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CONFORMATIONAL PROPERTIES OF CHITO-OLIGOSACCHARIDES: TITRATION, OPTICAL ROTATION, AND CARBON-13 N.M.R. STUDIES OF CHITO-OLIGOSACCHARIDES

SATOSHI TSUKADA AND YASUO INOUE
Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo (Japan)

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ABSTRACT

Several chito-oligosaccharides [β-(1→4)-linked oligosaccharides of 2-amino-2-deoxy-D-glucose] have been studied. A semi-empirical treatment of titration and rotation data indicates that the glycosidic torsion-angles ($\Delta\Phi$ and $\Delta\Psi$) are all closely similar throughout the oligomers and also in the polymer (chitosan), in their fully protonated forms. The microscopic, thermodynamic dissociation-constants are evaluated from data based on the ^{1}H-n.m.r. titration of chitobiose at 25°. We also measured the ^{13}C-chemical shifts in chitobiose. With the exception of the C-1 and C-4′ resonances of chitobiose, the observed chemical shifts are in good agreement with those calculated from δ (chitobiose) = δ (cellobiose) + δ (2-amino-2-deoxy-glucose) − δ (glucose). The discrepancy is utilized for further conformational analysis. On the basis of these observations and of the exoanomeric effect, we consider that these deviations can be accounted for by a change in the glycosidic torsion angle ($\Delta\Psi$) of chitobiose from that of cellobiose.

INTRODUCTION

Polysaccharides having 1e,4e-linkages, for example, cellulose and chitin, are important structural materials in Nature. For examining the differences in the conformational features brought on by substitution of the 2-OH group in the glucose residues in cello-oligosaccharides by the 2-NH_3^+ group, we have undertaken conformational studies on several chito-oligosaccharides, β-(1→4)-linked oligomers of 2-amino-2-deoxy-D-glucopyranose. Several methods have been exploited for imposing restrictions on conformations in aqueous solution. First, potentiometric titration of several chito-oligosaccharides, [D-GlcNH$_3^+$-β(1→4)-D-GlcNH$_3^+$-β-(1→4)-]$_n$ in which n = 1, 2, 3, 4, 5, and 6, was used to analyze the effects of chain alterations in the chito-oligosaccharide upon the ionization of its amino group, as titration data for chitobiose and its higher oligomers studied here have not been reported before. Secondly, by combining the proton nuclear magnetic resonance (^{1}H-n.m.r.) titration data, based on the analysis of the pD-dependent chemical shifts

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of chitobiose, and the macroscopic, overlapping pK values, the microscopic thermodynamic dissociation constants of chitobiose have been determined. The overall shape of the simplest-chito-oligosaccharide, chitobiose, in solution is defined by the two dihedral angles $\Delta\Phi$ and $\Delta\Psi$. A schematic diagram of chitobiose is given in Fig. 1, together with the atom numbering and the torsion angles relevant for this discussion. We also use the term *linkage conformation*, which has been previously defined by Rees[1,2] to denote a particular pair of values for $\Delta\Phi$ and $\Delta\Psi$. Next, the molecular rotations of chitobiose and higher chito-oligosaccharides have been measured to impose certain restriction on the linkage conformations, based on Rees' method of linkage rotation[1], and have indicated that the glycosidic torsion-angles, $\Delta\Phi$ and $\Delta\Psi$, per inter-residue seem to be similar throughout the β-(1→4)-linked oligomers and even in the polymer of 2-amino-2-deoxy-D-glucose, in their protonated forms. It would be desirable to obtain further information by other methods of spectroscopy, and the development of carbon-13 nuclear magnetic resonance (^{13}C-n.m.r.) techniques should be useful in deriving approximate values of $\Delta\Phi$ and $\Delta\Psi$. Recently, ^{13}C-n.m.r. spectroscopy has been shown to be applicable to the study of the linkage conformation of disaccharides[3-7]. We have estimated the average linkage-conformation of chitobiose by considering how the linkage conformation (or the C-1 and C-4′ chemical-shift values) is influenced on passing from cellobiose to the configurationally related chitobiose.

In the absence of an X-ray crystal structure for chitobiose and higher chito-oligosaccharides, the conformation based on the data obtained in this study should be considered tentative. Nevertheless, all of the results seem to indicate that chito-oligosaccharides and chitosan all have highly extended, backbone conformations and are constrained to adopt $\Delta\Phi$ and $\Delta\Psi$ values within the narrow area indicated in this paper.

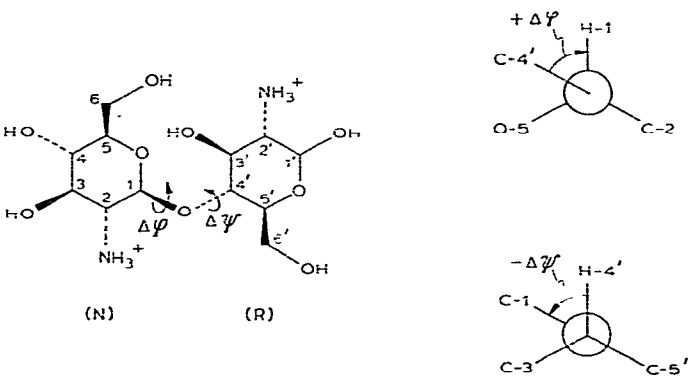


Fig. 1. Schematic diagram of chitobiose. Note that the atom numbers in the reducing residue are primed. The definition of the two torsional angles about glycosidic linkages is also shown: The 0° torsional angle about the C-1–O bond ($\Delta\Phi$) is represented by the C-1–H-1 bond *cis*-planar to the O–C-4′ bond with respect to rotation about the C-1–O bond ($\Delta\Phi$). The 0° angle about the O–C-4′ bond ($\Delta\Psi$) is represented by the O–C-1 bond *cis*-planar to the C-4′–H-4′ bond with respect to rotation about the O–C-4′ bond. The angles $\Delta\Phi$ and $\Delta\Psi$ are considered positive for a right-handed rotation; when viewing along any bond, the far bond rotates clockwise relative to the near bond.

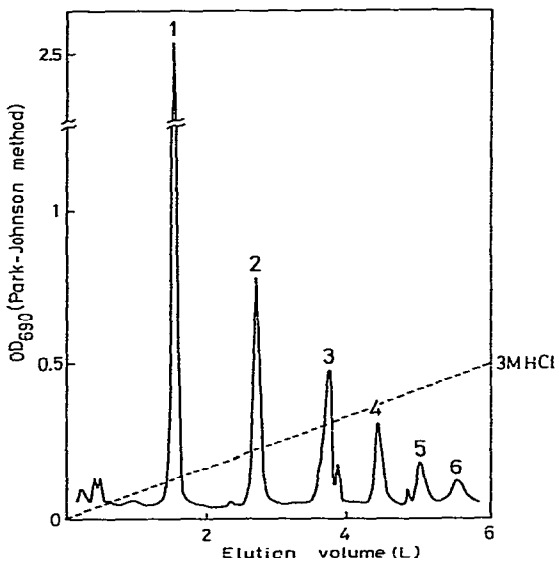


Fig. 2. Chromatographic separation of chitosan hydrolyzate [GlcNH$_2$-β-(1→4)]$_n$ by gradient ion-exchange according to the chain length. The ordinate expresses absorbance at 690 nm by the method of Park and Johnson (see text for details).

