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Acid hydrolysis of chitosans ☆

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Abstract

The hydrolysis of the *O*-glycosidic linkages (depolymerization) and the *N*-acetyl linkage (de-*N*-acetylation) of partially *N*-acetylated chitosans were studied in dilute and concentrated HCl. The rate of hydrolysis of the glycosidic linkages was found to be equal to the rate of de-*N*-acetylation in dilute acid, while the glycosidic linkages was hydrolysed more than 10 times faster than the *N*-acetyl linkage in concentrated HCl. This can be explained by assuming that the hydrolysis of the *N*-acetyl linkage is a S_N2 reaction (rate-limiting step: addition of water to the carbonium ion) while the hydrolysis of the glycosidic linkages is a S_N1 reaction where the rate-limiting step is the formation of the carbonium ion. The specificity of the acid-catalysed cleavage of the different chitosan glycosidic linkages in concentrated HCl was such that the linkages between two acetylated units (**A–A**) and between an acetylated and a deacetylated unit (**A–D**) was cleaved with about equal rate and three orders of magnitude faster than the other two linkages (**D–A** and **D–D**). The activation energies for acid hydrolysis of two almost fully de-*N*-acetylated chitosans ($F_A = 0.002$ and $F_A < 0.0003$) were determined to be 152.2 ± 8.1 and 158.1 ± 9.8 kJ mol^{-1}, respectively, representing the activation energy for hydrolysis of the **D–D** glycosidic linkage in chitosans. The activation energies for acid hydrolysis of two partially *N*-acetylated chitosans ($F_A = 0.47$ and $F_A = 0.62$) were determined to be 130.4 ± 2.5 and 134.3 ± 3.1 kJ mol^{-1}, respectively, representing the activation energy for hydrolysis of the **A–A** and **A–D** glycosidic linkage in chitosans.

Keywords: Chitosan; depolymerization; de-*N*-acetylation; reaction mechanisms; specificity; activation energies

1. Introduction

Chitosans may be considered as a family of linear binary copolymers of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit), where the A- and D-units have been shown to be randomly distributed along the chains in water-soluble, partially *N*-acetylated chitosans prepared from chitin by alkaline de-*N*-acetylation (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991a,b). Water-soluble chitosans can be prepared with fractions of *N*-acetylated units (F_A) from 0 to 0.6 (Anthonsen, Vårum, & Smidsrød, 1993; Nordtveit, Vårum, & Smidsrød, 1994).

Chitosans can be chemically depolymerized by different mechanisms, e.g. acid hydrolysis, oxidative–reductive and nitrous acid depolymerization. The nitrous acid depolymerization reaction mechanism has been found to be specific in the sense that HONO attacks the amino group of **D**-units, with subsequent cleavage of the following glycosidic linkage. By determining the identity and the relative amounts of the new non-reducing ends of nitrous acid depolymerized chitosans, it was concluded that the **D–A** and **D–D**-linkages in partially *N*-acetylated chitosans were cleaved with about equal rate by nitrous acid depolymerization (Vårum, Holme, Izume, Stokke, & Smidsrød, 1996a). The rate of acid hydrolysis of the glycosidic linkages in model compounds as methyl-2-acetamido-2-deoxy-β-D-glucopyranose and methyl-2-amino-2-deoxy-β-D-glucopyranose has been determined, and it was shown that the *N*-acetylated methyl-glucosaminide was hydrolysed much faster than the corresponding de-*N*-acetylated sugar, although a direct comparison of the rate constants at the same experimental conditions was not reported (Moggridge & Neuberger, 1938).

In this paper we have undertaken a detailed study of the acid hydrolysis of partially *N*-acetylated chitosans, including rate of de-*N*-acetylation relative to rate of depolymerization, specificity with respect to hydrolysis of the four different glycosidic linkages in chitosans, degradation rates as a function of F_A, and activation energies.

