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^{13}C-N.m.r. studies of the acetylation sequences in partially *N*-deacetylated chitins (chitosans)*

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ABSTRACT

Chitosans obtained under homogeneous conditions of *N*-deacetylation, with degrees of *N*-deacetylation between 46% and 94%, were depolymerised and their 125-MHz ^{13}C-n.m.r. spectra have been interpreted. The sequence of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) residues influenced the chemical shifts, and the diad and triad frequencies have been calculated. Chitosans that were *N*-deacetylated under homogeneous and heterogeneous conditions gave values for the diad and triad frequencies that were consistent with a random arrangement of GlcN and GlcNAc residues.

INTRODUCTION

Chitosan, prepared commercially by *N*-deacetylation of chitin, is composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose residues and may be considered as a binary heteropolysaccharide. ^{1}H-N.m.r. spectroscopy has been used to determine the degree of *N*-acetylation (d.a.) and the diad frequencies of chitosans[1]. Due to their long-range sensitivity to conformation, particular ^{13}C resonances may have different chemical shifts, depending upon the nature of the neighbouring units. For alginate, another binary heteropolysaccharide, the biofunctional properties and technical applications are correlated strongly with the composition and sequence of the monomeric units[2].

Chitosan may be *N*-deacetylated under homogeneous or heterogeneous conditions[3], and earlier reports suggested that the product obtained under the latter conditions had a more blockwise distribution of *N*-acetyl groups[4]. Diad frequencies, determined by ^{1}H-n.m.r spectroscopy[1], showed that chitosans prepared under homogeneous conditions had a random distribution of *N*-acetyl groups, whereas samples prepared under heterogeneous conditions had a frequency of the GlcNAc–GlcNAc diad that was slightly higher than for a random distribution. However, where the binary heteropolysaccharide has a blockwise distribution of the units[5], it is possible that the diad frequencies could be consistent with a Bernoullian arrangement. Such a block structure

* High-field N.m.r. Spectroscopy of Partially *N*-Deacetylated Chitins (Chitosans), Part II. For Part I, see ref. 1.

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can be revealed only by measuring triad frequencies. We now report the ^{13}C-n.m.r. spectra of partially *N*-deacetylated chitins, and the diad and triad frequencies of samples obtained under conditions of homogeneous and heterogeneous *N*-deacetylation.

EXPERIMENTAL

Materials. — Chitin, isolated[6] from fresh shrimp shell, was milled in a hammer mill to pass through an 80-mesh sieve. Chitosan fractions with different degrees of *N*-acetylation (d.a.) were prepared as described[1]. The d.a. was determined from the areas of the resonances for C-1,5,6.

Samples were degraded[1] to a $\overline{d.p.}_n$ between 14 and 20.

N.m.r. spectroscopy. — The samples were dissolved in D_2O in 5-mm tubes at pD 4 (70 mg/mL). The deuterium resonance was used as a field-frequency lock and the chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-d_4 (0.00 p.p.m.). 125-MHz ^{13}C-n.m.r. spectra were recorded with a Bruker WM-500 spectrometer, using 64K data points and a spectral width of 11 363 Hz. A pulse angle of 52° was used, with a recycling time of 1.4 s. The spectra were recorded at 90° in order to diminish the viscosity and thereby the line width. The relative intensities of ^{13}C resonances were determined by a computer program developed by O. E. Bakøy in our laboratory.

RESULTS AND DISCUSSION

The 125-MHz proton-decoupled ^{13}C-n.m.r. spectrum (Fig. 1) of a 54% *N*-acetylated chitosan (F_A 0.54) contains multiplets that reflect the sequence of GlcN (D) and GlcNAc (A) units. The spectrum showed considerable complexity, and the resonances were identified on the basis of heteronuclear (^{13}C–^{1}H) shift-correlated, 2D-n.m.r. experiments on chitosan samples with degrees of acetylation within the range 54% (F_A 0.54) to 6% (F_A 0.06). The $NCOCH_3$ resonance from the A units appeared at low field (176.9 p.p.m.).

