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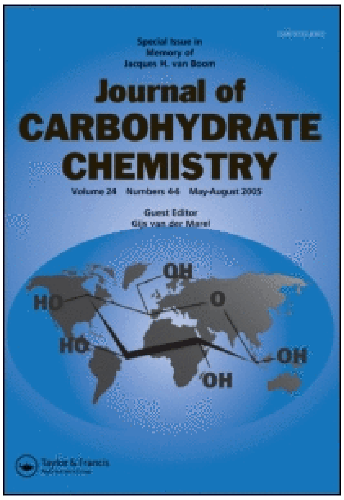

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
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To cite this Article Mazeau, Karim , Pérez, Serge and Rinaudo, Marguerite(2000) 'Predicted Influence of *N*-Acetyl Group Content on the Conformational Extension of Chitin and Chitosan Chains', Journal of Carbohydrate Chemistry, 19: 9, 1269 — 1284

To link to this Article: DOI: 10.1080/07328300008544150

URL: http://dx.doi.org/10.1080/07328300008544150

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J. CARBOHYDRATE CHEMISTRY, 19(9), 1269–1284 (2000)

PREDICTED INFLUENCE OF *N*-ACETYL GROUP CONTENT ON THE CONFORMATIONAL EXTENSION OF CHITIN AND CHITOSAN CHAINS

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Received August 1, 1999 - Final Form September 13, 2000

ABSTRACT

Conformational analysis of chitosan molecules has been performed using the MM3(92) force field to investigate the role played by the acetamido groups on the stiffness of these chains. A high dielectric constant value was needed to model an aqueous environment and to reproduce the distribution of the *N*-acetyl glucosamine group orientation that is observed by NMR. Disaccharidic fragments, differently substituted at C2, were selected as models for chitin and chitosan chains. Their conformational space has been explored by means of adiabatic mapping of the glycosidic Φ,Ψ torsion angles. Although the overall features of all the potential energy surfaces created appear similar, the accessible conformational space of a glycosidic bond is affected by the nature of the substituent at C2 on the non-reducing residue of the disaccharide unit. This is illustrated by the differences in the calculated partition functions together with the predicted average homonuclear and heteronuclear coupling constants. Computed maps were used to predict polymeric unperturbed dimensions, characteristic ratio and persistence length of idealized chitin and chitosan chains, by Monte Carlo methods. Pure chitosan is predicted to be more coiled than pure chitin chains. At low *N*-acetyl group contents, chain extension appears to be dependent on the degree of substitution. Average chain dimensions increase monotonically for increases in content up to 60% of *N*-acetyl groups, but show no significant variation at higher contents. For molecules consisting of 50% amino and 50% *N*-acetylated residues, random, alternate and block patterns of substitution have been investigated. It has also been shown that the spatial extension of the polymer chains is dependent on the primary structure. Comparison with the literature experimental data is difficult because of the extreme diversity of the reported conformationally dependent values. However, such study provides a unique insight into the dependence of these two factors (degree of acetylation and distribution of acetyl groups) on the stiffness and flexibility of different chitin and chitosan chains.

INTRODUCTION

Chitin is one of the most widespread of the naturally occurring polysaccharides. It is a structural polysaccharide found in the exoskeleton of many arthropods. It is composed of β(1→4)-D-glucosamine units with a variable degree of *N*-acetylation (DA). Whereas chitin is an insoluble material in aqueous solution, chitosan, the partially acetylated form of chitin (DA < 60%) becomes soluble in aqueous acid.

The technological applications of chitosan include uses in the food, pharmaceutical, agricultural and cosmetics industries. For rational uses of chitosan, designed for specific applications, it is essential to understand its solution behavior. The question of the intrinsic stiffness of these polymers is still under discussion. They are known to behave in solution as worm-like chains characterized by a persistence length (Lp), but no definite value is accepted at this time. Nevertheless, stiffness is the most important characteristic related to the intrinsic viscosity of the polymer in solution and its thickening properties. Besides the inherent flexibility of the β(1→4) glycosidic linkage, the conformation of the chain is likely to be influenced by several factors including pH and ionic strength. Unfortunately, the influence of the DA on the solution conformation of chitosan in solution remains unclear; this is especially due to the change in polymer solubility in a given solvent as DA varies.[1] There is contradictory experimental literature data on this particular aspect. For example it has been shown, using viscometry and light scattering, that when the degree of acetylation increases, the stiffness of the chain can increase[2-4] or decrease,[1] or even have no effect on chain extension in a limited range of *N*-acetylation (DA< 20%[5] or DA<40%[6]). A general criticism of all the experimental work is that the limited data is not sufficient for discriminating between the effects of polyelectrolyte, excluded volume, solubility and chain stiffness in relation to different degrees of *N*-acetylation. Furthermore, this polymer has a strong tendency to associate, which perturbs experimental measures. The aim of this *study was to investigate how the nature of the functional group at the C-2 position on the* monomeric unit influences the conformational behavior of the glycosidic linkage on disaccharide model compounds, and how this in turn influences the chain expansion for some models of chitin and chitosan molecules.