☆ Preliminary results of this work were presented at the 7th International Chitin and Chitosan Conference in Lyon (September 3–5, 1997) and at the XII International Carbohydrate Symposium in San Diego, CA (August 9–14, 1998).

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2. Methods

2.1. Characterization of chitosans

Fully water-soluble chitosans with different F_A-values were prepared by homogeneous de-*N*-acetylation of chitin (Sannan, Kurita, & Iwakura, 1976). The chitosan with $F_A = 0.002$ was prepared by further heterogeneous de-*N*-acetylation of a chitosan with $F_A = 0.01$. The chitosan with $F_A < 0.0003$ was prepared by further heterogeneous de-*N*-acetylation of the chitosan with $F_A = 0.002$. F_A-values were determined as previously described (Vårum et al., 1991a). However, the F_A-values of the more extensively de-*N*-acetylated chitosans were determined from the relative areas of H-1 of **D**-units and the three methyl protons of the *N*-acetyl group, which makes it possible to determine very low F_A-values with reasonable accuracy provided that spectra with a high signal to noise ratio are recorded. Intrinsic viscosities (ionic strength: 0.1 M) of the chitosan hydrochlorides were determined as previously described (Draget, Vårum, Moen, Gynnild, & Smidsrød, 1992). The characterization of the chitosan samples are given in Table 1.

2.2. Methods to follow the depolymerization and de-N-acetylation of chitosans

Two different methods were used: (i) NMR spectroscopy to determine the identity of the new reducing and non-reducing ends (**A**- or **D**-units) (Ishiguro, Yoshie, Sakurai, & Inoue, 1992; Vårum, Holme, Izume, Stokke, & Smidsrød, 1996b); and (ii) decrease in viscosity to determine rates of degradation as a function of the chemical composition (F_A) of the chitosans (Nordtveit et al., 1994).

2.3. Determination of rate of de-N-acetylation versus rate of depolymerization in dilute acid

A chitosan with F_A of 0.49 (see Table 1) was acid hydrolysed in 0.1 M HCl (83°C) for 10–103 h (chitosan concentration: 10 mg/ml). The reaction was stopped by increasing the pH to 4.5, the sample was lyophilized, dissolved in D_2O and analysed by proton NMR spectroscopy. Degrees of scission (α-values) were determined from the integral of all resonances from H-1 and dividing by the integral of the resonances of the reducing end protons of H-1. The α-reducing and the β-reducing end protons of a deacetylated unit are found at 5.43 and 4.94 ppm, respectively, while the α-reducing and the β-reducing end protons of H-1 of an acetylated unit are found at 5.18 and 4.69 ppm, respectively (Ishiguro et al., 1992). Since the β-reducing end protons from both **A**- and **D**-units are only partly resolved from the internal H-1, we calculated the integral of the β-reducing end protons from the tautomeric equilibrium between the α- and β-anomer, which is 60% α- and 40% β-anomer (Tsukada & Inoue, 1981). However, the relatively high ionic strength in the solvent when obtaining the proton NMR spectra (4.1 M NaCl) of the chitosans depolymerized at 12.08 M HCl (see Fig. 2) were found to affect the equilibrium ratio between α- and β-anomer. In order to determine the tautomeric equilibrium ratio, we obtained proton NMR spectra of *N*,*N*-diacetyl chitobiose in D_2O-solutions containing 4.1 M NaCl. The tautomeric equilibrium between α- and β-anomer were determined to be 50% α- and 50% β-anomer, which was used to calculate the β-reducing end protons of the chitosans depolymerized in 12.08 M HCl.