Fig. 2 shows the region (expanded) for C-1 resonances of these samples and reveals seven relatively well-resolved resonances that reflect the triad frequencies. These resonances can be explained only by assuming that the C-1 resonances of D and A are sensitive to both nearest-neighbour units. The resonances associated with the four possible D-centered triads (DDD, ADD, DDA, and ADA) appear at ~ 100 p.p.m. since these signals are dominant at low d.a. (F_A 0.06). The resonances associated with the four possible A-centered triads (AAA, DAA, AAD, and DAD) appear at ~ 103 p.p.m. since these signals become dominant at a high d.a. (F_A 0.54). These identifications were confirmed by 2D-heterocorrelated n.m.r. spectroscopy (COSY). The chitosan samples with various d.a. values were prepared under homogeneous conditions of *N*-deacetylation, which give products with a nearly random distribution of *N*-acetyl groups[1].

Identification of the D-centered triads (δ 99.5–100). — The dominant line for a chitosan with F_A 0.06 (almost complete *N*-deacetylation) must be associated with the

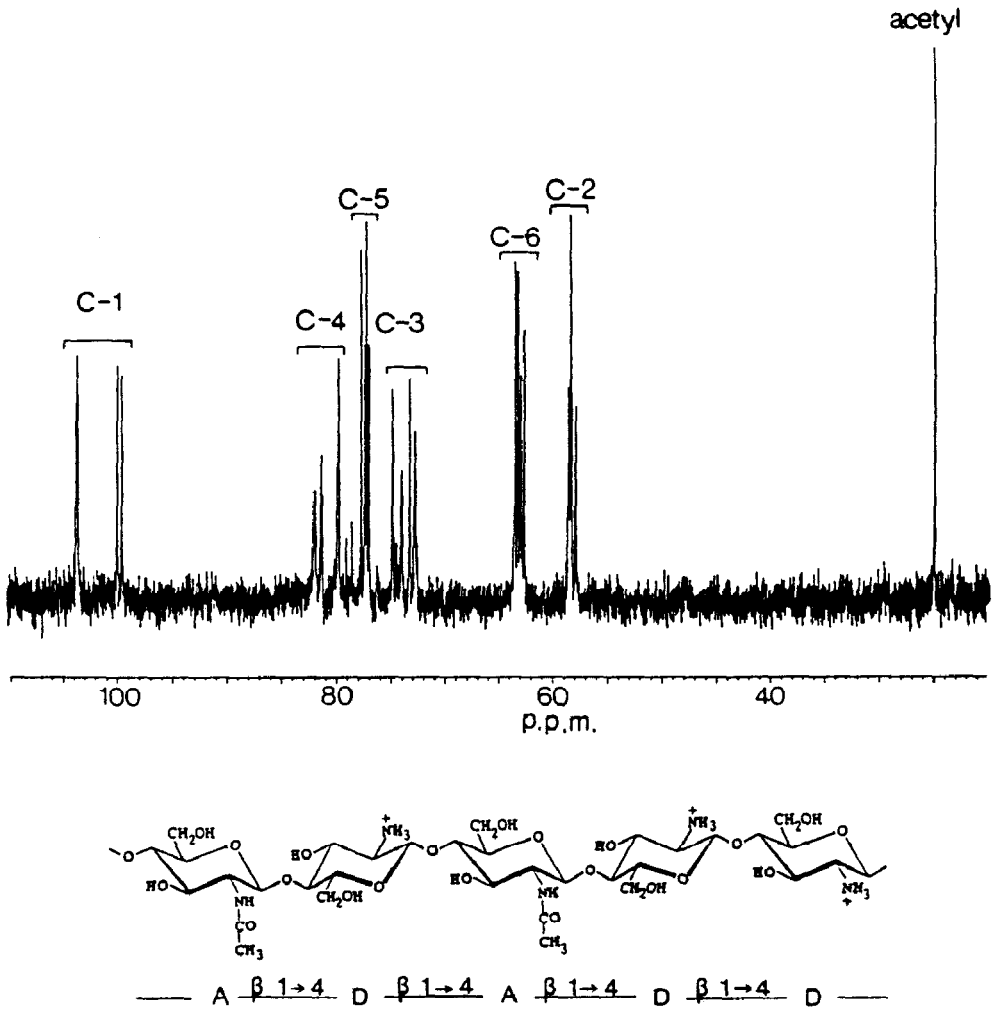

Fig. 1. ^{13}C-N.m.r. spectrum (125 MHz) of a solution (70 mg/mL) of chitosan in D_2O (at pD 4 and 90°).