METHODS

All possible combinations of the two monosaccharides 2-amino-2-deoxy-β-D-glucopyranose (glucosamine) and 2-acetamido-2-deoxy-β-D-glucopyranose (*N*-acetylglucosamine) that occur in chitin and chitosan chains were investigated. This gives four (1→4) linked disaccharides: 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-β-D-glucopyranose (**C1**), 2-amino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose (**C2**), 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-β-D-glucopyranose (**C3**), and 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose (**C4**). These dimeric segments are shown in Fig 1.

The following torsion angles describe the relative orientations of a pair of contiguous residues: Φ = O-5'—C-1'—O-4—C-4 and Ψ = C-1'—O-4—C-4—C-3. For calculations of the (C,H) coupling constants, the torsion angles: ΦH = H-1'—C-1'—O-4—C-4, ΨH = C-1'—O-4—C-4—H-4 are used. The orientation around the C-5'—C-6' and C-5—C-6 bonds is defined by ω' = C-4'—C-5'—C-6'—O-6' and ω = C-4—C-5—C-6—O-6 respectively. The orientation of the acetamido group is described by two torsion angles χ_1 = C-1—C-2—N—C-7 and χ_2 = C-2—N—C-7—C-8.

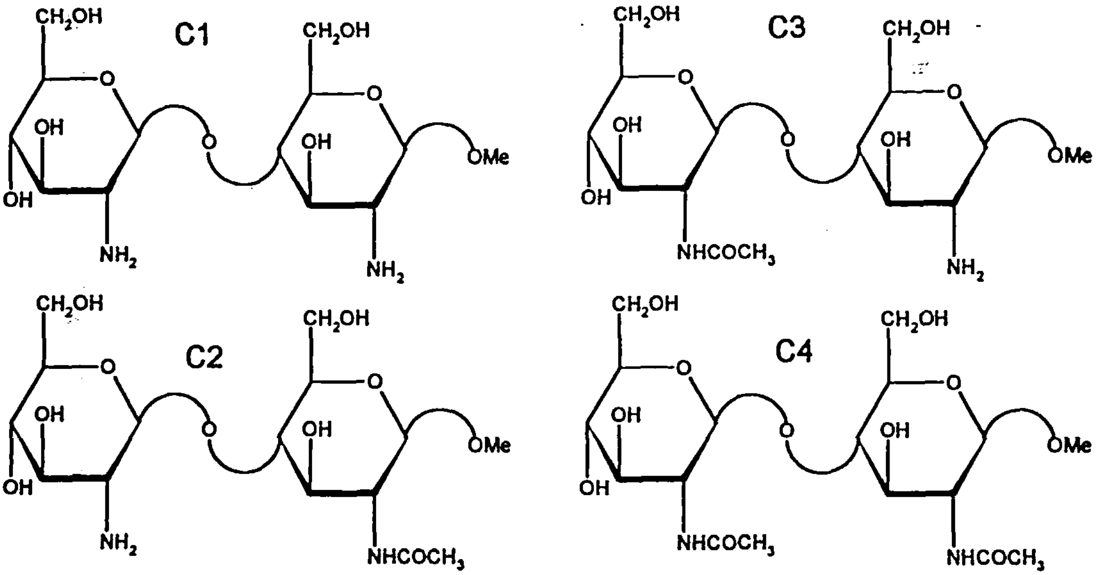

Figure 1. Schematic representation of the disaccharides.