Table 1
Characterization of chitosans. The fraction of acetylated units (F_A) was determined from proton NMR spectroscopy (Vårum et al., 1991a) and the intrinsic viscosities ([η]) of the chitosans (hydrochloride salt) as previously described (Draget et al., 1992). Number-average molecular weights (M_n) were calculated from the Mark–Houwink–Sakurada equation as reported by Anthonsen et al. (1993)

F_A	[η] (ml/g)	M_n
< 0.0003	400	80,000
0.002	670	190,000
0.16	560	135,000
0.30	690	170,000
0.36	780	190,000
0.47	920	210,000
0.49	1200	280,000
0.62	970	210,000

2.4. Determination of rate of de-N-acetylation versus rate of depolymerization in concentrated acid

The chitosan with F_A of 0.49 was dissolved by gentle shaking (4 h) in concentrated (37%) HCl at 0°C (chitosan concentration: 20 mg/ml), and the dissolved sample was hydrolysed in concentrated HCl (12.08 M) at 30°C for 1–4 h. The reaction was stopped by cooling the sample to 0°C and diluting it with an equal volume of 12.08 M NaOH (0°C) to reach a final pH of 4.5. The sample was lyophilized, dissolved in D_2O (ionic strength of 4.1 M) and analysed by proton NMR spectroscopy.

2.5. Determination of rate of depolymerization in dilute acid by viscosity assay

The kinetics of the acid depolymerization reaction was followed by determining the decrease in the relative viscosity as a function of time (Nordtveit et al., 1994; Vårum, Holme, Izume, Stokke, & Smidsrød, 1997). The kinetics of the acid depolymerization reaction as a function of temperature was determined at an acid concentration of 0.4 M (HCl) and a chitosan concentration of 1.5 or 2 mg/ml. The decrease in the viscosity of the partially *N*-acetylated chitosan samples at pH 4.5 and ionic strength of 0.4 M was used as a control, and the degradation rate of this sample was always less than 5% (usually less than 2%) of the sample in 0.4 M HCl. These degradation experiments were performed directly in a capillary Ubbelohde viscosimeter (chitosans with F_A of 0.47 and 0.62) water bath with temperature control from 20 to 80°C. However, the much lower degradation rate

constants of the highly de-*N*-acetylated chitosans ($F_A = 0.002$ and $F_A < 0.0003$) made it necessary to use a different design of the degradation experiments for these chitosans. In this case the degradation experiments were performed at higher temperatures (75–120°C, where the experiments above 100°C were performed in a pressure cooker) using prolonged times of degradation.

The degradation rates are reported as rate constants (k), as defined in the following equation (Tanford, 1961):

$$1/\overline{DP}_n = (1/\overline{DP}_n)_0 + kt$$

where DP_n and $(DP_n)_0$ are number-average degrees of polymerization and t is the time. Through the Mark–Houwink–Sakurada equation intrinsic viscosities are converted to number-average molecular weights (Anthonsen et al., 1993) for chitosans with different F_A-values, and by dividing with the molecular weight of the monomer (197 for the de-*N*-acetylated units as the hydrochloride salts and 203 for the *N*-acetylated units) the number-average degree of polymerization is obtained. The rate constant are then obtained directly from the slope of the linear plots of degree of scission (α, equal to $1/\overline{DP}_n$) versus time (see Figs. 7–9).

3. Results and discussion

3.1. Determination of the rate of de-N-acetylation relative to the rate of depolymerization in dilute acid

When studying acid hydrolysis of chitosans, one complicating factor is that hydrolysis of the *N*-acetyl linkage (de-*N*-acetylation) may occur in addition to hydrolysis of the *O*-glycosidic linkage (depolymerization). Therefore, the rate constants of de-*N*-acetylation, k_{acetyl}, relative to the rate constants of depolymerization, k_{glyc}, was determined in dilute acid solution (0.1 M HCl at 83°C) by measuring the change in F_A and the degree of scission (α) of a high-molecular weight chitosan ($F_A = 0.49$, see Table 1) upon acid degradation.

