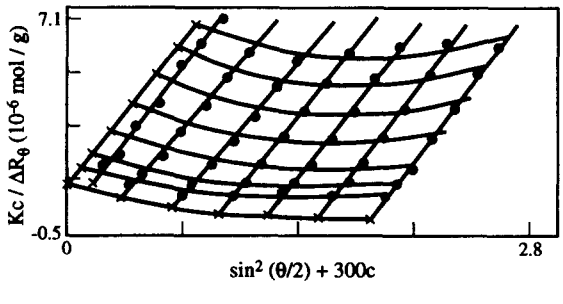

Fig. 7. Zimm plot of chitosan with $F_A = 0$ and $[\eta]_{0{\cdot}1} = 655$ ml/g including high chitosan chloride concentrations (0·05–0·61 g/100 ml). $M_w = 6{\cdot}8 \times 10^5$ g/mol, $R_g = 120$ nm and $A_2' = -3 \times 10^{-4}$ ml/mol^2 (dn/dc values from Wang *et al.*, 1991). Concentrations are plotted as g/ml.

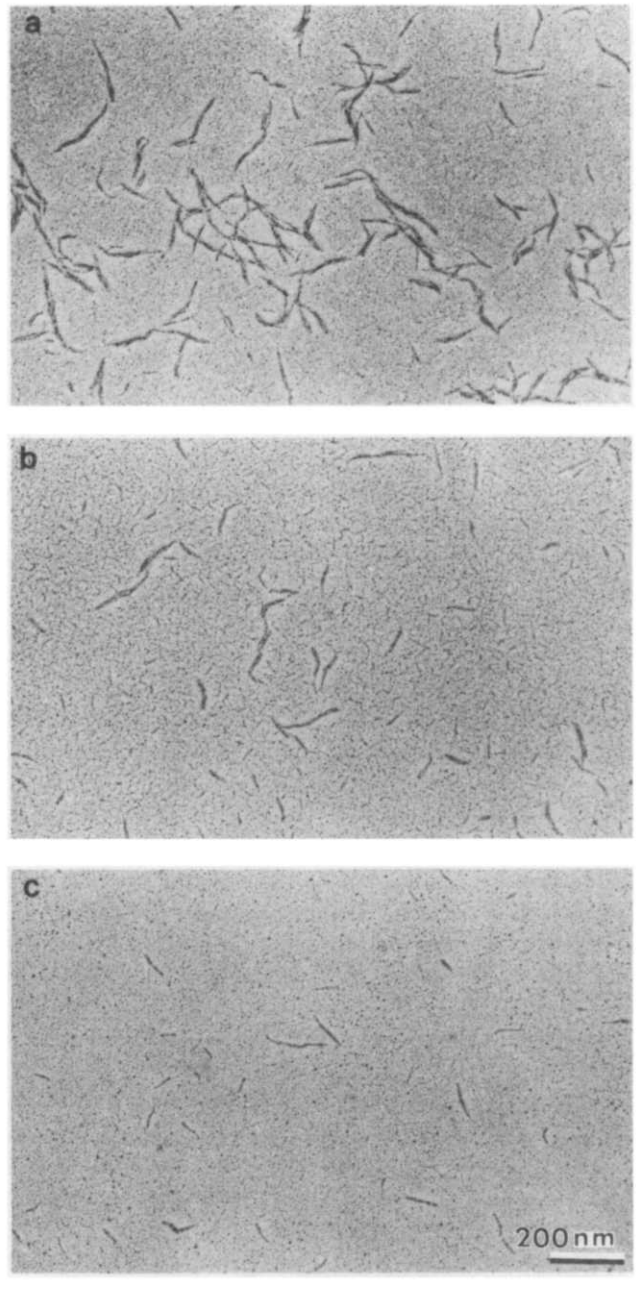

Fig. 8. Supramolecular structures of chitosan with $F_A = 0{\cdot}01$, $[\eta]_{0{\cdot}1} = 830$ ml/g. Solutions of (a) 0·03 g/100 ml, (b) 0·01 g/100 ml, and (c) 0·003 g/100 ml.

between chitosan chains and in 1 M NaCl to suppress longer range electrostatic effects. Measurements were done at 60°C following reports by Domard and Rinaudo (1983) suggesting that this had an effect on the light scattering results. Muzzarelli *et al.* (1987) stated that the chitosan solutions should be left to rest for some time before being studied by light scattering in order to result in positive A_2' values. None of these devices had any significant effect on the light scattering behavior of the samples studied here.

Due to the natural occurrence of chitin in coexistence with protein in, e.g. crustacean shells, it might be speculated that aggregation is caused by protein impurities leading to cross-links between the polysaccharide chains. The protein content in the chitosan fractions was measured and found to be at most 1% (w/w). Therefore, an acidic proteinase from a *Rhizopus* species having a broad substrate specificity (Sigma; Matsubara & Feder, 1971) was added to the chitosan solutions. The isoelectric point of the proteinase was about 5, i.e. higher than the pH of the chitosan solution to suppress attractive electrostatic interaction with the chitosan. The protein in the solution was subsequently extracted with phenol. A Zimm plot characteristic of concentration dependent aggregates was retained.