EXPERIMENTAL

Materials. — Graded hydrolysis of chitosan (completely *N*-deacetylated chitin) was employed for preparation of chito-oligosaccharides. Chitosan (2 g), was partially hydrolyzed with ~11M hydrochloric acid (500 mL) for 42 h at 53°. The partial hydrolyzate was then applied to a column (4 × 31 cm) of pre-equilibrated Dowex 50W × 2 (H^+), after removal of hydrochloric acid by a rotary evaporator under diminished pressure. The column was then eluted with 6 L of a 0.01–3.0M hydrochloric acid linear gradient at a flow rate of ~70 mL/h. Fractionation according to chain length yielded six discrete peaks. The results are shown in Fig. 2. The material of peak 1 was identified as 2-amino-2-deoxy-D-glucose. The material in peak 2 was isolated and characterized by ^{1}H- and ^{13}C-n.m.r. methods. Experiments were performed to determine the chain length of the various components: pooled fractions were analyzed for GlcN content and for reducing sugar by the method of Park and Johnson[8]. All other reagents were of analytical grade.

Methods. — Potentiometric titrations were performed under nitrogen in a magnetically stirred, thermostatted vessel by using a Radiometer expanded-scale pH meter Model PHM 26 (Radiometer, Copenhagen) fitted with an internally shielded, Radiometer combination electrode No. GK2301C. The pH meter was calibrated with 0.05M potassium hydrogenphthalate and 0.05M sodium borate solutions at 25° before and after each set of measurements. A solution (2–3mM, 3.50 mL) in carbon dioxide-free, glass-distilled water was titrated with standard 60mM sodium

hydroxide (carbonate-free) in steps of one-twentieth equivalent by using an all-glass, micrometer syringe. The apparent pK values, defined as $pK_{app} = pH + \log(1 - \alpha)/\alpha$, where α is the degree of dissociation, were calculated by fitting the observed data to the simple Henderson–Hasselbalch equation[9]. Calculations of pK_1 and pK_2 values (see Appendix for the definition of K_1 and K_2) of chitobiose from titration curves were made by the iterative, least-squares method described previously[10]. The dissociation constants thus obtained were converted into the thermodynamic, macroscopic dissociation-constants by the equation:

$$pK_1^T = pK_1 - \frac{1.535\sqrt{I_{1/4}}}{1 + 1.6\sqrt{I_{1/4}}} \quad \text{and} \quad pK_2^T = pK_2 - \frac{0.512\sqrt{I_{3/4}}}{1 + 1.6\sqrt{I_{3/4}}},$$

where $I_{1/4}$ is the ionic strength at $\alpha = 1/4$ or the one-fourth-point of the titration (that is, for a 2.76mM titration, $I_{1/4}$ is 3.49×10^{-3}), and $I_{3/4}$ is the ionic strength at $\alpha = 3/4$ (that is, for a 2.76mM titration, $I_{3/4}$ is 2.80×10^{-3}). The end-point of the second equivalent in the titration of chitobiose was determined by finding the point at which the second derivative of the titration curve was equal to zero.

^{1}H-N.m.r. spectra were obtained with a Bruker 270-MHz instrument operating in the Fourier-transform mode at a probe temperature of 25 ± 1°. The concentration of chitobiose was 0.1mM. Chemical shifts are reported relative to the methyl resonance of sodium 4,4-dimethyl-4-silapentane-1-sulfonate. The ionic-strength correction was applied to the pK data obtained from the ^{1}H-n.m.r.–pD titration of chitobiose by using thermodynamic constants, K_1^T and K_2^T, obtained from the potentiometric titration.

^{13}C-N.m.r. spectra were obtained with a Bruker WH-270 spectrometer operating at a frequency of 67.9 MHz. The Fourier-transform mode was used with proton decoupling. ^{13}C-Chemical shifts were measured relative to 1,4-dioxane, but are reported in parts per million relative to tetramethylsilane. The ^{13}C nuclei of 1,4-dioxane are 67.4 p.p.m. less shielded than those of tetramethylsilane. Chitobiose was recorded in D_2O at a concentration of ~20 mg per mL (50mM; 11000 accumulations).

RESULTS AND DISCUSSION

Potentiometric titration studies of chito-oligosaccharides. — The chito-oligosaccharides have been prepared[11], but their dissociation constants have not yet been reported. The potentiometric titrations were performed at 25 ±0.1° by the addition of carbonate-free 0.06M sodium hydroxide at an ionic strength identical with that of the sample solution.

The degree of dissociation, α, defined as the fraction of amino group dissociated, was determined for each addition of titrant. Titration data thus obtained are shown in Fig. 3 as $pK_{app} = pH + \log(1 - \alpha)/\alpha$ *vs.* α. The dissociation of 2-amino-2-deoxy-D-glucose cation may be described by a single dissociation constant (K) as expressed by:

$$pK = pH + \log\frac{1 - \alpha}{\alpha}.$$

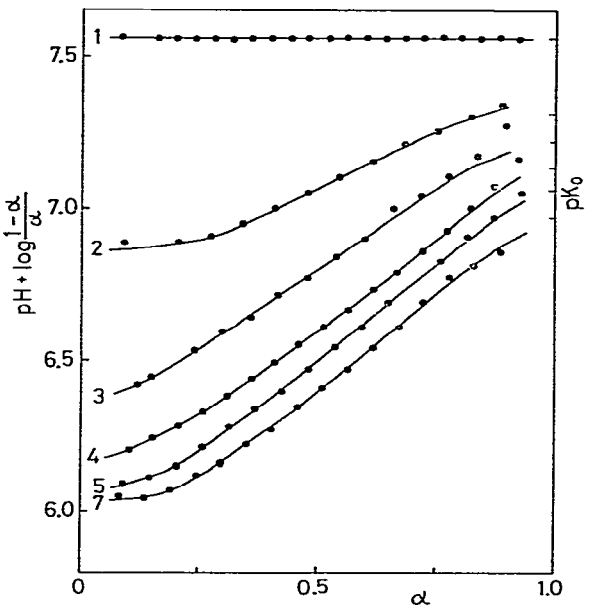


Fig. 3. The dependence of the apparent dissociation constant of 2-amino-2-deoxy-D-glucose (curve 1) and chito-oligosaccharides (curves 2, 3, 4, 5, and 7 for $[GlcNH_3^+\text{-}\beta\text{-}(1\rightarrow4)]_n$ having n = 2, 3, 4, 5, and 7, respectively) on the degree of dissociation. (Concentration: 2.00, 2.76, 3.13, 3.63, 2.56, and 2.43mM for 2-amino-2-deoxy-D-glucose, chitobiose, chitotriose, chitotetraose, chitopentaose, and chitoheptaose, respectively.)

In the ionization of chito-oligosaccharides, additional work is required to ionize the chargeable groups against the existing charge(s) on the neighboring residue(s) and hence, the intrinsic, macroscopic dissociation-constant may be written as[12,13]:

$$pK_0 = pH + \log\frac{1-\alpha}{\alpha} - \frac{0.434}{RT}\left(\frac{\partial G_{ion}}{\partial\alpha}\right).$$

For chito-oligosaccharides or chitosan, the pK_{app} value may then be defined as:

$$pK_{app} = pK_0 + \frac{0.434}{RT}\left(\frac{\partial G_{ion}}{\partial\alpha}\right) = pH + \log\frac{1-\alpha}{\alpha}.$$

Plots of observed pK_{app} against α have been found useful in recognizing conformational effects on the overall free-energy change ($\partial G_{ion}/\partial\alpha$). As shown in Fig. 3 for the chito-oligosaccharides, which do not seem to undergo a conformational change during titration, pK_{app} is[12] a monotonic, increasing function of α.