From standard starting geometries of the disaccharides, MM3(92)[8] adiabatic potential (Φ,Ψ) maps were computed. The conformational space was explored by rotating the residues around Φ and Ψ on a 10° grid. At each point of the grid, several possible orientations of the exocyclic groups were taken into account and geometry optimization was carried out while constraining the Φ and Ψ torsion angles. At most 81 starting geometries are needed to account for the three staggered orientations of each hydroxymethyl group (referred as *gg*, *gt* and *tg)*, the two hydrogen-bonding networks (clockwise and counterclockwise) of both the secondary hydroxyl groups and the amino group. For the amino group three possible orientations are possible; two of them imply a clockwise orientation whereas the remaining one occurs for counterclockwise orientation. The preferred orientations of the acetamido group were assessed for the monosaccharide unit by systematically changing the χ_1 and χ_2 torsion angles and by using two different dielectric constants of 4 and 78. From this result, only the two lowest energy orientations of the acetamido groups were considered for the computation of the adiabatic maps. Isoenergy contours are then plotted by interpolation of 1 kcal/mol within a limit of 8 kcal/mol. The exact positions of the minima were obtained after additional unconstrained minimizations.

Ensemble average molecular characteristics were evaluated from the whole maps using statistical mechanics methods. The relative population P_i of each conformer is given by the Boltzmann distribution. Each molecular parameter or conformation-dependent experimental observable was calculated for each conformational microstate (M_i) and the ensemble average parameter M was calculated by $M=\Sigma P_i M_i$

Mean values of the vicinal heteronuclear C,H three bond and one bond coupling constants were then calculated using a Karplus type relationship[9] and published equations.[10,11]

The energy values were used to calculate the partition function of the molecule being studied and the Boltzmann factors needed in carrying out Metropolis Monte Carlo[12] generation of statistical chains, as described in the following section.

The computational strategy and the various assumptions used in the method have been described in detail elsewhere[7] and will be only summarized here. The generation of chitin and chitosan chains was achieved by using a normalized probability associated with each Φ_i, Ψ_j state that corresponds to the MM3(92) potential energy surface of the disaccharide. Ensemble-average chain properties are determined by conformational sampling. The size of the polysaccharide chain is characterized by the dimensionless characteristic ratio quantity. It is defined by the mean-square end-to-end distance ($<r^2>$) normalized by the number (x) of residues of the chain and the square of the virtual bond linking two glycosidic oxygens (L^2). The virtual bond length is 5.44Å.[7] In the limit of infinite chain length the C_x becomes equal to characteristic ratio C_∞ with $C_\infty = \lim C_x = \lim <r^2>/xL^2$.

Another indicator of chain stiffness is the persistence length Lp. It is defined as the average projection of the end-to-end vector of an infinite chain in the direction of the first segment of the chain.

The typical calculations involve averaging for 3000 molecules containing 2000 residues each. Such calculations were repeated ten times at a temperature of 50°C since this temperature was chosen for our experimental work. The sequence of monomers was random, in blocks or alternate.

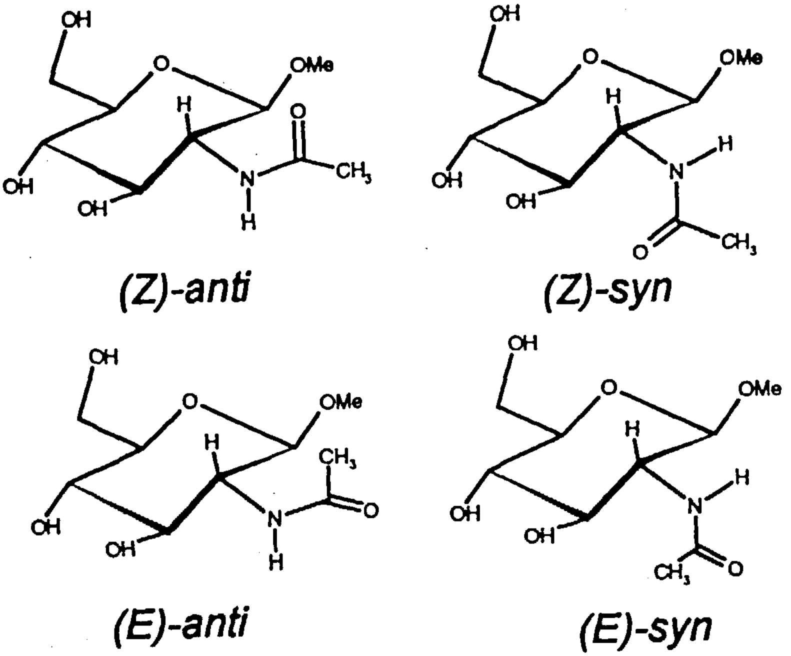

Figure 2. Schematic representation of the different possible conformations of the acetamido group.

RESULTS AND DISCUSSION

Conformation of the N-acetyl-D-glucosamine.

