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Chitosans from *Euphausia superba*. 1: Solution properties

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A technological development of chitosan (copolymer of *N*-acetylglucosamine and glucosamine) is based on the knowledge of parameters such as composition, types of sequence, chain length and distribution. In addition to the sample source, these factors are related to preparation and purification methods.

In this connection the authors examined chitosan samples having the same origin (*Euphausia superba*), but different degrees of acetylation, as well as materials which were subjected to regeneration and beating processes. In addition, the authors prepared chitosan samples of different degrees of polymerization by hydrolysis of commercial chitosan of high degree of acetylation under different pH and temperature conditions.

The samples were characterized in 0·1N CH_3COOH (HAc) – 0·2M NaCl by intrinsic viscosity ([η]) and light scattering techniques.

The viscometric behaviour of the starting chitosans and of all depolymerization products follows an expected pattern. On the other hand, light scattering shows important differences among samples having identical [η], but obtained under different hydrolysis conditions. A tentative explanation is suggested for the discrepant responses.

INTRODUCTION

Chitosan is the *N*-deacetylated product of chitin but the name does not identify a characteristic product, rather a product family, each product differing from each other for the degree of deacetylation, type of sequence arrangement, chain length and molecular weight distribution. Thus, chitosan can be considered as a copolymer of poly-*N*-acetyl-D-glucosamine and poly-D-glucosamine. Its structural characteristics depend on the method of deacetylation used (Kurita *et al.*, 1977) as well as on the conditions of regeneration from its acid solutions (Struszczyk, 1987).

The present work is a systematic light scattering study of chitosans from *Euphausia superba*, both at the molecular and supramolecular level, to gain insight into their dilute solution behaviour (Part 1) in relation to their structural features in the solid state (Part 2, Focher *et al.*).

Besides commercial chitosan samples having various degrees of acetylation, preparations were also studied which were obtained by regeneration, beating and depolymerization processes. Light scattering technique is greatly affected by the presence of almost negligible amounts of very high molecular weight material. Thus, unlike viscometry, it can detect macromolecular heterogeneities (i.e. microgels, multimers). This fact could be partially responsible for the disagreement in literature reports concerning the solution properties of chitosan (Kienzle-Sterzer *et al.*, 1986).

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EXPERIMENTAL

Materials

Samples of commercial chitosans from *Euphausia superba* (Antarctic krill) were kindly supplied by Sea Fishering Institute, Gdynia, Poland.

The degree of acetylation (Ac %), specified by the producer and confirmed via UV (Muzzarelli & Rocchetti, 1985), is 42% for chitosan A and 28% for chitosan B. The preparation of chitosan C (Ac $\simeq$ 5%) from chitosan B followed with minor modifications the method proposed in the literature (Domard & Rinaudo, 1983). In order to induce structural modification of chitosan without changing its molecular weight and degree of acetylation, chitosan A_B was prepared by beating a 10% aqueous dispersion of chitosan A with laboratory PFI mill at 25°C for 24 h (Centola & Borruso, 1962).

Chitosan samples were regenerated by treatment with 1% acetic acid (HAc) and neutralization with 1N NaOH, at 1% polymer concentration. After prolonged washing, the samples were recovered by lyophilizing the slurry (A_R, B_R, C_R and $(A_B)_R$).

Chitisan samples of different degrees of polymerization were prepared by hydrolysis of commercial chitosan A, using different pH and temperature conditions: 1% and 10% HAc refluxing and 0·6N HCl at 50 and 25°C. Polymer concentration was 1%.

The depolymerization, performed as illustrated in Fig. 1, was monitored by viscosity measurements. The effect of temperature as well as the importance of the acid catalyst are clearly shown. The effect of acetic acid concentration is illustrated in the inset, where the fastest kinetics are reported. The specific initial rate constants (K) evaluated according to the equation

$$[\eta]_t = [\eta]_0 e^{-kt} \tag{1}$$

are respectively 0·09 h^{-1} in 1% HAc reflux, 0·11 h^{-1} in 10% HAc reflux, 0·23 h^{-1} in 0·6N HCl at 50°C and 0·003 h^{-1} in 0·6N HCl at 25°C.

The samples were recovered by precipitation with ~1N NaOH, except for samples 7 and 11 (Table 1), which precipitate to an extent of only 10–20% under alkaline pH. To isolate sample 7 the reaction mixture was made basic (pH 9–10), dialysed against water (cutoff of 1000 *D*) and finally lyophilized (yield 68%). Sample 11 was recovered by direct lyophilization of the hydrolysed solution.