The anomer region of the proton NMR spectra of the chitosan depolymerized in 0.1 M HCl is shown in Fig. 1. The α-anomeric reducing end protons resonate at 5.43 ppm (D-unit) and at 5.19 ppm (**A**-unit) (Ishiguro et al., 1992), while the internal **D**- and **A**-units resonate at 4.9 and 4.6 ppm, respectively (Vårum et al., 1991a). Both deacetylated and acetylated reducing ends appear in the spectrum, although the acetylated reducing ends at 5.19 ppm are dominating (see Fig. 1). However, in addition to hydrolysis of glycosidic linkages, hydrolysis of the *N*-acetyl linkage (de-*N*-acetylation) is also evident as seen from a systematic decrease in F_A with increasing time of hydrolysis. The results are shown in Table 2 where F_A, the change in F_A (ΔF_A), the number-average degree of polymerization (DP_n) and the degree of scission ($\alpha = 1/DP_n$) is shown as a function of time of hydrolysis. The rate of de-*N*-acetylation is proportional to ΔF_A and the rate of hydrolysis is proportional to α, and as ΔF_A and α are almost equal at all times of hydrolysis, we can conclude that k_{acetyl} and k_{glyc} are very similar.

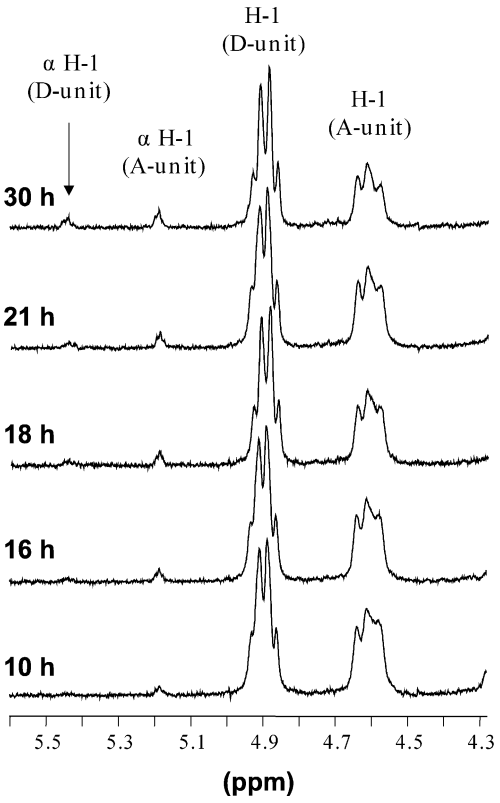

Fig. 1. Anomer region of the proton NMR spectra of solutions of chitosans (10 mg/ml) in D_2O (pD 3 and 90°C) which were depolymerized in 0.1 M HCl.

3.2. Determination of the rate of de-N-acetylation relative to the rate of depolymerization in concentrated acid

Previous results where fully *N*-acetylated oligosaccharides were prepared by hydrolysis of chitin indicate that the rate of hydrolysis is much higher than the rate of de-*N*-acetylation when the hydrolysis is performed in 11 M HCl (Rupley, 1964). The anomer region of the proton NMR spectra of the chitosans which are depolymerized in 12.08 M HCl are shown in Fig. 2, and the spectra show exclusively the α-anomeric reducing end protons of acetylated units at 5.19 ppm (acetylated unit) with no detectable

Table 2
Characterization of the chitosan with F_A of 0.49 (see Table 1) upon hydrolysis in 0.1 M HCl at 83°C. F_A, change in F_A (ΔF_A), number-average degree of polymerization ($\overline{DP}_n$) and degree of scission (α) are given as a function of time

Time (h)	F_A	ΔF_A	$\overline{DP}_n$	α
10	0.463	0.027	41.5	0.024
16	0.445	0.045	23.2	0.043
21	0.435	0.055	19.1	0.052
30	0.403	0.087	12.9	0.078