The orientations of the exocyclic groups of the *N*-acetyl-D-glucosamines were established as a function of the dielectric constant values. In principle the acetamido group can adopt four different conformations referred to as (*Z*)-*anti*, (*Z*)-*syn*, (*E*)-*anti* and (*E*)-*syn* (Fig 2). In this nomenclature, (*Z*) and (*E*) designates the staggered orientations of the N—C bond while *syn* and *anti* designates the orientations around the C-2—N bond. For the other pendant groups, both clockwise and counterclockwise orientations of the secondary alcohol groups, as well as the three staggered orientations (*gg*, *gt* and *tg*) of the hydroxymethyl group, were considered.

In Table 1 the occurrence of the different conformations of the exocyclic groups at the different dielectric constant values are reported. A low dielectric constant value favors an equilibrium between clockwise (46%) and counterclockwise (54%) conformation of the

Table 1. Molar fraction of the different staggered orientations of alcohol groups, the *Z*, *E*, *syn*, *anti* conformers of the acetamido group in *N*-acetyl glucosamine as a function of the dielectric constant.

	Clockwise			Counterclockwise		
ε	*gg*	*gt*	*tg*	*gg*	*gt*	*tg*
4	4	1	95	44	48	1
78	25	30	45	33	44	22

	syn	*anti*	*Z*	*E*
4	8.8	91.2	39.5	60.5
78	3.0	97.0	63.0	37.0

secondary hydroxyls. All three conformations of the hydroxymethyl are observed: the *gg* and *gt* in the counterclockwise arrangement and the *tg* in the clockwise one. The (*E*)-anti conformation is preferred for the acetamido group. High dielectric constant values (ε=78) strongly favor the counterclockwise arrangement of the secondary hydroxyls, the population of the *gg*, *gt* and *tg* conformers is 33:44:22. For the acetamido group, the anti conformer is preferred for more than 90% of the equilibrium mixture. The *Z* conformer is the most abundant.

Experimental NMR data favors the equilibrium mixture of the hydroxymethyl groups together with the dominant population of the (*Z*)-anti conformation.[13,14] The use of a dielectric constant of 78 better reproduces these preferences. Moreover such a dielectric constant value is generally used to mimic water solvation. Therefore this value was used for the remaining simulations.

Molecular modeling of the disaccharides

The conformational spaces of the four disaccharides of Figure 1 are needed to build chitin and chitosan chains. Their MM3(92) adiabatic (Φ,Ψ) potential energy surfaces are presented in Figure 3. As expected, these potential surfaces look like those published for