Since chitosan hydrochlorides undergo acid catalysed depolymerization in the solid state, all samples, except 11, are isolated and stored as the free bases.

Each sample was characterized by elemental analysis and Ac% (UV method). Ac% values are reported in Table 1. Hydrolysis of glycosidic linkages occurs without involving deacetylation when using HAc (samples 1-5). An increase in the degree of acetylation, such as found for sample 7, is probably related to the isolation process.

Depolymerization performed with HCl causes simultaneous deacetylation, as indicated by compounds 10, 11 and 15. These findings are in agreement with the extensive depolymerization and deacetylation observed in hydrolysis experiments performed at room temperature in HCl at concentration higher than 0·6N.

Minor differences in the degree of acetylation were expected not to influence significantly the authors'

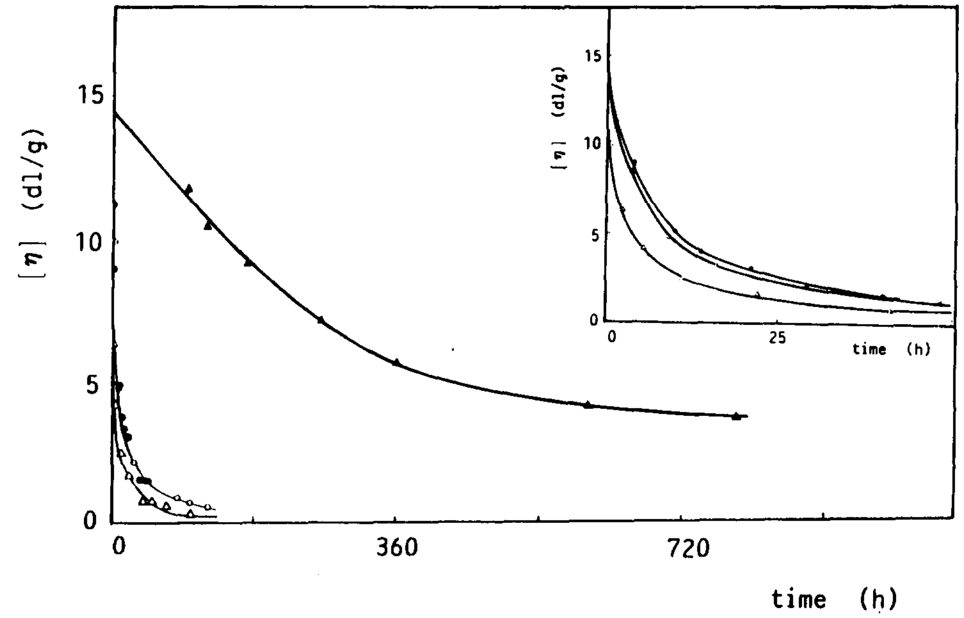

Fig. 1. Plots $[\eta]$ *versus* time of hydrolysis for commercial chitosan A. Polymer concentration 1%; (●) 1% HAc, reflux; (○) 10% HAc, reflux; (△) 0·6N HCl, at 50°C; (▲) 0·6N HCl, at 25°C.

Table 1. Depolymerization of chitosans A, A_B and B

Sample	Hydrolysis conditions			Ac % ± 0·7
1	1% HAc	reflux	20 min	41·6
2	1% HAc	reflux	10 h	41·0
3	1% HAc	reflux	14 h	40·3
4	1% HAc	reflux	21 h	41·5
5	1% HAc	reflux	37 h	42·2
6	10% HAc	reflux	29 h	46·0
7	10% HAc	reflux	145 h	52·5
8	0·6N HCl	50°C	12 h	42·0
9	0·6N HCl	50°C	41 h	39·9
10	0·6N HCl	50°C	71 h	35·3
11	0·6N HCl	50°C	100 h	33·0
12	0·6N HCl	25°C	101 h	42·8
13	0·6N HCl	25°C	177 h	40·5
14	0·6N HCl	25°C	367 h	40·0
15	0·6N HCl	25°C	818 h	38·6
	Depolymerization of chitosan A_B			
16	1% HAc	reflux	43 h	42·1
	Depolymerization of chitosan B			
17	1% HAc	reflux	49 h	28·0

results. In fact, the conformational rigidity of chitosan chains is moderately affected by different acetylation levels. More recently, a quantitative evaluation of these phenomena has been carried out in the authors' laboratories (Terbojevich *et al.*, 1991).

Samples 16 and 17 were prepared from chitosan A_B and B, respectively, under the hydrolysis conditions reported in Table 1.