The end effect is clearly indicated from the progressive decrease of the intrinsic pK_a (namely $pK_0 = \lim_{\alpha\to1} pK_{app}$) with chain length. The gradual decrease in pK_0 as the chain length increases is not unexpected, and is no doubt due to the fact that the intrinsic pK_a value of the internal residues should be lower than those of the terminal residues. Fig. 4A shows the plot of pK_0 *vs.* n for chito-oligosaccharides

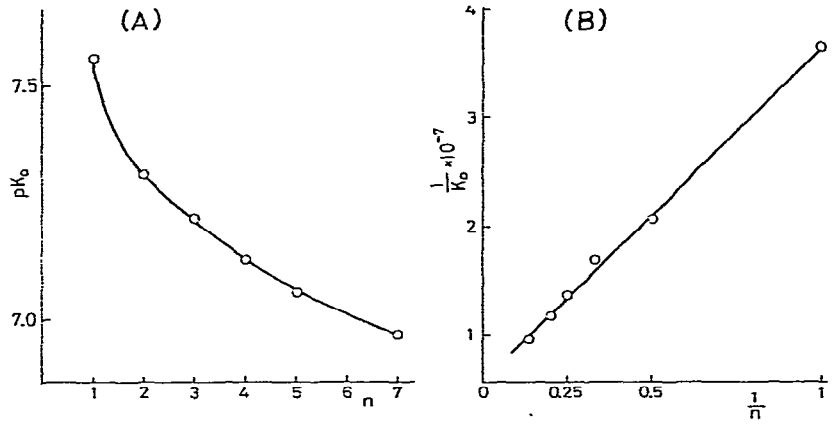


Fig. 4. (A) Dependence of the intrinsic dissociation constant of a chito-oligosaccharide on the chain length; (B) $1/K_0$ *vs.* $1/n$ for chito-oligosaccharides.

having chain lengths of 2, 3, 4, 5, and 7. After the hexasaccharide, lengthening of the sugar chain appears not to alter the pK_0 value significantly. This observation prompted us to the following considerations. For the nth macroscopic dissociation-constant (K_n) of the n-mer, the following expression may be written:

$$\frac{1}{K_n} = \frac{n-2}{k} + \frac{1}{k_N} + \frac{1}{k_R},$$

where k, k_N, and k_R are the intrinsic, microscopic dissociation-constants of the $(n-2)$ internal ionizable residues and of the non-reducing and reducing residues, respectively.

As

$$K_0 = nK_n,$$

then

$$\frac{1}{K_0} = \frac{1}{k} + \frac{1}{n}\left(\frac{1}{k_N} + \frac{1}{k_R} - \frac{2}{k}\right),$$

and thus, a plot of $1/K_0$ against $1/n$ would be linear, with slope equal to $1/k_N + 1/k_R - 2/k$ and intercept at $1/n = 0$ of $1/k$. Such a plot is shown in Fig. 4B and may be seen to approximate to a straight line having an intercept $1/k = 5.45 \times 10^6$, corresponding to $pk = 6.74$. The slope of the plot gave $1/k_N + 1/k_R - 1/k = 3.09 \times 10^7$ which, together with the known value of $1/k$, was used to obtain $1/k_N + 1/k_R = 4.18 \times 10^7$, that is, $\log(1/k_N + 1/k_R) = 7.62$. This result may be compared with the second macroscopic, stepwise dissociation-constant of chitobiose, $pK_2^T = 7.61$, which was obtained from the potentiometric titration data for chitobiose (Fig. 5) by a least-squares method with an iterative program[10] on a HITAC 8800/8700 computer. The agreement is good, in view of the differences in experimental error. The pk value of 6.74 should correspond to the pK_0 of the polymer (chitosan) at 25°. We have attempted

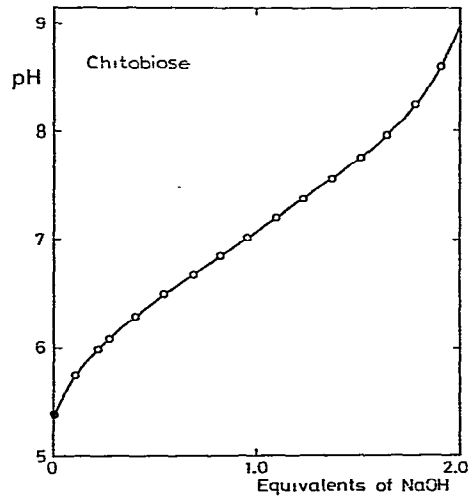


Fig. 5. Potentiometric titration-curve for chitobiose at 25°. Circles, experimental points; solid line, the computed curve.

to determine the ionization constant for chitosan, but have not yet succeeded because the conjugate-base form of chitosan is practically insoluble.

^{1}H-N.m.r. titration studies of chitobiose. — It is useful to consider the titration behavior of the simplest oligomer, chitobiose, where analysis of experimental data involves fewer uncertainties. We have measured the pD-dependence of selected proton chemical-shifts in chitobiose. Protonation of the amino group of GlcN was found to cause changes in the chemical shifts of the anomeric protons as well as in the chemical shifts of H-2. The signals of all three anomeric protons of chitobiose are observed individually between δ = 4.5 and 5.5 p.p.m. together with the separate signals for the axial protons at C-2. The pH-dependence of these selected ^{1}H chemical shifts reflects the state of ionization of 2-amino-2-deoxy-D-glucose residues in chitobiose. The ^{1}H chemical shifts of the anomeric protons [H-1, H-1′(β), and H-1′(α)] and H-2, H-2′(α), and H-2′(β) in the unprotonated and fully protonated forms of chitobiose are listed in Table I ["α" and "β" in parentheses denote "α configuration" and "β configuration" of the hydroxyl group at C-1′].

2-Amino-2-deoxy-D-glucose is known to exist in aqueous solution almost entirely in the $^4C_1$ pyranoid form[14]. In 1969, it was reported by Neuberger and Fletcher[15] that the two pyranoid anomers of the protonated forms of 2-amino-2-deoxy-D-glucose have different dissociation constants. The α anomer was found to be the stronger base. Later[16] they explained the difference in dissociation constants in terms of the anomeric effect. The dissociation constants of the two anomers of the protonated form of 2-amino-2-deoxy-D-glucose are related to the mutarotational equilibrium-quotients in the following manner[15]: $k\ (\alpha)/k\ (\beta) = K_{t_2}/K_{t_1}$, where $k\ (\alpha)$ and $k\ (\beta)$ are the two dissociation constants ("α" and "β" denote the α anomer and β anomer, respectively), and K_{t_1} and K_{t_2} are the equilibrium quotients for the

TABLE I

CHEMICAL-SHIFT VALUES FOR CHITOBIOSE (SELECTED PROTON CHEMICAL SHIFTS FOR THE DIAMINO FORM AND DIAMMONIUM CATION ARE SHOWN)

Proton	*Chemical shift (p.p.m.)*	
	Diammonium form (dication)[a]	*Diamino form (neutral)*[b]
H-1	4.85	4.46
H-2	3.13	2.63
H-1′(α)	5.44	5.19
H-1′(β)	4.96	4.58
H-2′(α)	3.34	2.74
H-2′(β)	3.06	(2.65)

[a]Values at pD 4.16. [b]Values at pD 9.31.