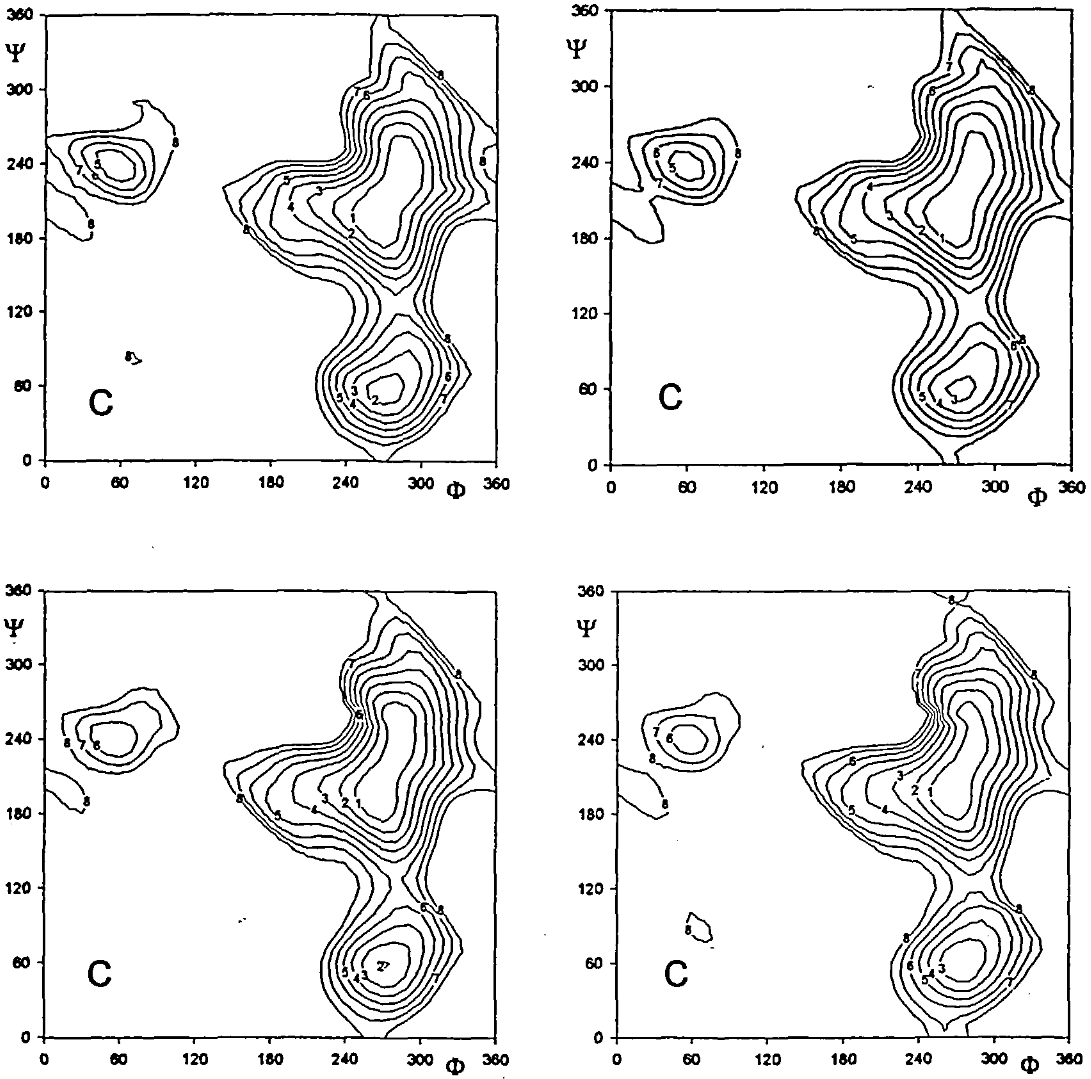

Figure 3. Conformational (ϕ,Ψ) energy maps of the disaccharides.

equatorially-equatorially (1→4) linked disaccharides such as cellobiose[15,16] and chitobiose.[17] Therefore we will only summarize their key features.

Rotations about the ϕ torsion angle are restricted to two different low-energy values in the *-synclinal* domain for the main area and in the *+synclinal* domain for the secondary minimum, as dictated by the exo-anomeric effect.[18] Much more flexibility is exhibited by

Table 2. Geometric (°) and energetic (kcal/mol) features of the lowest energy minimum of each energy well for the four disaccharides.

C1	1	2	3	C2	1	2	3
φ	272.2	273.5	60.1	φ	272.4	273.7	60.2
ψ	188.1	56.3	239.0	ψ	187.6	54.7	237.5
ω'	68.3	68.3	69.0	ω'	68.1	68. 4	68.1
ω	68.5	71.7	63.0	ω	294.0	71.9	296.8
τ	117.0	120.2	118.3	τ	116.9	120.8	118.1
DE	0.0	1.42	4.24	dE	0.0	2.38	4.26

C3	1	2	3	C4	1	2	3
φ	271.1	273.0	59.4	φ	270.0	2732.9	58.2
ψ	188.8	56.2	240.0	ψ	188.8	54.5	239.6
ω'	68.7	68.6	68.2	ω'	69.1	68.9	68.3
ω	68.1	71.4	296.3	ω	293.4	71.6	296.4
τ	116.6	119.9	119.2	τ	117.0	120.8	118.7
DE	0.0	1.62	5.05	dE	0.0	1.70	5.15

the ψ torsion angle, which can span almost the entire angular range, by surmounting a barrier no greater than 9 kcal/mol. Both the adiabatic energy surfaces and the slight difference in the geometrical parameters between the minima suggest a limited effect of the nature of the different substitutions on atoms C-2 and C-2', for 1→4 linked β-D-glucopyranosides (Table 2). All maps show a large potential well (delimited by the first contour) extended in the Ψ direction that encompasses the lowest energy minima. It corresponds to extended conformations near the twofold helix. Libration motion within this surface seems easy for those four compounds. However, the total area covered by the upper contour shows dissimilarity between the four maps. It is larger for **C1** and **C2** and lower for **C3** and **C4**. This suggests that the bulkiness of the acetamido group will affect the flexibility of the adjacent glycosidic bond toward the reducing end. There is a less pronounced effect when the substituant is located on the reducing unit; this is probably due to its spatial location, far from the glycosidic bond.

Concerning the two other low-energy wells, helical propagations from the geometry of the minima predict the four-fold (minimum at Φ,Ψ values of 300°, 60°) and the six-fold helix chains (minimum at Φ,Ψ values of 60°, 300°). Acetylation on the reducing end has no effect, whereas on the non-reducing end it weakly destabilizes the conformations within the region at (60°, 300°). For the four-folded conformers, only **C2** is affected by the *N*-acetylation.