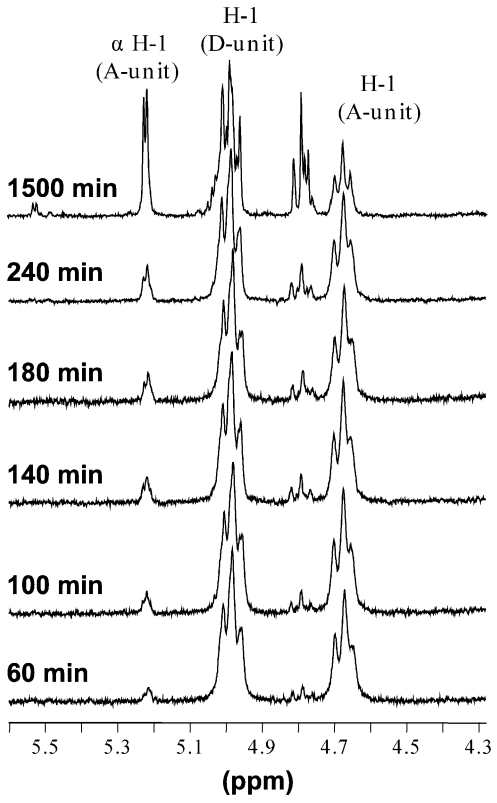

Fig. 2. Anomer region of the proton NMR spectra of solutions of chitosans (7 mg/ml) in D_2O (pD 3 and 90°C) which were depolymerized in 12.08 M HCl. The ionic strength of the solutions are much higher (4 M NaCl) than in Fig. 1.

de-*N*-acetylated reducing end units from 60 to 240 min of hydrolysis. However, when the hydrolysis in concentrated HCl was extended to 103 h ($\overline{DP}_n$ of 3.5), the reducing end of de-*N*-acetylated units could also be detected. For this extensively depolymerized sample, the α-anomeric reducing end protons of acetylated units was calculated to 92% while the α-anomeric reducing end protons of de-*N*-acetylated units was 8%, meaning that the rate of hydrolysis is more than 10 times higher than the rate of de-*N*-acetylation in concentrated acid (12.08 M). The results are presented in Table 3 where F_A, the change in F_A (ΔF_A), the number-average degree of polymerization ($\overline{DP}_n$) and the degree of scission (α) is shown as a function of time of hydrolysis, and the degree of scission increases linearly with time.

Table 3
Characterization of the chitosan with F_A of 0.49 (see Table 1) upon hydrolysis in 12.08 M HCl at 30°C. F_A, change in F_A (ΔF_A), number-average degree of polymerization (DP_n) and degree of scission (α) are given as a function of time

Time (min)	F_A	ΔF_A	$\overline{DP}_n$	α
60	0.440	0.050	20.8	0.048
100	0.455	0.035	16.2	0.062
140	0.464	0.026	11.4	0.088
180	0.476	0.014	9.00	0.111
240	0.484	0.006	7.81	0.128

The reason for the apparently systematic increase in F_A from 0.440 (60 min) to 0.484 (240 min) with time is presently not known. One possibility could be a decrease in the solubility (at the high ionic strength (4.1 M) of the NMR solutions) of more highly *N*-acetylated oligomers at the lowest α-values, which gradually are solubilized upon hydrolysis. A control experiment was performed where the least degraded sample (60 min) was dialysed against distilled water for 4 h, lyophilized, dissolved in D_2O and reanalysed by proton NMR spectroscopy (at an ionic strength of 0.1 M). The characterization of the sample revealed an increase in F_A to 0.482 together with an increase in $\overline{DP}_n$ to 29.3, suggesting that the low F_A-values of the less degraded sample are related to the high ionic strength. Whatever reason to the low F_A-values determined for the least degraded samples, it seems safe to conclude that there is a high specificity in the acid hydrolysis reaction in concentrated acid, as the new reducing ends are clearly dominated by acetylated units.

Our results are in full agreement with previous results (Rupley, 1964) who studied the depolymerization of chitin in concentrated acid (11 M HCl), and found that the increase in ninhydrin colour (assumed to measure the amount of deacetylated units) proceeded at about one-tenth of the change of reducing-group concentration.