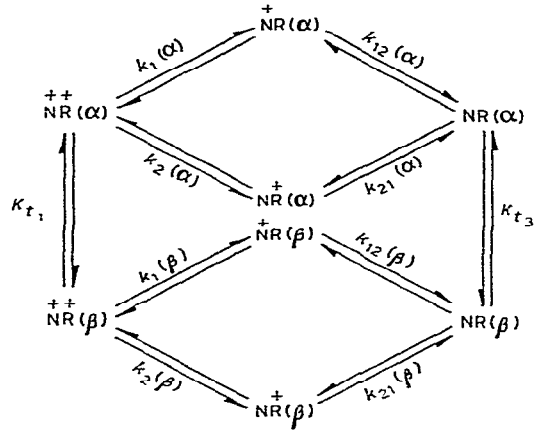
tautomeric equilibrium, β anomer ⇄ α anomer and for the equilibrium, protonated β anomer ⇄ protonated α anomer, respectively.

To furnish experimental evidence for the anomeric distribution at equilibrium for the amino sugars, we investigated the effect of dimerization and protonation on the anomeric equilibrium-quotient. We have shown that dimerization and protonation both give rise to significant increases of the amounts of the α anomers. It has been shown[15] that, in GlcN, the α anomer is preponderant in acid solution. From the ^{1}H-n.m.r. data for GlcN, the predominant forms are the α and β anomer in acidic and basic solutions, respectively, and we estimated the degree of predominance as $[\overset{+}{\mathrm{R}}(\alpha)]/[\overset{+}{\mathrm{R}}(\beta)] = 1.22$ and $[\mathrm{R}(\alpha)]/[\mathrm{R}(\beta)] = 0.58$ at 25° from the data at pD 5.82 and 9.52, respectively. These values are close to the corresponding values of 1.14 and 0.414 obtained from titration data for 2-amino-2-deoxy-D-glucose hydrochloride by Neuberger and Fletcher. Similar ^{1}H-n.m.r. measurements for chitobiose at pD 4.16 and 9.31 have also shown the α anomer to be the preponderant form in acid solution: $[\overset{++}{\mathrm{NR}}(\alpha)]/[\overset{++}{\mathrm{NR}}(\beta)] = 2.33$ and $[\mathrm{NR}(\alpha)]/[\mathrm{NR}(\beta)] = 0.87$ at 25°.

Incidentally, it is known[17] that both the α- and β-(1→4)-linked oligomers of glucose resemble D-glucose in their anomeric ratio at equilibrium. However, a quite different behavior is shown by the protonated form of chitobiose; namely, the relative proportions of α and β isomers of the reducing-end varies on proceeding from monomer to dimer, and the α anomer is greater in protonated chitobiose than observed in $GlcNH_3^+$. We suggest that the increase in proportion of the α anomer in the dimer, relative to the monomer, is the consequence of steric constraints introduced by the neighboring GlcN residue. Chito-oligosaccharides $[\text{D-GlcNH}^+\text{-}\beta\text{-}(1\rightarrow4)]_n$ having $n \geqq 3$ resemble the cationic form of chitobiose in their anomeric ratio at equilibrium, but differ from $GlcNH_3^+$.

The chemical shifts of H-1′(α), H-1′(β), H-1, H-2′(α), H-2′(β), and H-2 of

chitobiose as a function of pD are shown in Fig. 6. The pD dependence of the chemical shift is due to the following reactions:



$k_2(\alpha) = k_2(\beta)$ and $k_{12}(\alpha) = k_{12}(\beta)$

Three sets of apparent, microscopic pK values, (*i*) $pk_1^{obs}(\alpha)$ and $pk_{21}^{obs}(\alpha)$, (*ii*) $pk_1^{obs}(\beta)$ and $pk_{21}^{obs}(\beta)$, and (*iii*) $pk_2^{obs}(\alpha,\beta)$ and $pk_{12}^{obs}(\alpha,\beta)$, are observed for the ammonium groups on going from $\overset{+}{\underset{+}{NR}}$ to NR through a pair of isomers of monoprotonated species, when the H-1′(β) [or H-2′(α)], H-1′(α), and H-1 (or H-2) chemical shifts are monitored, respectively. Actually, from plots of apparent pK against the degree

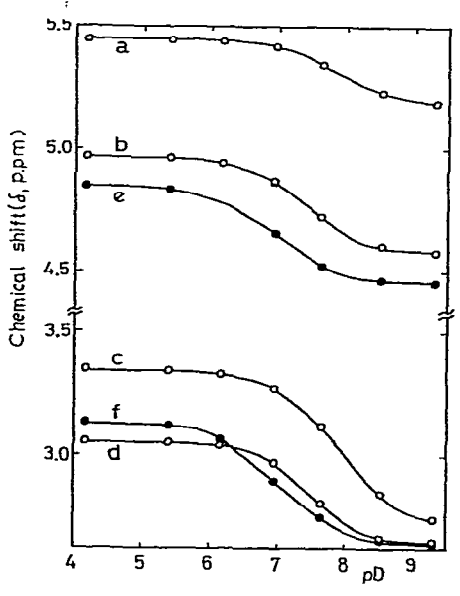


Fig. 6. pD-Dependence of the chemical shifts of (a) H-1′(α); (b) H-1′(β); (c) H-2′(α); (d) H-2′(β); (e) H-1; and (f) H-2.

of dissociation, it is evident that the proton chemical-shifts of $\delta_{H\text{-}1'(\beta)}$ and $\delta_{H\text{-}2'(\alpha)}$ both reflect the ionization of the amino group of the reducing end over the pD range 4–9. Also, within experimental error, the same apparent microscopic pk value (7.84 $\pm$0.01) is observed from each of the two curves as $pk_1^{obs}(\alpha)$ and $pk_{21}^{obs}(\alpha)$. Similarly, plots of apparent pK values, based on $\delta_{H\text{-}1}$ and $\delta_{H\text{-}2}$, *vs.* degree of neutralization, gave $pk_2^{obs}(\alpha,\beta) = pk_{12}^{obs}(\alpha,\beta) = 7.03 \pm 0.05$. Finally, the data based on $\delta_{H\text{-}1'(\alpha)}$ and $\delta_{H\text{-}2'(\beta)}$ yield $pk_1^{obs}(\beta) = pk_{21}^{obs}(\beta) = 7.41 \pm 0.03$ (See Appendix).

To obtain the thermodynamic dissociation-constants, activity corrections must be applied to the foregoing data by using the thermodynamic, macroscopic, stepwise dissociation-constants, K_1^T and K_2^T, and the equilibrium quotients, K_{t_1} and K_{t_3}, for the equilibria $\overset{-\,+}{NR}(\beta) \rightleftarrows \overset{+\,+}{NR}(\alpha)$ and $NR(\beta) \rightleftarrows NR(\alpha)$. First, the thermodynamic values, $k_{21}^T(\alpha)$, $k_{21}^T(\beta)$, and $k_{12}^T(\alpha,\beta)$, may be obtained from estimates of the ionic strength at the limit of the degree of neutralization being unity. The steps in the calculation are as follows:

Letting Δ_1 be the correction term for the activity function, the foregoing, observed constants may be related to the thermodynamic constants by

$$pk_{21}^{obs}(\alpha) = pk_{21}^T(\alpha) + \Delta_1,$$

$$pk_{21}^{obs}(\beta) = pk_{21}^T(\beta) + \Delta_1,$$

and $$pk_{12}^{obs}(\alpha,\beta) = pk_{12}^T(\alpha,\beta) + \Delta_1.$$

The second, macroscopic dissociation-constant, K_2^T, may be written as

$$K_2^T = \frac{k_{12}^T(\alpha,\beta)\,[K_{t_3}k_{21}^T(\alpha) + k_{21}^T(\beta)]}{k_{12}^T(\alpha,\beta) + k_{21}^T(\beta) + K_{t_3}[k_{12}^T(\alpha,\beta) + k_{21}^T(\alpha)]}.$$