Because of the above-mentioned similarities and differences between compounds differently substituted on carbon 2 and 2', it is expected that the DA together with the distribution of the monomeric units should have some influence on the predicted hydrodynamic parameters of long chains, as we discuss later.

Dissimilarities also appear in the calculated averaged values that are reported in Table 3. As expected from inspection of the maps: the partition function differs. **C1** is the most flexible disaccharide and **C4** the most rigid one; **C2** and **C3** are in between. The averaged values of the $<\Phi_H>$ glycosidic torsion angles are almost identical while $<\Psi_H>$ shows differences. The predicted carbon-proton coupling constants corresponding to the Ψ angle vary significantly from structures **C1** to **C4**.

Conformational statistic of the chains.

From the potential energy surfaces of the parent disaccharides, normalized Boltzmann abundances of each conformational microstate at 50°C could be calculated. Subsequently 3000 chains, having a degree of polymerization of 2000 each, were generated according to a Metropolis Monte Carlo procedure. This configurational sampling, repeated ten times, allows the computation of average properties such as the mean square of the end-to-end distance of the chain, which in turn allows the computation of both characteristic ratio (C_∞) and persistence length (Lp).

Idealized homopolymers of chitosan and chitin chains were generated. Figure 4 shows the chain length dependence of the characteristic ratio. Both curves converge toward their asymptotic limit at a relatively low degree of polymerization, underlying the

Table 3. Predicted averaged characteristic properties: partition function Q (deg^2), virtual torsion angle values (°) coupling constants (Hz), and relative abundance of the three staggered orientations of the hydroxymethyl groups, of reducing end (primed) and non reducing end (unprimed).

	C1	C2	C3	C4
Q	7.9	6.0	6.1	5.2
$<\phi H>$	36.6	34.6	36.2	34.0
$<\psi H>$	337.9	331.5	341.9	333.5
$<{}^3J_{H\text{-}1'—C\text{-}4}>$	3.58	3.74	3.63	3.82
$<{}^3J_{C\text{-}1'—H\text{-}4}>$	4.06	3.79	4.10	3.79
$<{}^1J_{C1'\text{-}H1'}>$	169.50	162.48	162.51	162.48
$<{}^1J_{C4\text{-}H4}>$	146.68	146.71	146.65	146.71
Xω'*gt*	100	93	99	100
Xω'*tg*	0	0	0	0
Xω'*gg*	0	7	1	0
Xω*gt*	93	50	46	13
Xω*tg*	0	0	0	0
Xω*gg*	7	50	54	87

stiffness of these chains. The estimated asymptotic limit of the characteristic ratio is 32.8 for chitosan and 38.9 for chitin, indicating that chitin favors a more extended conformation when compared to chitosan. A much greater calculated C∞ value of 70 was proposed[17] from an adiabatic map that was computed by using 36 initial geometries and a dielectric constant of 4. In the present work 81 initial conformations and a dielectric constant of 78 were used. As a consequence, the relative energies of the predicted minima are different. In our study, folded conformations are predicted to have lower relative energies, they are therefore of higher importance in the equilibrium mixture. On the other hand different temperatures were used. At higher temperatures (our study), the total accessible space is larger. In the Monte Carlo conformational sampling of polymeric chains, the generated chains show greater flexibility and tortuosity, and thus yield smaller C∞ values.