DDD sequence and probably with another D-centered triad, as this line gradually becomes broader as the d.a. increases. *N*-Deacetylation initially introduces single D units which are identified by ADA triads. As the reaction proceeds, blocks of contigous D units (DDD) are formed, and their termination gives rise to the triads ADD and DDA. Therefore, the downfield line at $\delta \sim 99.5$, which is dominant at F_A 0.54 but minor at F_A 0.27, must be due to the triad ADA. The upfield line at $\delta \sim 99.5$ must be due to the termination (DDA) or beginning (ADD) sequence of D blocks, as it becomes gradually more dominant at lower d.a., and it has been identified tentatively as due to DDA. Consequently, the ADD triad must be in the DDD triad, as this line probably contains another triad (as noted above). The identification of the triads ADD and DDA may be interchanged, but their frequencies will be identical in a long chain. It is likely that the

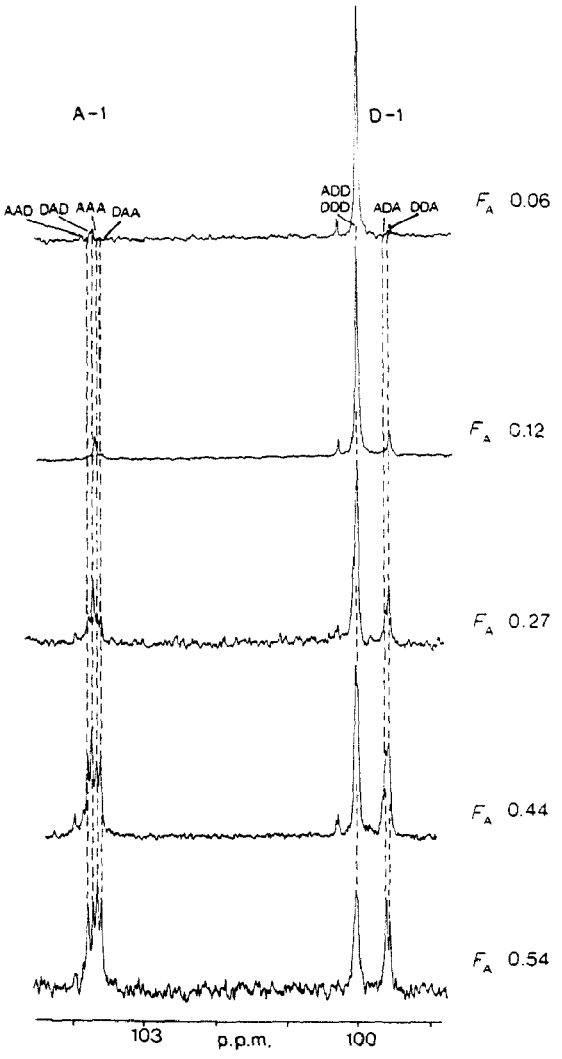

Fig. 2. ^{13}C-N.m.r. spectra in the region for C-1 resonances of chitosans with different d.a.

resonances of C-1 are most sensitive to the following residue in the chain, which is the basis for the proposed identification of DDA and ADD triads. The weak resonance at 100.3 p.p.m. is probably due to an end signal, as this line shows no change as the d.a. is varied.

Identification of the A-centered triads (δ 103.6–103.7). — The dominant line at F_A 0.54 must be due to the AAA triad (second line from the right in Fig. 2), since its relative intensity gradually becomes the smallest as the d.a. decreases. As *N*-deacetylation proceeds, the probability of finding a single *N*-acetylated unit (DAD) increases, thereby identifying the third line from the right as due to the DAD triad. Consequently, the high- and low-field C-1 resonances of an A residue must be the termination (AAD) and

beginning (DAA) sequence of A blocks. On the assumption that the C-1 resonances are most sensitive to the following residue in the chain, the DAA and AAD triads were assigned tentatively as indicated in Fig. 2.

Identification of resonances of C-2/6. — Figs. 3 and 4 show the expanded region for C-3,4,5 and C-2,6 resonances, respectively, for chitosans with different d.a. values. It is likely that the C-3/6 resonances are affected most by the preceding residue in the chain and those of C-1,2 by the following residue. The assignment of the four diad frequencies from the C-5 (Fig. 3) and C-6 resonances (Fig. 4) is based on the fact that the A*A* diad dominates at high d.a. while the D*D* diad dominates at low d.a. The assignment of diads D*A* and A*D* is tentative and may be interchanged. The same diad sequence is obtained from the C-5,6 resonances [D*D* shifts downfield when the preceding D unit is replaced by an A unit (A*D*), and A*A* shifts upfield when the preceding A unit is replaced by a D (D*A*)].