This equation may be written in the form:

$$K_2^T = \frac{k_{12}^{obs}(\alpha,\beta)\,[K_{t_3}k_{21}^{obs}(\alpha) + k_{21}^{obs}(\beta)]}{k_{12}^{obs}(\alpha,\beta) + k_{21}^{obs}(\beta) + K_{t_3}[k_{12}^{obs}(\alpha,\beta) + k_{21}^{obs}(\alpha)]} \cdot 10^{\Delta_1},$$

and thus

$$\Delta_1 = -\,pK_2^T - \log \frac{k_{12}^{obs}(\alpha,\beta)\,[K_{t_3}k_{21}^{obs}(\alpha) + k_{21}^{obs}(\beta)]}{k_{12}^{obs}(\alpha,\beta) + k_{21}^{obs}(\beta) + K_{t_3}[k_{12}^{obs}(\alpha,\beta) + k_{21}^{obs}(\alpha)]}.$$

Similarly, by using the correction term Δ_0 (at lower pD where the degree of dissociation approaches 0), for $pk_1^{obs}(\alpha)$, $pk_1^{obs}(\beta)$, and $pk_2^{obs}(\alpha,\beta)$, the thermodynamic constants may be written as

$$pk_1^{obs}(\alpha) = pk_1^T(\alpha) + \Delta_0,$$

$$pk_1^{obs}(\beta) = pk_1^T(\beta) + \Delta_0.$$

and $$pk_2^{obs}(\alpha,\beta) = pk_2^T(\alpha,\beta) + \Delta_0.$$

TABLE II

MICROSCOPIC, THERMODYNAMIC DISSOCIATION-CONSTANTS OF CHITOBIOSE AT 25° AND SOME RELATED DATA FOR 2-AMINO-2-DEOXY-D-GLUCOSE AND CHITOBIOSE

	Chitobiose	*2-Amino-2-deoxy-D-glucose*
Dissociation constant	$pk_1^T(\alpha) = 7.28$ $pk_1^T(\beta) = 6.85$ $pk_2^T(\alpha,\beta) = 6.47$ $pk_{12}^T(\alpha,\beta) = 6.89$ $pk_{21}^T(\alpha) = 7.70$ $pk_{21}^T(\beta) = 7.27$	$pk^T(\alpha) = 7.74$ $pk^T(\beta) = 7.41$
	$pK_1^T = 6.38$ $pK_2^T = 7.61$	
Anomeric quotient	$K_{t_1} = [\overset{-}{N}R(\alpha)]/[\overset{+}{N}R(\beta)] = 2.33$ $K_{t_3} = [\overset{-}{N}R(\alpha)]/[\overset{+}{N}R(\beta)] = 0.87$	$K_{t_1} = [\overset{+}{R}(\alpha)]/[\overset{+}{R}(\beta)] = 1.22$ $K_{t_2} = [\overset{+}{R}(\alpha)]/[\overset{+}{R}(\beta)] = 0.58$

The corresponding equation is:

$$\Delta_0 = -pK_1^T - \log \frac{K_{t_1}[k_1^{obs}(\alpha) + k_2^{obs}(\alpha,\beta)] + k_1^{obs}(\beta) + k_2^{obs}(\alpha,\beta)}{1 + K_{t_1}}.$$

With the information concerning K_1^T, K_2^T, K_{t_1}, and K_{t_3}, and the values given here, the microscopic, thermodynamic dissociation-constants have been obtained as listed in Table II. Related data for 2-amino-2-deoxy-D-glucose and chitobiose are also presented.

The additional work required to add H^+ against the repulsive forces of the neighboring residue may be estimated as $\Delta pk_{intrinsic} = pk_{21}^T - pk_1^T = 0.42 \pm 0.04$. This experimental value of k_1^T/k_{21}^T for chitobiose leads to a value of 7.6–8.1 Å for the distance separating the ammonium ion from the amino group[18]. This value appears reasonable. The value estimated for a fully extended molecule is about 8 Å, and the present pK determination suggests an intramolecular hydrogen-bond between O-5 and H-3′ in chitobiose. The ^{13}C-n.m.r. evidence also supports this view (as discussed in the following sections, independent evidence for a consistent conclusion are available from ^{13}C-n.m.r. spectroscopy and optical-rotation measurements). It should be noted that the value of $\log(1/k_N + 1/k_R) = 7.62$, as determined from a plot of $1/K_0$ against $1/n$, is entirely in agreement with the second, macroscopic dissociation-constant of chitobiose, $pK_2^T = 7.61$. This result indicates that, in chito-oligosaccharide homologs, the ammonium groups of the neighboring sugar residues are sufficiently removed from each other as to have a negligible electrostatic effect on its dissociation.

Optical rotation of chito-oligosaccharides. — Evidence has accumulated in recent years that the short-range, favored conformation of a polymer is close to that

of the related dimer or oligomers[19]. Thus, conformations generally accepted[1,20] for such homopolymers as cellulose and chitin are quite close to those of the corresponding disaccharides[1,21]. There is as yet a paucity of crystal data on oligomeric structures related to polysaccharides. Although the crystalline conformations of cellobiose[22] and *N,N'*-diacetylchitobiose[23] have been described, the structure of chitobiose has not yet been reported. Chitobiose has an equatorial–equatorial β-(1→4)-linkage between 2-amino-2-deoxy-D-glucose residues, as with cellobiose. Like cellulose, chitosan may be expected to adopt an extended-ribbon conformation with a two-fold screw axis, and adjacent GlcN residues may be joined by hydrogen bonds to each O-5.

In characterizing the linkage conformation of chito-oligosaccharides we have first utilized the relationship, suggested by Rees[2], between the optical rotation at a single wavelength, say, at the sodium D line (5890 Å), for a series of chito-oligosaccharides, and the conformation at the glycosidic linkage as expressed in torsion angles about the C-1–O and O–C-4′ bonds. The projections given in Fig. 1 describe the torsion angles around the glycosidic bonds, C-1–O and O–C-4′ (for the atom numbering, see also Fig. 1). The angles H-1–C-1–O–C-4′ and C-1–O–C-4′–H-4′ are denoted in this paper as the conformational angles $\Delta\Phi$ and $\Delta\Psi$ according to the convention of Rees; these define the relative orientation of contiguous residues.

Within the context described by Rees, the "linkage rotation", $[\Lambda]_D$, at 589 nm may be defined by:

$$[\Lambda]_D = [M_{NR}]_D - ([M_{MeN}]_D + [M_R]_D),$$

where $[M_{NR}]_D$, $[M_{MeN}]_D$, and $[M_R]_D$ are the molecular rotation of chitobiose, the methyl glycoside of the non-reducing (N) residue having the same anomeric configuration as the disaccharide, and the reducing group (R) at the sodium D line. The rotations of the reducing, higher chito-oligosaccharides may be treated in the same way as that of chitobiose. Thus, for a chito-oligosaccharide, the linkage rotation may be defined by:

$$[\Lambda]_D = [M_n]_D - \{(n-1)[M_{MeGlcNH_3^+}]_D + [M_R]_D\},$$

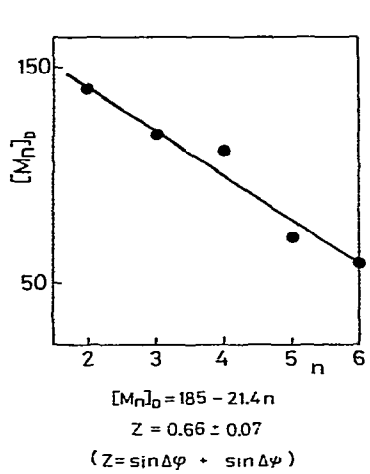
where $[M_n]_D$ is the molecular rotation for the chito-oligosaccharide, and n is the chain length ($n \geqq 2$), $[M_{MeGlcNH_3^+}]_D$ is the contribution of methyl 2-amino-2-deoxy-D-glucoside, and $[M_R]_D$ is the rotation of the reducing residue. For the present, we assume that mutarotational equilibrium is the same for all chito-oligosaccharides used [see p. 9].