In one experiment TTHA (triethylene tetraamine hexaacetic acid) 10 mM was added to the 0·02% (w/v) chitosan solution ($F_A = 0{\cdot}6$, pH 6·7) in order to bind any divalent cations present in the solution. The addition of TTHA had no effect on the low angle light scattering results, suggesting that divalent or multivalent cations are not the cause of aggregation.

It has also been suggested that aggregate formation in degraded chitosans is dependent on the specific hydrolysis conditions used (Terbojevich *et al.*, 1992). The increase in M_w/M_n with decreasing mean molecular size seen in Tables 2 and 3 might suggest that the nitrous acid procedure used for degrading the samples (Allan & Peyron, 1989) has an effect on the aggregation behavior. However, the reactive aldehyde group formed at each cleavage site was in all instances reduced with $NaBH_4$. In addition, two non-degraded samples, i.e. those with the largest $[\eta]_{0\cdot1}$ in Tables 2 and 3, yielded Zimm plots qualitatively similar to those for the degraded samples. This indicates that the procedure used for degrading the samples is not the origin of the aggregation behavior.

In order to investigate the possible influence of the *N*-deacetylation procedure on the light scattering behavior of the chitosan, two samples were prepared in our laboratory by homogeneous *N*-deacetylation. These samples were not degraded with nitrous acid. Both samples were also found to give negative A_2' values.

Most of the reports in the literature reporting positive A_2' have used chitosans with relatively high $[\eta]_{0\cdot1}$. This might indicate that the aggregating material is more easily removed (e.g. by filtering), when the average molecular weight of the unimer chitosan chain is large. It should be mentioned that for a sample with high DP ($[\eta]_{0\cdot1} = 1010$ ml/g, $F_A = 0{\cdot}1$), the LALLS experiment disclosed a positive A_2' in 0·1 M HAc, pH 3 with 0·2 M NaCl, whereas in the solvent used preferentially in this study (0·02 M HAc/NaAc, pH 4·5 with 0·1 M NaCl), the A_2' measured by LALLS was negative. Moreover, a sample having lower DP yielded negative A_2' in 0·2 M HAc, pH 3. Also, it should be mentioned that recent experiments indicate that slightly positive A_2' values are obtained for chitosan in ammonium acetate, pH 4·5. Ammonium acetate has been reported to have a hydrogen bond destabilizing effect (Domard & Rinaudo, 1984).

In conclusion, the results reported in this study reveal the presence of concentration dependent aggregates in solutions of chitosan in acetic acid. Comparisons of light scattering and osmotic pressure measurements on the samples suggest, however, that only a small fraction of the total weight of the samples contributes to the high molecular weight aggregates. The aggregation process might be a closed or an open type of association. Classification of the mechanism of association or the shapes of the aggregates according to Burchard's recent categories (1993) is not possible from the present data, although the electron micrographs in Fig. 8 do suggest a fibrillar assembly of the chitosan chains.

The chemical and physical nature of the sites leading to association is unknown. Relatively long sequences of *N*-acetylated units represent one possible kind of association site, inasmuch as the insolubility of native (fully *N*-acetylated) chitin under the conditions of the present experiments suggests strong association of chitin-like chain segments. With a random, i.e. Bernoullian, distribution probability for the occurrence of several consecutive homopolymeric units. Let us assume, for example, that a *minimum* of five consecutive units of GlcNAc is necessary for a chain to engage in chitin-like interchain association. Then the fraction of all sequences of five consecutive residues which are homopolymeric in GlcNAc is $(F_A)^5$. Taking, for example, $F_A = 0{\cdot}10$ and a mean degree of polymerization of 10^3, we thus find that one chain in 10^2, or 1%, can be expected to contain an association site of the minimum requisite length. Accounting for all sequences of GlcNac of length 5 or greater increases the estimated fraction of chains with 10^3 residues containing chitin-like association sites to 1·11%. Longer chains will have a proportionately higher probability of association; shorter ones a lower probability.

We see, therefore, that even a 'homogeneous' chitosan sample with a random distribution of the monomeric residues contains the heterogeneity of structure required to allow a few percent of the material to participate in the postulated chitin-like association process. Moreover, some deviations from the random distribution of monomer units, arising perhaps from the *N*-deacetylation process used, are entirely possible within the inher-

ent precision of the NMR measurements used to determine the statistical distribution. If such deviations from Bernoullian statistics exist, they might be in the direction of greater block-like character in the sequence distribution. This example is presented not to advocate a particular mode of chain association, but rather to illustrate how the association might occur in a sample that may from the chemical point of view appear quite homogeneous.

The results also illustrate the importance of giving careful descriptions of the experimental procedures for preparing solutions for light scattering. The results from light scattering experiments might differ considerably depending, for example, on the clarification procedure employed. It is the mean molecular weight of the array of dissolved material at the end of the clarification process that is measured, and interpretation of the results evidently depends on a full description of the sample preparation. Combined techniques such as HPSEC-LALLS-RI and HPSEC-MALLS-RI seem especially well suited to characterization of aggregating polysaccharide systems.

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