Preparation of polymer solutions

To prepare chitosan solutions, the material was added, under stirring, to 0·1N acetic acid. After solubilization of the sample, sodium chloride was added to reach the desired ionic strength (0·2 M) and the solution was centrifuged at 25 000 *g* for 1 h.

Instruments and methods

Viscosities were determined using a multigradient suspended-level Ubbelohde viscometer at 25·0 ± 0·1 °C with 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 $[\eta]$ of 8–10 dl g^{-1}. Higher viscosities were extrapolated to zero flow-velocity gradient (Terbojevich *et al.*, 1967). Intrinsic viscosity data ($[\eta]$) were calculated by the Huggins equation (Huggins, 1942).

Light scattering measurements were performed at 25 °C using a Sofica Model 42 000 photometer with cylindrical cells immersed in toluene. Non-polarized laser light (633 nm) was used, covering scattering angles ϑ between 30° and 150°. A Rayleigh ratio $R_{90°} = 8{\cdot}96 \times 10^{-6}$ cm^{-1} at 633 nm was used for calibration of the instrument with benzene (Millaud & Strazielle, 1979). Solutions and solvents were clarified by additional centrifugation at 25 000 *g* for 3 h. The light scattering data were plotted as c/H_θ versus $\sin^2 \theta/2$ or versus c, where c is the concentration of polymer in g ml^{-1}. H_θ is defined as

$$H_\theta = \frac{(I - I_0)\sin\vartheta}{1 + \cos^2\vartheta} \tag{2}$$

where I_0 and I are intensity of scattered light for solvent and solution, respectively. Molecular weight (M_w), second virial coefficient (A_2) and radius of gyration (R_G) were calculated by Zimm plots, according to the literature (Terbojevich *et al.*, 1985*a*).

Values of d*n*/d*c* were determined using a Chromatix CMX-16 refractometer, at 633 nm.

The values obtained by the authors are dependent on the degree of acetylation. In 0·1N HAc–0·2M NaCl they were 0·180 ± 0·006 g^{-1} ml for chitosan A, 0·183 ± 0·005 g^{-1} ml for chitosan B and 0·201 ± 0·006 g^{-1} ml for chitosan C.

Light scattering and d*n*/d*c* measurements before and after extensive dialysis of the polymer solution against the solvent showed negligible differences, indicating that the results were not affected by non-equilibrium of the chemical potential.

RESULTS AND DISCUSSION

Sample A is only partially (~50%) soluble in 0·1N HAc: even after prolonged stirring part of the material, although highly swollen, cannot be dissolved. This fact does not appear to be related to the presence of chitosan having heterogeneous composition, as the degree of acetylation of the soluble fraction (42%) and the elemental analysis are the same as in the starting material. The intrinsic viscosity of the soluble fraction is reported in Table 2. When sample A at a concentration of 1% is treated with 1% HAc and regenerated *in toto* with 1N NaOH, its solubility in 0·1N HAc is practically complete. The acetyl content is not modified, whereas the $[\eta]$ value is somewhat lower in comparison to the soluble fraction of before regeneration (Table 2). A similar effect is brought about by the beating process. Sample A_B is completely soluble in 0·1N HAc and the corresponding intrinsic viscosity is quite close to the one found for A_R.

The commercial sample B having a degree of acetylation of 28% is, on the contrary, freely soluble in acidic media, probably due to its higher content in charged groups. The $[\eta]$ in saline solution is similar to the values found for samples A_R and A_B (Table 2).

To shed light on the chemical and structural modifications occurring upon regeneration, samples A_B and B were submitted to the same procedure. Regeneration causes for both samples a lowering in $[\eta]$. This is possibly associated with moderate depolymer-

Table 2. Intrinsic viscosity of chitosan samples in 0·1N HAc–0·2M NaCl

Chitosan sample	$[\eta]$ (dl g^{-1})
A	23·5
A_R	16·6
A_B	17·0
$(A_B)_R$	15·2
B	18·1
B_R	14·8
C_R	8·1
1	11·2
2	5·4
3	3·7
4	3·2
5	2·0
6	2·2
7	0·5
8	2·6
9	1·2
10	0·9
11	0·3
12	8·7
13	5·8
14	5·2
15	3·4
16	2·4
17	2·7

ization in the acidic medium, which is favoured by the relatively high acetyl content of the samples (Terbojevich *et al.*, 1989).

Since the preparation of sample C, having low acetyl content, was carried out under experimental conditions (Domard & Rinaudo, 1983) differing from those employed for the production of industrial chitosans (Muzzarelli, 1977), no homogeneous comparison can be made with samples A and B. This is, however, possible among regenerated materials. The $[\eta]$ value relative to C_R is reported in Table 2.