The decrease in k_{acetyl} relative to k_{glyc} from high to low acid concentration can be explained by different reaction mechanisms, as schematically shown in Fig. 3. It is assumed that the hydrolysis of the *N*-acetyl linkage is a S_N2 reaction where the rate-limiting step is the addition of water to the carbonium ion (Fig. 3a), implying that k_{acetyl} decreases with increasing acid concentration (decreasing water concentration). In contrast, the hydrolysis of the glycosidic linkage is assumed to be a S_N1 reaction where the rate-limiting step is the formation of the carbonium ion, implying that k_{glyc} is not dependent on the water concentration (Fig. 3b).

3.3. Specificity in the acid hydrolysis reaction in concentrated acid

The results presented in Fig. 2 and Table 2 show, in addition to the finding that the rate of hydrolysis is about 10 times higher than the rate of deacetylation, that there is a high specificity in the acid hydrolysis reaction of the different glycosidic linkages in partially *N*-acetylated chitosans. The new reducing ends are dominated by acetylated units, which means that the rates of acid hydrolysis of the **D–D** and **D–A** glycosidic linkages must be vanishingly lower than the rate of cleavage of the **A–A** and **A–D** linkages. In order to get information on any possible difference between the rate of acid hydrolysis of the **A–A** and the **A–D** glycosidic linkages, we determined the identity of the new non-reducing ends. Fig. 4 shows part of the ^{13}C NMR spectrum (carbons 4 and 5) of the acid hydrolysed chitosan ($F_A = 0.49$), showing the new

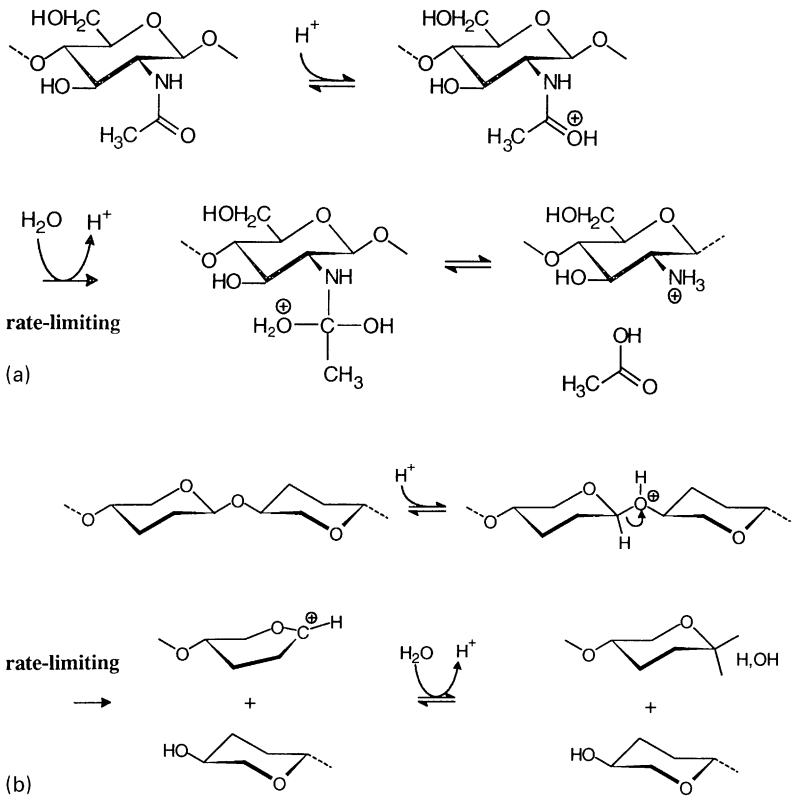

Fig. 3. Schematic illustration of the proposed reaction mechanisms for the acid-catalysed hydrolysis of the *N*-acetyl linkage and the glycosidic linkage in chitosans. (a) Hydrolysis of the *N*-acetyl linkage (S_N2 reaction). (b) The most widely accepted mechanism for hydrolysis of the glycosidic linkage (S_N1 reaction).