The linkage rotation may be related to the parameters, $\Delta\Phi$ and $\Delta\Psi$, defined in Fig. 1, for the conformation in solution by the relationship[2]

$$[\Lambda]_D = (n-1)\{105 - 120(\sin\Delta\Phi + \sin\Delta\Psi)\}.$$

Equating this relation to the foregoing equation, we have:

$$[M_n]_D = [M_R]_D + 120(\sin\Delta\Phi + \sin\Delta\Psi) - 58 - n\{120(\sin\Delta\Phi + \sin\Delta\Psi) - 58\}.$$



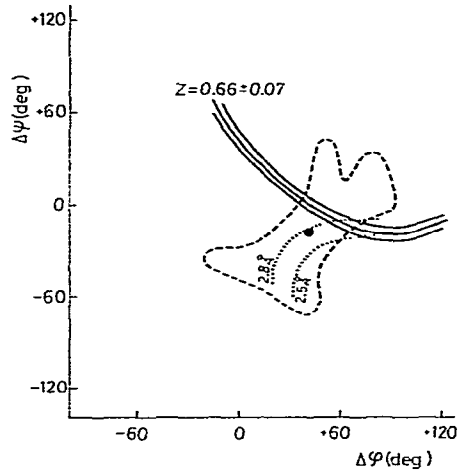


Fig. 7. Molecular rotation of chito-oligosaccharides as a function of the degree of polymerization, pH 4.

Fig. 8. Restriction of chitobiose conformations by the relation $\sin\Delta\Phi + \sin\Delta\Psi = 0.66 \pm 0.07$ (The curves of iso $z = \sin\Delta\Phi + \sin\Delta\Psi$ are given by solid curves). The limits of O-5...O-3′ hydrogen bonding, assuming that this requires the O...O distance to be 2.5–2.8 Å, are shown by dotted curves. The energy map for cellobiose[24] is shown in terms of the 5 kcal.mol^{-1} contour for comparison (broken line). The crystal structure of β-cellobiose[22] is marked as ●.

Thus, a plot of $[M_n]_D$ against the chain length ($n \geqq 2$) would be linear, with the slope equal to $120(\sin\Delta\Phi + \sin\Delta\Psi) - 58$. This correlation has been observed for a series of chito-oligosaccharides, and it is therefore likely that the linkage contributions to optical rotation would be additive in the chito-oligosaccharide system*. From the results shown in Fig. 7, the value of $\sin\Delta\Phi + \sin\Delta\Phi = 0.66$, with standard deviation = ± 0.07, was obtained. This would seem to impose certain restrictions on the solution conformations. In other words, the conformations at the glycosidic linkage appear to be restricted to within the angular range that fulfils the requirement of $\sin\Delta\Phi + \sin\Delta\Psi = 0.66 \pm 0.07$. The range of possible $\Delta\Phi$ and $\Delta\Psi$ values for, say, chitobiose is represented in the $\Delta\Phi\Delta\Psi$ map (Fig. 8). This map also includes for comparison the energy map for cellobiose as reported by Rees and Smith[24] and the likelihood of hydrogen bonding between O-3′ and O-5.

A part of the area restricted by the interrelation between $\Delta\Phi$ and $\Delta\Psi$ also satisfies the allowed range in the conformation map constructed[24] by energy calculations for cellobiose. The crystal conformation of β-cellobiose[22] is within the 5 kcal. mol^{-1} contour. As judged from the conformational map shown in Fig. 8, the freedom of rotation is comparatively higher around the C-1–O bond (namely, a wider range of $\Delta\Phi$) than around the O–C-4′ bond (a limited range of accessible $\Delta\Psi$). On changing

*The results are also consistent with the fact that there is no chromophoric complication in the chito-oligosaccharide series, in contrast to such chromophoric sugars as oligo- and poly-(acetamido sugars) and -uronic acids[2].

TABLE III

CARBON-13 CHEMICAL SHIFTS OF CHITOBIOSE(NR), δ IN P.P.M.

Carbon atom no.	*Reducing end (R)*		*Carbon atom no.*	*Nonreducing end (N)*
	α	β		
1′	89.74	93.43	1	98.57
2′	55.00	57.54	2	56.77
3′	68.80	71.16	3	72.77
4′	77.41	77.51	4	70.45
5′	70.85	75.41	5	77.20
6′	60.91	61.12	6	61.12

$\Delta\Phi$ from $+30^c$ to $+65°$, $\Delta\Psi$ changes from $+10°$ to $-15°$. Also, when $\Delta\Phi = +50°$ and $\Delta\Psi = -6°$, the distance O-3′ . . . O-5 is ~2.9 Å and this decreases to 2.7 Å for $\Delta\Phi = +40°$ and $\Delta\Psi = -18°$. Through the range $\Delta\Phi = +40°$ to $+55°$ and $\Delta\Psi = +1°$ to $-9°$, the distance O-3′ . . . O-5 decreases from 3.1 to 2.7 Å. In the latter conformation, there is still a weak, intramolecular, hydrogen bond between O-5 and the -3′-hydroxyl group.

It would also be interesting to determine optical rotation for chito-oligosaccharides in their free amino form. Unfortunately, unprotonated forms of 2-amino-2-deoxy-glucose oligomers and the corresponding polymer are too insoluble for studying the rotations as a function of degree of polymerization.

By using the value of $\sin\Delta\Phi + \sin\Delta\Psi$ obtained here, it is possible to make a rough estimate of the expected rotation of chitosan from the relationship, $\lim_{n\to\infty}[\alpha_n]_D = \lim_{n\to\infty}[M_n]_D \times 100/162n$. The limiting value thus obtained is $-13.2°$, in fair agreement with the observed specific rotation of chitosan, $-17°$. Both the linear relationship between molecular rotation and degree of polymerization for a series of chito-oligosaccharides and the similarity in the observed and estimated values of $[\alpha]_D$ for chitosan indicate that the linkage conformation (the glycosidic torsion-angles) is nearly the same for oligo- and poly-[β-(1→4)-linked 2-amino-2-deoxy-D-glucose hydrochloride] in aqueous solution. Thus, there seems to be no additional interaction in chitosan that is absent from chitobiose.

The foregoing optical-rotation procedure (namely, the Rees method) alone cannot, however, yield a precise determination as to which particular part of a restricted area is likely to be favored. One parameter, namely, $z = \sin\Delta\Phi + \sin\Delta\Psi$, is of course insufficient to determine the two independent quantities, $\Delta\Phi$ and $\Delta\Psi$. Further restrictions of the conformations of chitobiose would be desirable by combining this optical-rotation approach with other methods utilized for study of the conformational properties of oligosaccharides in solution. Additional information necessary to derive approximate values of $\Delta\Phi$ and $\Delta\Psi$ may be obtained by ^{13}C-n.m.r. spectroscopy.