The viscometric behaviour of all depolymerization products (Tables 2 and 3) follows an expected pattern and the samples exhibit a progressive viscosity drop on increasing reaction times.

For a better understanding of the effects caused by regeneration, beating and depolymerization on the solution properties of chitosan samples having the same origin but different acetylation degrees, the above materials were submitted to light scattering measurements in aqueous acetic acid in the presence of NaCl. Measurements with commercial chitosan A were not reproducible, due to incomplete solubilization of the sample which did not allow one to obtain solutions free of undissolved material, in particular microgels. This is not the case for samples A_R and A_B, which were examined at concentrations lower than c^* ($c^* \simeq 1/[\eta]$) to avoid association phenomena connected to entanglement process (Terbojevich *et al.*, 1986). Plots c/H_ϑ versus $\sin^2\vartheta/2$ at different concentrations are parallel and linear. The slope $d(c/H_0)/dc$, related to the virial coefficient A_2, is positive. These results confirm the absence of rather high M_w species (microgels), as well as of small multimers formed by the majority of the polymer chains (associated species) (Terbojevich *et al.*, 1985*b*). The molecular parameters obtained from Zimm plots (Fig. 2) are well comparable and in agreement with the viscosity data ($M_w = 1 \times 10^6$, $A_2 = 1{\cdot}5 \times 10^{-3}$, $R_G = 1450$ Å). Since these samples were prepared by solubilization of the commercial material with completely different processes, the presence of stable multimers (aggregates) in solution can be reasonably ruled out. Samples A_B and A_R are thus molecularly dispersed in solution. The same conclusion can be drawn for sample B (Fig. 2), which behaves quite similarly to A_B and A_R ($M_w = 7 \times 10^5$, $A_2 = 1{\cdot}9 \times 10^{-3}$, $R_G = 1240$ Å).

Table 3. Light scattering data for some representative chitosan samples in 0·1N HAc–0·2M NaCl

Sample	Hydrolysis conditions	% Ac	$[\eta]$	$d(c/H_0)/dc$
3	1% HAc, 14 h, reflux	40·3	3·7	$\simeq 0$
15	0·6N HCl, 818 h, 25°C	38·6	3·4	>0
5	1% HAc, 37 h, reflux	42·2	2·0	<0
16	1% HAc, 43 h, reflux	42·1	2·4	<0
17	1% HAc, 49 h, reflux	28·0	2·7	$\simeq 0$
6	10% HAc, 29 h, reflux	46·0	2·2	>0
8	0·6N HCl, 12 h, 50°C	42·0	2·6	>0
7	10% HAc, 145 h, reflux	52·5	0·5	$\simeq 0$
11	0·6N HCl, 100 h, 50°C	33·0	0·3	>0

Plots c/H_ϑ versus $\sin^2\vartheta/2$ are shown in Fig. 3 for sample C_R at various concentrations. They differ remarkably from the corresponding plots obtained using samples A_R, A_B and B. Curved and non-parallel lines, as well as the intercepts at $\vartheta = 0$ indicate the presence of associated species in solution, which increase in size on increasing concentration. The apparent M_w, obtained by extrapolation to $c = 0$ is about 5×10^5. Since C_R was prepared by deacetylation of sample B under conditions at which depolymerization processes are not expected to occur, the above extrapolation could well correspond to the true M_w of C_R. The presence at equilibrium of associated forms in highly deacetylated chitosan is likely related to a reduced solubility of polyelectrolytes systems with high charge density in saline media (the authors used 0·2M NaCl).

Light scattering measurements were finally performed on depolymerized chitosans, having about 42% acetylation (Table 1 and Experimental section).

Samples 1-5, depolymerized under mild conditions, present high dissymetry in all cases. A linear dependence in the plot c/H_ϑ versus $\sin^2\vartheta/2$ is observed for the examined samples at low concentrations ($c \simeq 0{\cdot}3 \times 10^{-3}$ g ml^{-1}), while at higher concentration a pronounced curvature appears in the same plot for samples 2, 3 and 4, which makes extrapolation to $\vartheta = 0$ rather uncertain (Fig. 4).