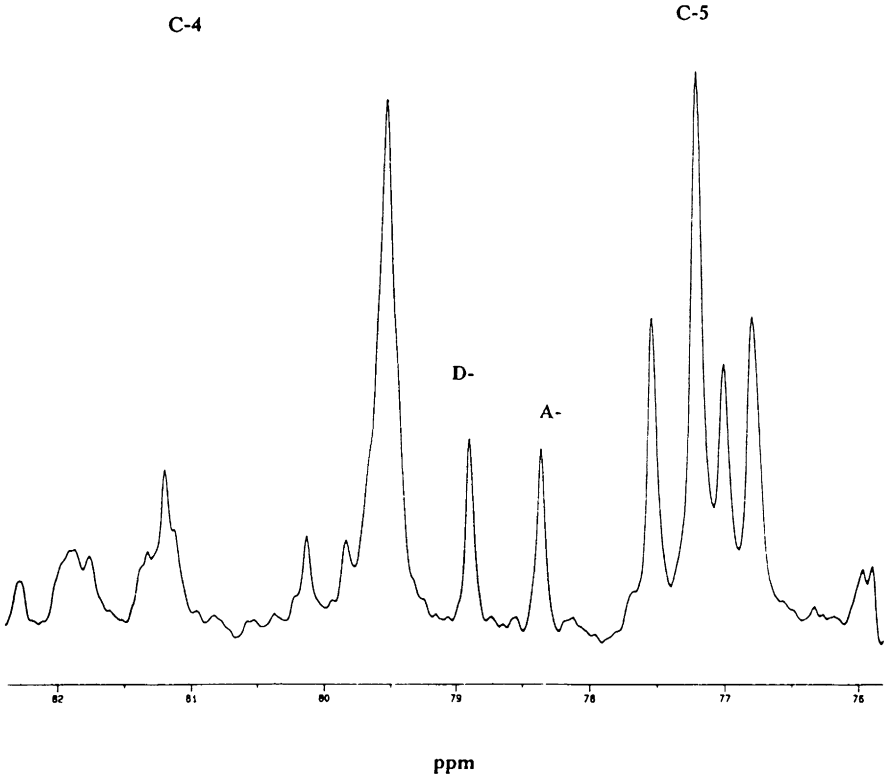

Fig. 4. Part of the ^{13}C NMR spectrum (carbons 4 and 5) of the chitosan ($F_A = 0.49$) depolymerized in 12.08 M HCl, showing the new non-reducing ends at 78.9 ppm (D-units) and 78.5 ppm (A-units).