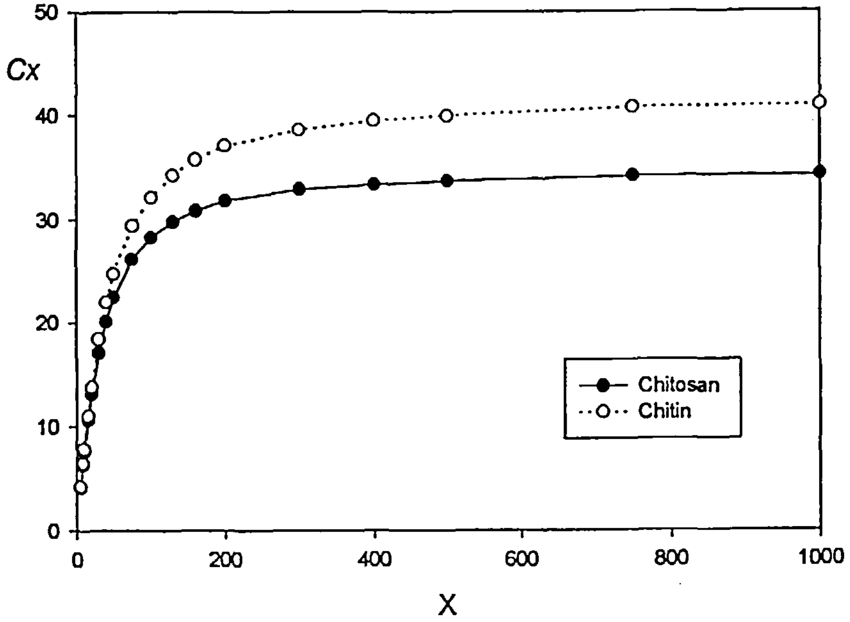

Figure 4. Chain length x dependence on *Cx* for chitosan (unbroken line) and chitin chains (dashed line).

To address the question of the effect of the degree of *N*-acetylation on chain dimensions, chains differing in the amount of *N*-acetyl groups (10, 20, ...90%) were generated assuming a random distribution of the substituents. The dependence of C∞ on DA for the chitosan and chitin chains is shown in Fig. 5. Several points of interest emerge from this dependence. First, at low *N*-acetyl content, between 0 and 60%, the calculated characteristic ratios are sensitive to the *N*-acetyl group content and the values increase as the amount of *N*-acetylated residue increases. Second, values are not significantly different for the range of *N*-acetylation between 60 and 100% for which the curve shows a plateau indicating that, in this range of acetylation, the chain extension is globally the same. The persistence length shows the same behavior. It is predicted to be 90 Å for a DA of 0% and it increases monotonically to its limiting value of 125 Å for 60% DA. Further increases of the *N*-acetyl group content do not perturb the persistence length value.

The distribution of *N*-acetyl groups along the polysaccharide chain has been analyzed. In that respect, chitosans can be classified in three categories. Those derived

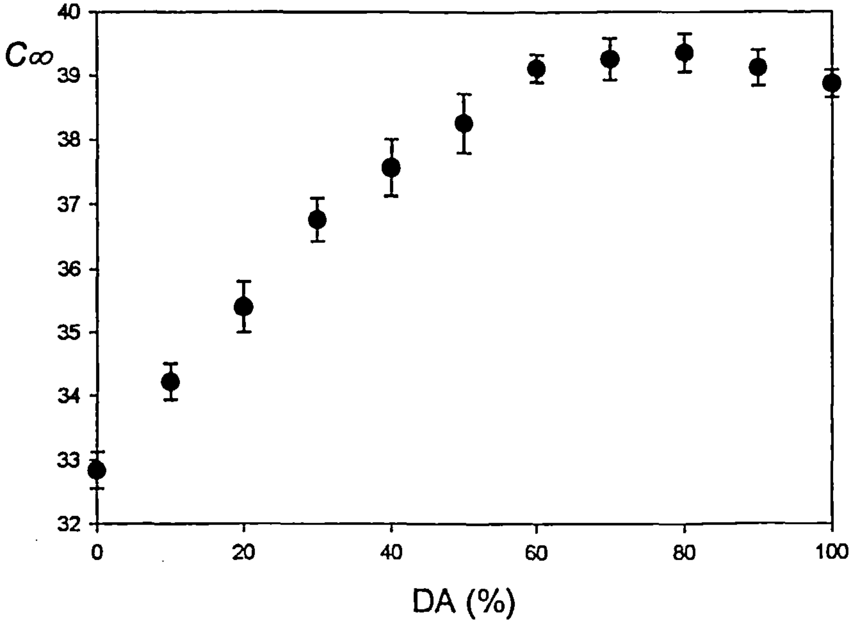

Figure 5. Characteristic ratio as a function of the degree of acetylation (%).

from chitin by deacetylation under homogeneous or heterogeneous conditions, and those derived from chitosans by partial acetylation under homogeneous conditions. Partially *N*-acetylated chitosans derived from highly *N*-deacetylated chitosans can be considered as random-type copolymers whereas moderately *N*-deacetylated chitosans were considered as block-type copolymers.[19] This is in accord with ^{1}H and ^{13}C NMR spectroscopic results which disclosed that the distribution of *N*-deacetylated residues in the chain was random.[20,21] To investigate how the monomeric distribution influences the chain properties, calculations have been performed for chains having 50% glucosamine and 50% *N*-acetylglucosamine residues with different patterns of monomeric distribution, random, alternate or in blocks. C∞ of alternate AB copolymers increases by 15% whereas it decreases by only 2% for the A_2B_2 ones when compared to the random (Bernouillian) chains. Furthermore, there is no significant variation of the characteristic ratio for copolymers having from 2 to 20 consecutive glucosamine and *N*-acetylglucosamine monomers. This suggests that the chain dimensions would be the same for samples having either a random or a block substitution pattern of the *N*-acetyl groups. In contrast, the alternate AB copolymer is stiffer when compared to the others.