Extrapolation to $c = 0$ (Fig. 5) gives different values

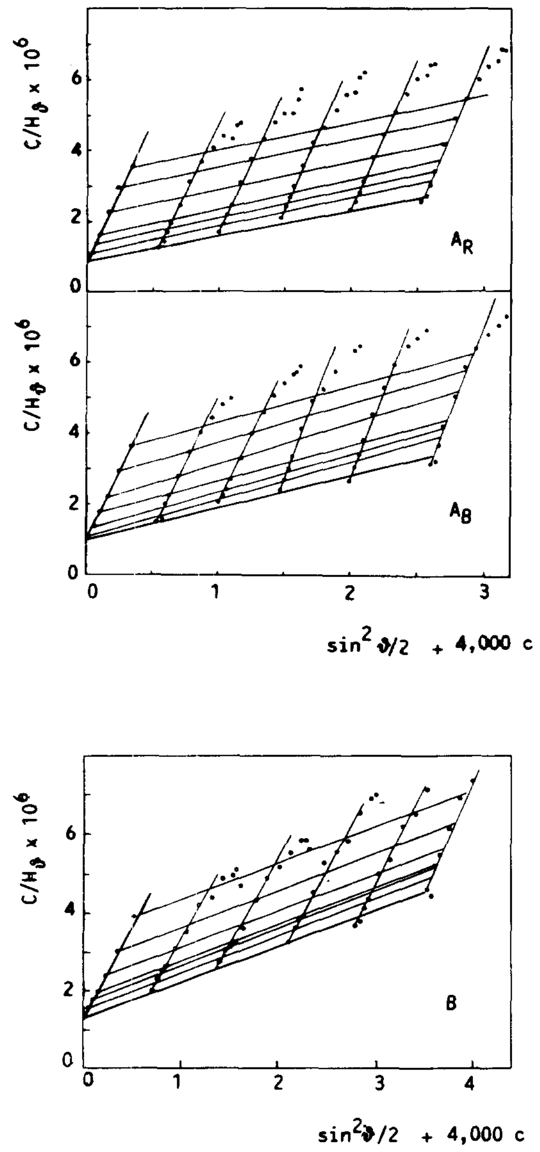

Fig. 2. Zimm plots of chitosans A_R, A_B and B.

for the various samples, which are not simply related to the differences in molecular weight, expected on the basis of the viscosity data. The second virial coefficient is positive ($\simeq 10^{-3}$) for sample 1, whereas it is much lower or zero for samples 2–4 and finally becomes negative for sample 5. Only following short hydrolysis are chitosan chains molecularly dispersed. On the contrary, multimerization phenomena occur in samples 2–5, having lower $[\eta]$.

To examine the effects of hydrolysis conditions on the properties of depolymerized chitosans, at 42% Ac, sample 6 was prepared and isolated. It exhibited $[\eta]$ values close to those of sample 5.

Light scattering measurements show again a linear angular dependence. However, c/H_0 values are substantially increased ($5–7 \times 10^{-6}$ as compared to $1–2 \times 10^{-6}$) and the second virial coefficient is positive (Fig. 6). The behaviour is similar to compounds A_R and 1. The molecular weight is close to 2×10^5, much lower than the value corresponding to sample A_R ($1{\cdot}0 \times 10^6$). This result rules out the possibility that chitosan chains are associated even at relatively low DP values. It follows that the light scattering responses characteristic of associated materials have to be related to specific modifications occurring during hydrolysis with 1% HAc.

For sample 7, recovered by dialysis and lyophilization, light scattering data yield very low c/H_0 values ($2–3 \times 10^{-7}$) and a second virial coefficient close to 0. The results closely resemble those found for sample 5 and suggest that depolymerization in 10% HAc reduces, but does not eliminate, the phenomena observed after depolymerization at low HAc concentration.

As far as hydrolysis in 0·6N HCl at 50°C is concerned, a direct comparison can be made with the above data by examining samples 8–11, having $[\eta]$ in the range 2·6–0·3.