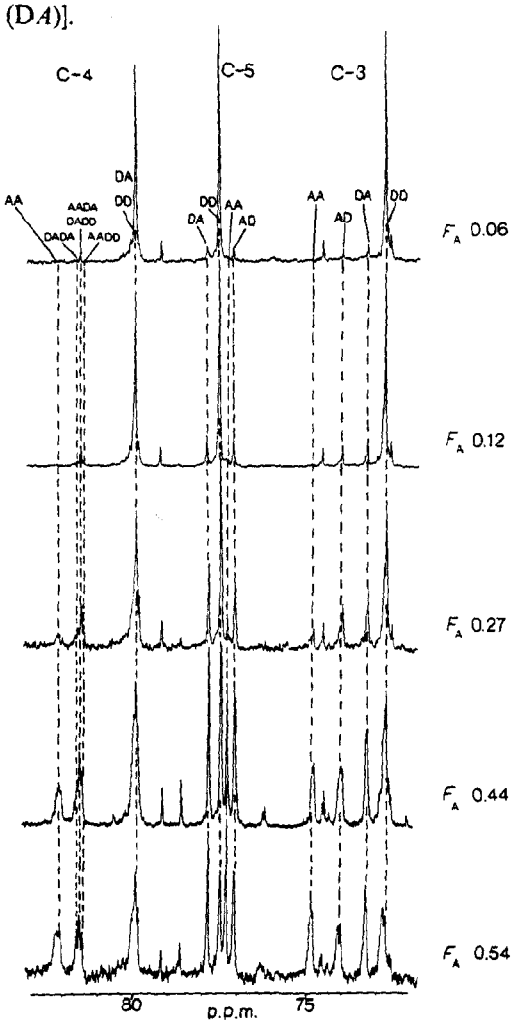

Fig. 3. ^{13}C-N.m.r. spectra in the region for C-3,4,5 resonances of chitosans with different d.a.

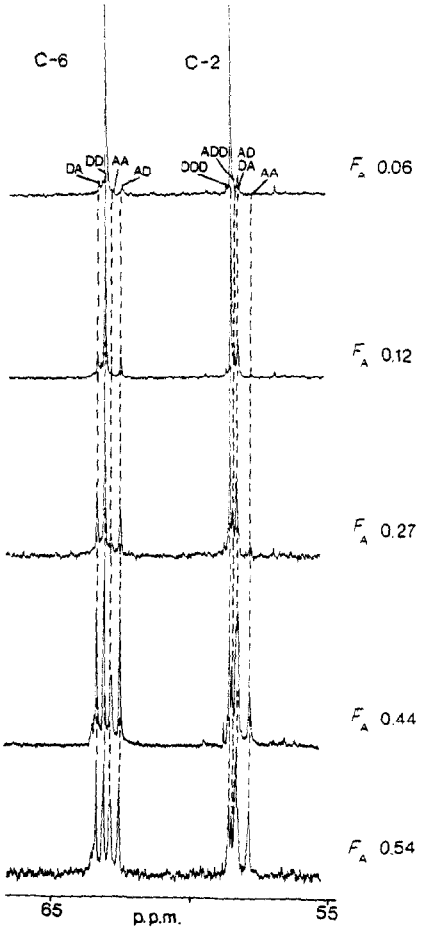

Fig. 4. ^{13}C-N.m.r. spectra in the region for C-2,6 resonances of chitosans with different d.a.

The identification of the C-3,4 resonances (Fig. 3) needs comment. The C-3 resonances give relatively broad lines, but their identification is straightforward. The C-4 resonances comprise three major lines, where those associated with the D*D* and D*A* diads occur most upfield (~79.8 p.p.m.), and those associated with the A*A* diad occur most downfield (~82 p.p.m.). However, the resonance associated with the A*D* diad (at 81.3 p.p.m.) is further split into three lines, and must contain information on tetrad sequences. The four possible tetrads are AA*D*A, AA*D*D, DA*D*A, and DA*D*D (A*D* is the diad from which they originate). Since two of the resonances are of similar intensity at all d.a. values, they must contain the same number of A and D units. The only candidates are AA*D*D and DA*D*A. The middle resonance at 81.3 p.p.m. must be due to DA*D*D together with AA*D*A, since its intensity is larger than those due to AA*D*D and DA*D*A at all d.a. Although the C-4 resonance does not give all four diads, it is the only resonance that contains information on tetrads.