TABLE IV

CALCULATED CHEMICAL SHIFTS[a] AND CHEMICAL-SHIFT DIFFERENCE ($\Delta\delta$)[b] IN CHITOBIOSE

Carbon atom no.	*Reducing end (R)*				*Carbon atom no.*	*Nonreducing end (N)*	
	α		β			δ	$\Delta\delta$
	δ	$\Delta\delta$	δ	$\Delta\delta$			
1′	89.8	+0.1	93.5	+0.1	1	100.3	+1.7
2′	55.1	+0.1	57.9	+0.4	2	56.9	+0.1
3′	69.0	+0.2	71.6	+0.4	3	72.8	0.0
4′	79.4	+2.0	79.4	+1.9	4	70.4	−0.1
5′	71.1	+0.2	75.3	−0.1	5	76.9	−0.3
6′	60.7	−0.2	60.7	−0.4	6	61.3	+0.2

[a] δ = δ (cellobiose) + δ (2-amino-2-deoxyglucose) − δ (glucose). [b] $\Delta\delta$ = δ (calculated) − δ (observed).

^{13}C-N.m.r. studies of chitobiose. — Previous investigations have determined the applicability of ^{13}C-n.m.r. spectroscopy to the study of problems of conformational analysis[3-7,25-28], and have furnished a basis from which the linkage conformation of disaccharides may be estimated[6]. The method of chemical-shift differences may be particularly useful in indicating the linkage conformation in oligosaccharides, if the linkage conformation of a related reference compound is known. We have therefore measured the ^{13}C chemical shifts in chitobiose (Table III). We are particularly interested in examining the influence of the glycosidic torsion-angles on the C-1 and C-4′ chemical-shift values in chitobiose as compared with those in cellobiose.

Assignment of signals. — The signals may be assigned to various carbon atoms from the spin–spin coupling constants, from the configuration of the substituents, and from the nature of the glycosidic bond between the GlcN monomers. Except for the C-6 signal at 61.12 p.p.m. and C-6′ signals at 60.91 (α) and 61.12 (β), which have been assigned independently by the appearance of a pair of triplets of relative intensity 1:2:1 in the undecoupled spectrum, and the anomeric signals, the other signals may be readily assigned by comparison of chemical shifts with those previously assigned to the corresponding carbon atoms in the monomeric units (2-amino-2-deoxy-D-glucose[29] and D-glucose[30]) and in cellobiose[30]. The chemical-shift changes that occur upon replacement of 2-OH by 2-NH_3^+ in cellobiose are of similar magnitude to the changes found in the monomer level, with the exception of the C-1 and C-4′ resonances of chitobiose. These observations may result from slight changes in steric constraints imposed on the glycosidic linkage as a result of replacement of the 2- and 2′-OH groups by the protonated amino group (-NH_3^+).

In accordance with the previously established evidence in hexoses[31] and the hexosamines[32], the chemical shifts of carbon atoms in the reducing group have been found to be dependent on the anomeric configuration, this dependence being specifi-

cally large in the ring carbon atoms (C-2′, C-3′, and C-5′). Here, the ^{13}C chemical shifts of chitobiose may be calculated empirically by assuming the substituent effects on the chemical shifts to be additive:

$$\delta^{\text{calculated}}_{\text{chitobiose}} = \delta^{\text{observed}}_{\text{cellobiose}} + \delta^{\text{observed}}_{\text{GlcN}} - \delta^{\text{observed}}_{\text{Glc}}.$$

Table IV gives a list of the calculated chemical shifts and the chemical-shift difference. The ^{13}C chemical shifts predicted for chitobiose show excellent agreement with those observed for most of the carbon atoms. The only significant differences in chemical shift between those observed and calculated are exhibited by upfield shifts of the carbon atoms involved in the interglycosidic linkages, namely, the C-4′(α), C-4′(β) and C-1 resonances by 2.0, 1.9, and 1.7 p.p.m., respectively, relative to the predicted chemical shifts (Fig. 9).

Substantial deviations in $\Delta\delta$ from ± 0.4 p.p.m. may thus be used to diagnose any significant alterations in the interglycosidic torsion angles, $\Delta\Phi$ and $\Delta\Psi$ (particularly $\Delta\Psi$) on passing from cellobiose to chitobiose. Deviations are in general rather small (within ± 0.4 p.p.m.) at all carbon atoms other than C-1 and C-4′. The utility of these chemical-shift data to determine the linkage torsion-angle $\Delta\Psi$ in chitobiose is considered. Lemieux and Koto have shown that C-1 chemical shifts depend on the glycosidic torsion-angle $\Delta\Psi$ in a series of substituted cyclohexyl α- and β-D-gluco-

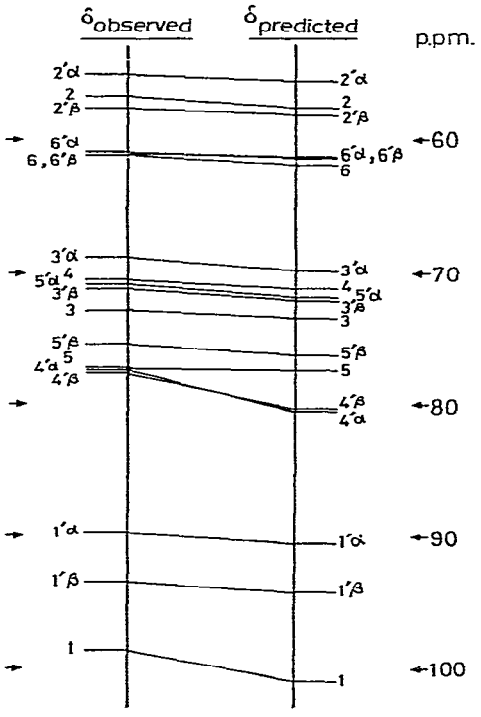


Fig. 9. Observed ^{13}C-n.m.r. chemical shifts (left) of chitobiose are shown, together with the predicted location of resonances (right).

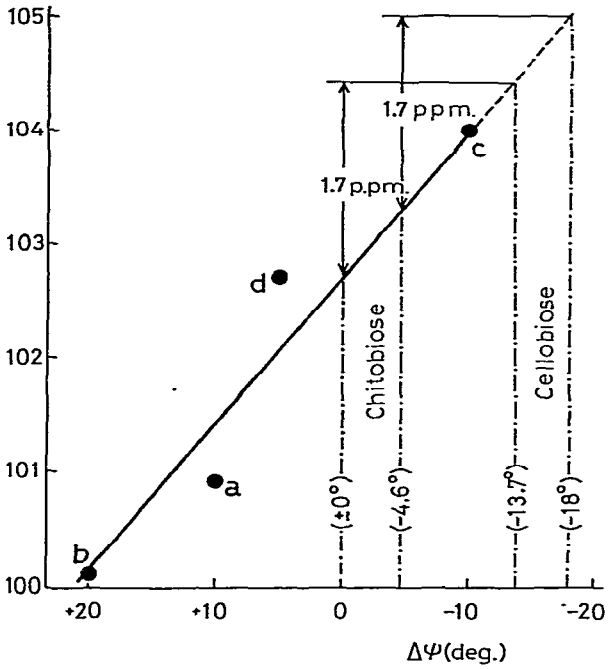


Fig. 10. ^{13}C Chemical shift of C-1 in substituted cyclohexyl β-D-glucosides *vs.* glycosidic torsion angle $\Delta\Psi$*. *a*, cyclohexyl β-D-glucoside; *b*, 2-methylcyclohexyl β-D-glucoside; *c*, 6-methylcyclohexyl β-D-glucoside; *d*, 2,6-dimethylcyclohexyl β-D-glucoside (data are taken from ref. 3). [*Based on the convention of Rees[2]; the torsion angle, $\Delta\Psi$, is related to the $\Psi^{C-2'} - 120°$ and $\Delta\Phi = \Phi^{O-5} + 120°$] the solid line has no theoretical significance.