The light scattering pattern of sample 8 closely

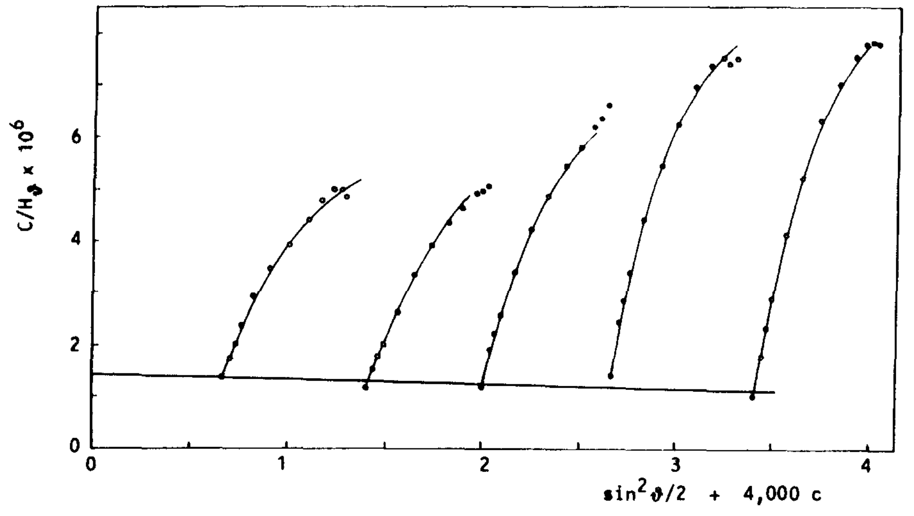

Fig. 3. Zimm plot of chitosan C_R.

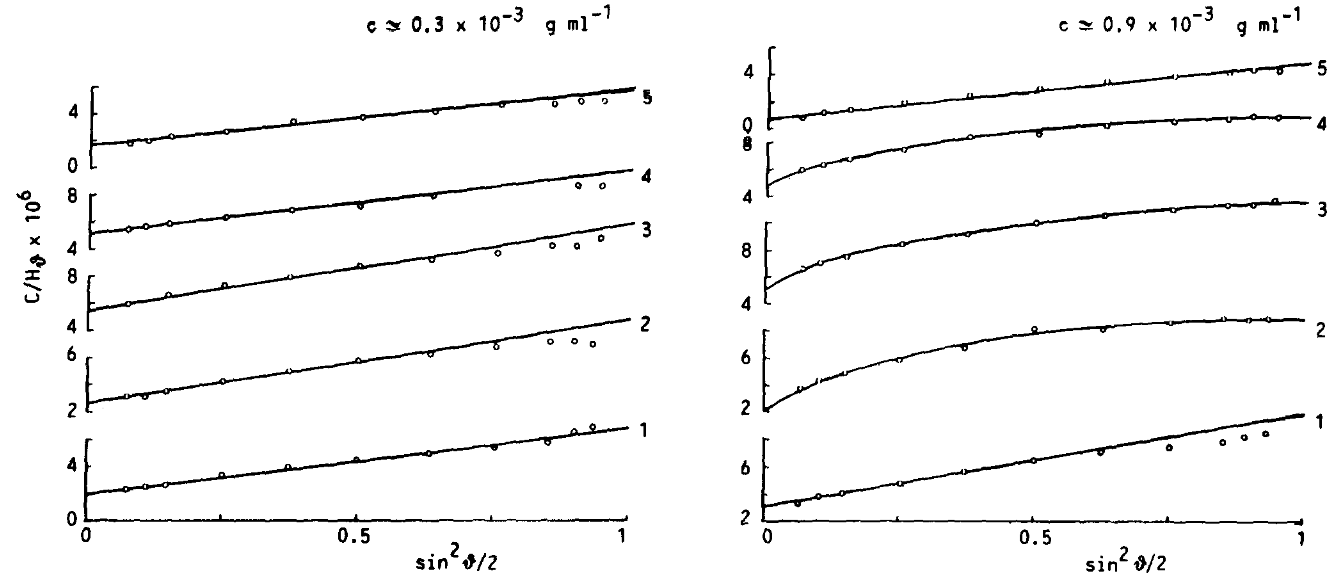

Fig. 4. Plots c/H_ϑ versus $\sin^2\vartheta/2$ of chitosans 1–5 at $c \simeq 0{\cdot}3 \times 10^{-3}$ g ml^{-1} and $c \simeq 0{\cdot}9 \times 10^{-3}$ g ml^{-1}.

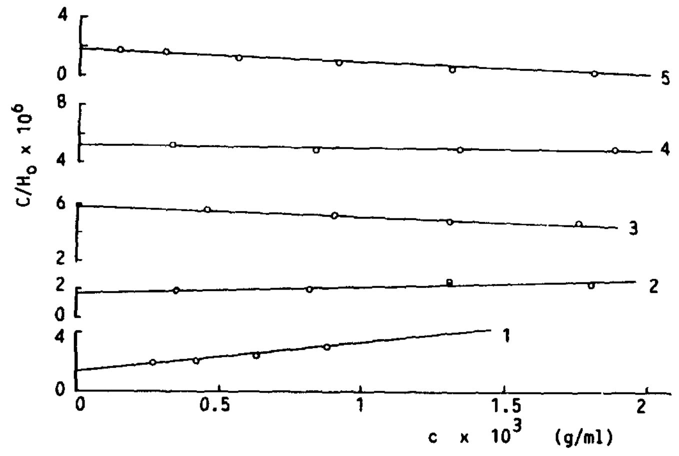

Fig. 5. Plots c/H_0 versus c of chitosans 1–5.