The resonances at 79.2 and 78.7 p.p.m. are probably due to end signals, as their intensities do not vary as the d.a. is changed.

C-2 gives four resonances but, from their relative intensities, they cannot be due to the four diads. The resonances associated with the *A*A diad appear most upfield (~57.8 p.p.m.). The broad middle resonance (~58.3 p.p.m.) is due to the diads *A*D and D*A*. The most downfield resonance (~58.5 p.p.m.) must be due to the DDD triad, since it is dominant at low d.a. The sharp middle resonance is most probably due to another D-centered triad. The possibilities are ADD, DDA, and ADA. Based on the intensity of the line at low d.a., ADA can be ruled out, and the tentative preference is for ADD.

Table I gives the chemical shifts of the various ^{13}C resonances.

Quantification of diad frequencies from the C-5,6 resonances. — The diad frequencies have been calculated from the C-5,6 resonances, each of which shows four relatively well separated lines that reflect the four diad frequencies (F_{AA}, F_{AD}, F_{DA}, and F_{DD}). The results are given in Table II. The results again show that chitosans prepared under conditions of homogeneous and heterogeneous *N*-deacetylation have a random distribution of A and D residues. ^{1}H-N.m.r experiments[1] indicated that chitosans obtained under heterogeneous conditions of *N*-deacetylation had a slightly more blockwise distribution compared to those prepared under homogeneous conditions, whereas the diad frequencies in Table II accord with a random distribution. The d.a. values calculated from the ^{13}C-n.m.r. spectra agreed with those calculated from ^{1}H-n.m.r. spectra. In addition, the internal consistency that concerned the equal intensity of all resonances of the same residue was satisfactory and indicated that the relative areas of the lines represented the relative occurrence in chitosan, as reported for the ^{1}H-n.m.r. spectra[1].

Quantification of triad frequencies. — A binary heteropolysaccharide with a blockwise distribution of the units may contain diad frequencies that are consistent with a Bernoullian arrangement[5]. By measuring the triad frequencies, such block structures may be ruled out, and Table III gives the triad frequencies for chitosans obtained under homogeneous conditions of *N*-deacetylation. The number-average block lengths from which all the singlets (*i.e.*, ADA and DAD) have been excluded[7] are also given in Table

TABLE I

Chemical shifts[a] of ^{13}C resonances for solutions of partially *N*-deacetylated chitosans in D_2O at 90° (pD 4)

Residue	*Sequence*	*Carbon atom*						
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*NAc*
A	AA	103.6	57.8	74.7	82.0	77.2	62.8	24.5
	AD	103.7	58.3	73.8	81.3	77.0	62.5	24.8
D	DD	100.0	58.5	72.6	79.8	77.3	63.1	—
	DA	99.5	58.3	73.1	79.8	77.7	63.4	—

[a] In p.p.m. downfield from the resonance of internal sodium 3-(trimethylsilyl)propionate-d_4.

TABLE II

Distribution of diad frequencies (from the C-5,6 resonances) in partially *N*-deacetylated chitosans as determined by ^{13}C-n.m.r. spectroscopy

Sample	F_{AA}	F_{AD}	F_{DA}	F_{DD}	$\bar{N}_A$	$\bar{N}_D$
N-Deacetylation under homogeneous conditions (aqueous 10% NaOH, 25°)						
1	0.28	0.24	0.23	0.24	2.3	2.0
random (F_A 0.54)	0.29	0.25	0.25	0.21	2.2	1.8
2	0.19	0.24	0.26	0.31	1.8	2.2
random (F_A 0.44)	0.19	0.25	0.25	0.31	1.8	2.2
3	0.08	0.17	0.22	0.53	1.6	3.3
random (F_A 0.27)	0.07	0.20	0.20	0.53	1.4	3.7
4	–	0.09	0.14	0.76	1.3	6.3
random (F_A 0.12)	0.01	0.11	0.11	0.77	1.1	8.0
5	0.00	0.05	0.08	0.87	1.1	11.8
random (F_A 0.06)	0.00	0.05	0.05	0.89	1.1	18.9
N-Deacetylation under heterogeneous conditions (aqueous 50% NaOH, 70°)						
20 min	0.28	0.27	0.21	0.24	1.9	2.3
random (F_A 0.51)	0.26	0.25	0.25	0.24	2.0	2.0
40 min	0.08	0.22	0.20	0.51	1.2	3.7
random (F_A 0.27)	0.07	0.20	0.20	0.53	1.4	3.7
80 min	0.02	0.15	0.10	0.73	0.7	9.0
random (F_A 0.10)	0.01	0.09	0.09	0.81	1.1	10.0