pyranosides[3]. They have argued the glycosidic torsion-angle ($\Delta\Psi$) plays some role in ^{13}C chemical shifts of C-1. Grant and Cheney first suggested the possible importance of hydrogen–hydrogen nonbonded interactions as an explanation for upfield shifts for a variety of spatially perturbed methyl groups[25]. The simplified model indicated that the steric effects may be explained with a slight charge polarization in the $H^{13}C$ bond[25,26]. Thus, for the β-glucosides, the more compressed the anomeric hydrogen atom, the more shielded the anomeric carbon atom. A plot of ^{13}C chemical shift *vs.* the glycosidic torsion-angle $\Delta\Psi$ assessed[3] by computer calculations according to the hard-sphere procedure, by keeping $\Delta\Phi$ at a fixed value of 55° is shown in Fig. 10 and demonstrates that a monotonic, empirical correlation may be drawn relating the chemical shifts and the torsion angles of substituted cyclohexyl β-D-glucopyranosides*. The compression of the anomeric hydrogen atom with H-4′ may also be suggested

*It is believed that the exoanomeric effect[3,33,34] plays a dominating role in establishing the favored orientation of an aglyconic carbon atom relative to the anomeric hydrogen atom, namely, the $\Delta\Phi$ torsion angle. In the absence of exceptional steric complications, β-D-glucopyranosides in solution may be expected[3,33] to adopt $\Delta\Phi$ angles near +50°. On the basis of the exoanomeric effect, the favored conformation for a glycosidic linkage may be considered to arise mainly from rotation only about the aglyconic carbon to glycosidic oxygen bond, that is, the $\Delta\Psi$ torsion angle.

as an explanation for shielding effects on the anomeric carbon atom (C-1) in a β-linked disaccharide system such as chitobiose and cellobiose.

If, indeed, a simple correlation as shown in Fig. 10 exists between $\Delta\Psi$ and the C-1 chemical shifts, with other factors playing a minor role, then these shifts may in turn be utilized to define the conformation of chitobiose in solution. We have noted a substantial upfield shift (1.7 p.p.m.) of the anomeric carbon (C-1) signal in chitobiose as compared with the expected C-1 resonance estimated from the data for cellobiose, cellulose, and 2-amino-2-deoxy-D-glucose. The simple, empirical correlation shown† in Fig. 10 would suggest a $\Delta\Psi$ torsion angle of 0–5° when we used the $\Delta\Psi$ value[19] —13.7° and (ref. 22) —18° for cellobiose as reference points. Thus, less-negative values of $\Delta\Psi$ are favored for chitobiose. This behavior is presumably due to the electrostatic repulsion between the ammonium groups (see p. 29).

It should be realized that the procedure for estimating $\Delta\Psi$ by using the δC-1 *vs.* $\Delta\Psi$ plot is only empirical, and the value derived is simply a rough estimate. Nevertheless, this treatment would seem to impose further restrictions, as there is no other precise method yet available for the present data.

ACKNOWLEDGMENT

We are indebted to Professor T. Miyazawa and his colleagues of the University of Tokyo for their aid in obtaining the ^{1}H- and ^{13}C-n.m.r. spectra.

APPENDIX

The following scheme representing the dissociation of chitobiose dication, $\overset{++}{\text{NR}}$, involves initial dissociation at one of the two protonated 2-amino-2-deoxy-D-glucose residues. This step is followed by the second dissociation to form NR.

$$\overset{++}{\text{NR}} \overset{K_1}{\rightleftharpoons} \begin{bmatrix} \overset{+}{\text{NR}} \\ \Updownarrow \\ \overset{+}{\text{NR}} \end{bmatrix} \overset{K_2}{\rightleftharpoons} \text{NR}$$

The reducing residue, R, of all species involved in this scheme is subject to the equilibrium, α anomer $\rightleftarrows$ β anomer. The macroscopic, stepwise dissociation-constants, K_1 and K_2, defined in the above scheme are thus

$$K_1 = \frac{a_H([\overset{+}{\text{NR}}(\alpha)] + [\overset{+}{\text{NR}}(\beta)] + [\overset{+}{\text{NR}}(\alpha)] + [\overset{+}{\text{NR}}(\beta)])}{[\overset{++}{\text{NR}}(\alpha)] + [\overset{++}{\text{NR}}(\beta)]}$$

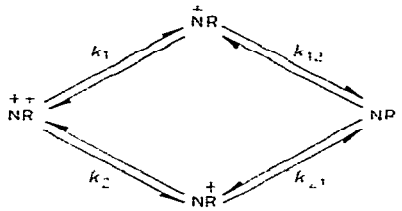
and

†We make no claim for a linear correlation between the C-1 chemical shifts and $\Delta\Psi$, and the solid line in Fig. 10 is only tentatively drawn.

$$K_2 = \frac{a_H([NR(\alpha)] + [NR(\beta)])}{[NR(\alpha)] + [NR(\beta)] + [NR(\alpha)] + [NR(\beta)]},$$

where a_H is the activity of the hydronium ion, and the square brackets represent the stoichiometric molar concentration of the species involved.

The microscopic ionization scheme of chitobiose may be represented schematically as



Letting the molar fractions of the four species in the mixture be P_{NR}^{++}, P_{NR}^{+}, P_{NR}^{+}, and P_{NR}, for the α anomer, these fractions are written as:

$$P_{NR(\alpha)}^{++} = \frac{[NR(\alpha)]}{[NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)]},$$

$$P_{NR(\alpha)}^{+} = \frac{[NR(\alpha)]}{[NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)]},$$

$$P_{NR(\alpha)}^{+} = \frac{[NR(\alpha)]}{[NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)]},$$

and

$$P_{NR(\alpha)} = \frac{[NR(\alpha)]}{[NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)] + [NR(\alpha)]}.$$

The fraction of molecules in which the amino group of the reducing end is not protonated, α, and the fraction of molecules in which the amino group of the reducing end is protonated, $1 - \alpha$, are then defined as:

$$\alpha = P_{NR(\alpha)}^{+} + P_{NR(\alpha)}$$

and

$$1 - \alpha = P_{NR(\alpha)}^{++} + P_{NR(\alpha)}^{-}.$$

Defining

$$pK(\alpha) = pD + \log \frac{1-\alpha}{\alpha},$$

the uncorrected, microscopic dissociation-constants may then be obtained:

$$\lim_{\alpha \to 0} \frac{1-\alpha}{\alpha} = \lim_{\alpha \to 0} \frac{P^{+-}_{NR(\alpha)} + P^{-}_{NR(\alpha)}}{P^{+}_{NR(\alpha)} + P_{NR(\alpha)}} = \frac{[D^+]}{k_1(\alpha)}$$

$$\lim_{\alpha \to 0} pK(\alpha) = pk_1^{obs}(\alpha).$$

Similarly,

$$\lim_{\alpha \to 1} pK(\alpha) = pk_{21}^{obs}(\alpha).$$

Here, the extent of dissociation may be determined directly from chemical-shift measurements.

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