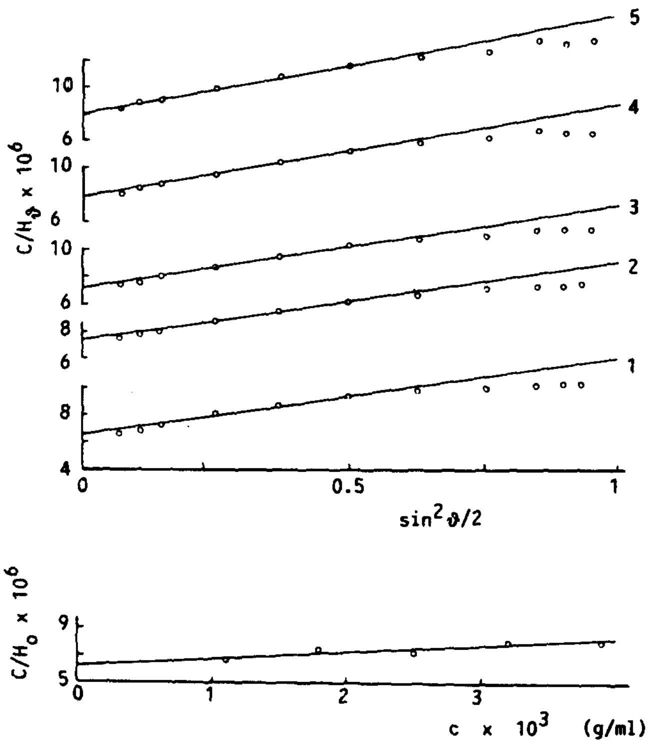

Fig. 6. Plots c/H_ϑ versus $\sin^2\vartheta/2$ at different concentrations (g ml^{-1} × 10^3): (1) 1·1; (2) 1·8; (3) 2·5; (4) 3·2; (5) 3·9; and plot c/H_0 versus c of chitosan 6.

resembles that of sample 6. On the contrary, the results obtained with materials having lower $[\eta]$ do not match those obtained with sample 7, as M_w values obtained by plotting $c/H_{90°}$ versus c (dissymetry $\simeq 1$ is present) are progressively reduced on lowering $[\eta]$. The M_w values range between 6×10^4 and 9×10^3. The second virial coefficient is positive and comparable to samples A_R, 1 and 6. The data obtained for samples 5 and 10 are reported in Fig. 7 for comparison.

It follows that the properties of depolymerized chitosans at 42% Ac are related to the hydrolytic conditions and the reaction in 0·6N HCl at 50°C eliminates high diffusion responses found for low $[\eta]$ samples obtained using HAc as the depolymerizing agent.

The slow depolymerization process in 0·6N HCl at room temperature allows the isolation of samples having constant degree of acetylation and intrinsic viscosity ranging from 8·7 and 3·4 dl g^{-1}. Unlike compounds 1–4, which have comparable $[\eta]$, samples 12–15 exhibit almost straight plots c/H_θ versus $\sin^2\theta/2$ and a progressive increase in the second virial coefficient, up to 2×10^{-3} in the samples having lowest viscosity.

Samples 16 and 17 obtained by depolymerization of chitosan A_B and B in 1% HAc were examined. They have $[\eta]$ values comparable to sample 5 (Table 2). Both derivatives show remarkable deviation from linearity,