Comparison with measured solution properties.

The solubility of chitosan in acidic solution is controlled by the pH, the DA and the distribution of the *N*-acetyl groups along the chain.[19] Different experimental approaches were proposed to access the polymer conformation, mainly from hydrodynamic and light scattering experiments. However, the role of the DA on the viscosity and chain dimensions from an experimental point of view is still under discussion. In the literature data,[22-24] the samples were more or less fractionated and characterized under different solvent conditions. In addition, many of these experiments were perturbed by the presence of aggregates.[22-24] The most effective approaches would be the study of different samples of chitosan under the same experimental conditions. Therefore large variations of the conformational behavior of the polymer may occur because of variations in the total charge of the polyelectrolyte in the absence of screening or due to the decrease of solubility when the degree of acetylation increases. Then, only the estimation of conformation dependent parameters for infinite ionic concentration is relevant. Those values reflect the conformation when all the long range electrostatic interactions are screened. This is a requisite to estimate the relationship between chain stiffness and *N*-acetyl content. In high salt solutions, the experimental measures should be good characteristics of the polymers, if their solubility remains high enough, and can be compared with the present atomic molecular modelling calculations performed for uncharged polymers.

The exponential a parameter of the Mark-Houwink equation is a viscometric constant that characterizes the average conformation of the polymer. It is dependent on the solvent, temperature, chemical structure of the polymer and on the concentration of simple electrolyte present. A higher a value corresponds to greater chain expansion. The value of a for a linear polymer having a random coil conformation is 0.5, when measured in theta conditions, and increases when the stiffness increases in the intermediate molecular weight range.[25]

By using viscometry, values of a=0.47, 0.62 and 0.95 for 0% DA, 15% and 60% *N*-acetylated chitosans respectively extrapolated to infinite salt concentration, have been reported.[3] Another study gave 0.81, 0.88, 0.96 and 1.12 for 0%, 9%, 16% and 31% *N*-acetylated samples in 0.2M CH_3COOH/0.1M CH_3COONa buffer.[4] Both results show the same trends: the more the *N*-acetyl content the greater the expansion. This is in good concordance with the present calculations. Note that absolute experimental values differ considerably but the differences in the experimental conditions might explain the differences observed. It must be mentioned that there is a strong discrepancy on the magnitude of the reported values in the literature.[26,27] Other experiments are needed to evaluate how the expansion of the chain varies with the degree of *N*-acetylation.

An alternative experimental measure of polymer chain extension is the persistence length, which indicates the distance through which the chain can be considered as rigid. The persistence length controls the radius of gyration determined by light scattering and the intrinsic viscosity due to the well-known contribution of the dimensions of the chains.[25]

The Lp values given in the literature are in the range 50 Å to 500 Å. Our calculation gives limiting values of 90 Å and 125 Å for a 0% *N*-acetylated chitosan and a pure chitin, respectively. The variations of Lp are equivalent to that of the characteristic ratio: Lp increases linearly for a degree of acetylation between 0% and 60%; then, no significant variation is observable above DA of 60%. Chitin is soluble in only a few organic solvents (DMAc-LiCl) or strong mineral acids. Using DMAc- 5% LiCl as a solvent, an Lp of 350 Å was given for chitin[29] and 220 ± 20 Å for chitosans[30] from the radius of gyration using the Benoit-Doty relation.[28] This difference suggests that chitosan is less rigid than chitin. For chitosan and carboxymethylchitins, a value of 120 Å was given by hydrodynamic experiments in large salt excess.[31] In another paper, an extremely low value of 25 Å was given for DA = 0% and 15%.[3] Based on multidetector steric exclusion chromatography, Lp was constant in the range of $0\%<DA<21\%$ for a molecular weight between 10^5 and 10^6; Lp was found equal to 50 Å from the radius of gyration analysis experiments and 80 Å from intrinsic viscosity measurements.[5] In recent experiments, in which chitosan was carefully purified, the multidetector steric chromatography gives an Lp value on the order of magnitude with the prediction given in this work; this study is in progress in this laboratory.[32]

The theoretical results from this work support the proposal that chitosan is semi-rigid with intrinsic persistence lengths between 90 and 125 Å, depending on the degree of acetylation.

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