TABLE III

Distribution of triad frequencies in partially *N*-deacetylated chitosans as determined by ^{13}C-n.m.r. spectroscopy

Sample	F_{AAA}	F_{AAD}	F_{DAA}	F_{ADA}	$F_{ADD}=F_{DDA}$	F_{DAD}	F_{DDD}	$\bar{N}_{D>1}$	$\bar{N}_{A>1}$
N-Deacetylation under homogeneous conditions (aqueous 10% NaOH, 25°)									
1	0.15	0.15	0.13	0.14	0.08	0.10	0.17	4.1	2.9
random (F_A 0.54)	0.15	0.13	0.13	0.13	0.12	0.12	0.10	2.9	3.2
2	0.10	0.12	0.12	0.12	0.12	0.14	0.17	3.5	2.9
random (F_A 0.47)	0.11	0.12	0.12	0.12	0.13	0.13	0.15	3.1	2.9
3	0.03	0.05	0.06	0.07	0.11	0.14	0.44	5.9	2.9
random (F_A 0.27)	0.01	0.05	0.05	0.05	0.14	0.14	0.39	4.7	2.4
4	0.01	0.02	0.01	0.01	0.09	0.09	0.68	9.3	2.0
random (F_A 0.12)	0.00	0.01	0.01	0.01	0.09	0.09	0.68	9.3	2.0
5	—	—	0.01	0.01	0.04	0.05	0.86	24.1	—
random (F_A 0.06)	0.00	0.00	0.00	0.00	0.05	0.05	0.86	18.5	2.0
N-Deacetylation under heterogeneous conditions (aqueous 50% NaOH, 70°)									
20 min	0.11	0.14	0.12	0.15	0.10	0.14	0.13	2.5	3.6
random (F_A 0.51)	0.13	0.13	0.13	0.13	0.12	0.12	0.12	2.9	3.1
40 min	0.02	0.07	0.06	0.11	0.13	0.12	0.36	4.8	2.6
random (F_A 0.27)	0.05	0.05	0.05	0.05	0.14	0.14	0.38	4.9	2.6
80 min	—	—	0.01	0.09	0.11	0.08	0.61	7.4	—
random (F_A 0.10)	0.00	0.01	0.01	0.01	0.08	0.08	0.73	11.1	2.0

III. For each fraction, the triad frequencies for a random distribution of triads have been calculated. The calculated triad frequencies and block lengths for chitosans prepared under homogeneous conditions of *N*-deacetylation are roughly consistent with a random arrangement of the units. Since the C-1 resonances are partly overlapped (see Fig. 2), the accuracy in the determination of the triad frequencies is at best ±15%.

Table III also contains the triad frequencies for chitosans prepared under heterogeneous conditions of *N*-deacetylation together with the number-average block lengths. The triad frequencies are roughly consistent with a random distribution of A and D units in accord with the results from ^{1}H-n.m.r. spectroscopy[1]. From the experimental triad frequencies, it is possible to calculate a number-average block length [$\bar{N}_{(>1)}$], from which all the singlets (*i.e.*, DAD and ADA) have been excluded[7]. The calculated block lengths are given in Table III, together with the corresponding values predicted by Bernoullian statistics.

It is concluded that, since both diad and triad frequencies show a Bernoullian distribution, the GlcNAc (A) and GlcN (D) residues are distributed randomly in chitosans prepared under homogeneous and heterogeneous conditions of *N*-deacetylation in the laboratory. However, preliminary investigations of commercial chitosans, which were *N*-deacetylated using a much less finely ground chitin than in our study, indicated that commercial fractions with lower d.a. (<30%) also had a random distribution of *N*-acetyl groups.

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