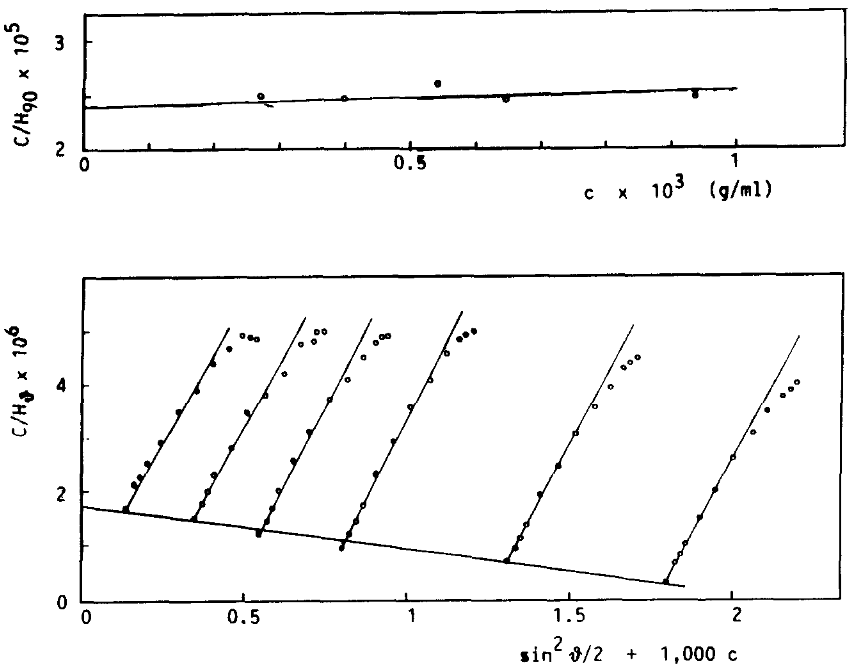

Fig. 7. $c/H_{90°}$ versus c of chitosan 10 and c/H_ϑ versus $\sin^2\vartheta/2 + Kc$ of chitosan 5.

and negative or very low virial coefficients. The analogy with compound 5 is immediate: neither beating nor lowering the degree of acetylation causes modifications in the light scattering response.

The most significant results obtained for some of the examined derivatives are reported in Table 3, where hydrolysis conditions, % Ac, $[\eta]$ values and the slope $d(c/H_0)/dc$, related to virial coefficient A_2 are listed. The present results clearly demonstrate that, while the intrinsic viscosity decreases steadily with time of hydrolysis in all experiments, the light scattering measurements are affected by other factors, such as nature of acidic catalyst and hydrolysis temperature.

As a tentative explanation it could be suggested that in commercial chitosans A and B, obtained by heterogeneous deacetylation of chitin, ordered regions are present, which are compact and insoluble, and involve polymer segments having a degree of acetylation higher than the average.

In fact, chitin has a crystalline structure and the deacetylation under heterogeneous conditions proceeds preferentially in the amorphous region to *c.* 70% of deacetylation, retaining the crystalline structure characteristic of chitin (Kurita *et al.*, 1977). Therefore, chitosans can be considered block-type copolymers, consisting of *N*-acetyl D-glucosamine and D-glucosamine units in various proportions. Light scattering measurements for sample A_R performed at polymer concentration $\simeq 1$ mg ml^{-1}, suggest that the amorphous region, having a large amount of ionic groups NH_3^+, favors deaggregation and complete solubilization of the crystalline areas to give molecularly dispersed solutions. This phenomenon does not occur at the higher concentrations ($\simeq 10$ mg ml^{-1}) at which the hydrolysis is carried out; thus, the properties of chitosan degradation products are related to the ability of the hydrolytic system to break ordered regions during the depolymerization process.

1% HAc, under reflux, leaves these regions substantially unaffected, while preferential depolymerization of the rest of the sample is observed. As hydrolysis proceeds, free crystallites so formed promote association and originate high molecular weight species, partially dissociable upon dilution (samples 2–5).

On the contrary, treatment in 0·6N HCl at 50°C involves total dissolution of the crystalline regions, as shown by the characteristics of all depolymerized samples, including those exhibiting very low intrinsic viscosity (samples 8–11).

Ordered regions are only partially affected during depolymerization in 10% HAc: thus, sample 6, for which the amorphous region is prevalent over the crystalline one, exhibits 'normal' light scattering response. On the contrary, in the highly depolymerized sample 7, species with very high M_w are present, which tend to precipitate, analogous to sample 5. During hydrolysis in 0·6N HCl at room temperature, the depolymerization process is faster than solubilization of crystalline regions. As a result, high diffusion centres are observed in samples 12 and 13, which are not present in samples 14 and 15. Finally, light scattering data relative to samples 16 and 17 suggest that neither beating nor further deacetylation is able to eliminate crystalline regions in commercial chitosans A and B.

CONCLUSIONS

The present viscometric and light scattering investigation shows that chitosan molecules having high degrees of *N*-acetylation (42–28%) are essentially non-associated in 0·1N HAc–0·2M NaCl within the whole range of molecular weights examined (10^4–10^6).

Depolymerization performed under mild conditions (1–10% HAc, reflux) may, in turn, cause multimerization phenomena. If the different light scattering behaviour is due to a structural heterogeneity, this could be possibly evidenced in the solid state. Such an analysis and the correlations between solid state and solution properties will be considered in the accompanying paper.

Highly deacetylated chitosan samples are associated in aqueous saline at acidic pH. However, the data at infinite dilution probably correspond to the true molecular weight.

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