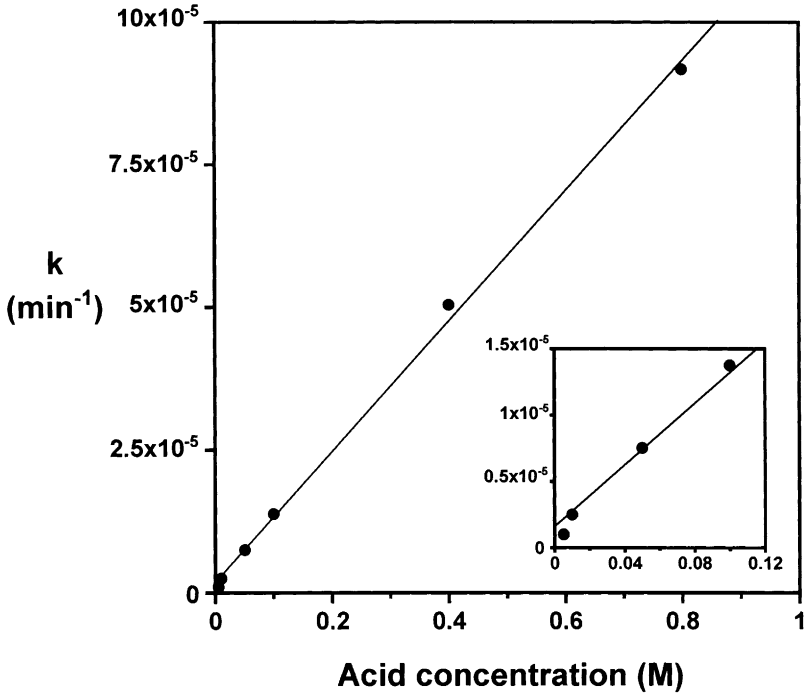

Fig. 5. Degradation rate constants (k) as a function of acid concentration (HCl), determined by the viscosity assay at a chitosan concentration of 1.5 mg/ml in 0.4 M HCl at 60°C.

non-reducing ends at 78.9 ppm (**D**-units) and 78.5 ppm (**A**-units).

The specificity of the reaction with respect to cleavage of the **A–A** and the **A–D** glycosidic linkages can be evaluated by comparing the F_A-value of the non-reducing units with that of the chitosan, when the chitosan has a random distribution of the acetylated and the deacetylated units. The chitosan has a F_A-value of 0.49 and the F_A-value of the non-reducing units were determined to be 0.51, which indicate that there is no

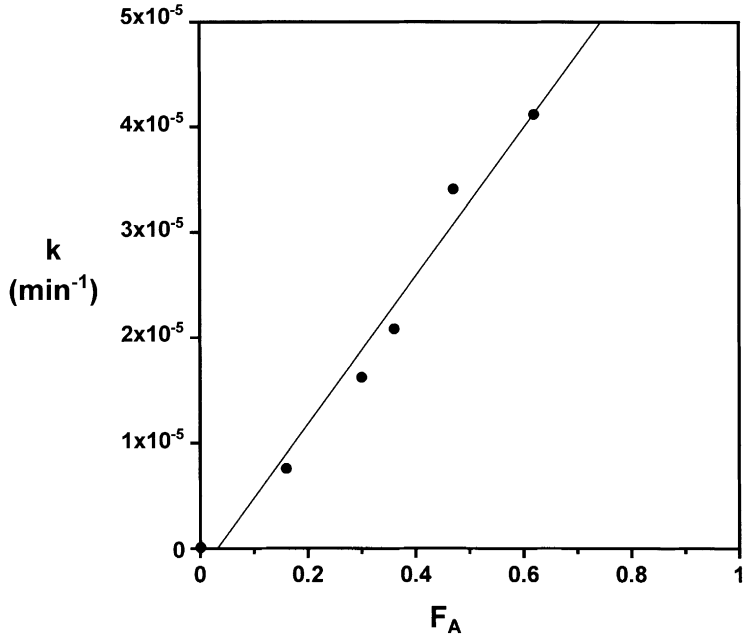

Fig. 6. Degradation rate constants (k) as a function of F_A of the chitosans, determined by the viscosity assay at a chitosan concentration of 1.5 mg/ml in 0.4 M HCl at 60°C. The estimated rate constant (equal to 5×10^{-8} min^{-1}) of the fully de-*N*-acetylated chitosan ($F_A < 0.0003$) is included in the figure, and was obtained from the extrapolated value at 60°C (see Fig. 8).

specificity with respect to cleavage of the **A–A** and the **A–D** glycosidic linkages.

3.4. Degradation rates as a function of acid concentration and F_A

The rate constants of acid depolymerization of a series of chitosans with different chemical composition (F_A) were measured by the viscosity assay (Nordtveit et al., 1994) in order to determine rates as a function of the chemical composition of the chitosans. The time course of degradation (plotted as the degree of scission (α) versus time) of the chitosans with F_A from 0.16 to 0.62 was linear, as demonstrated for the chitosan with F_A of 0.62 (see Section 3.5). The rate constants measured by the viscosity assay as a function of the concentration of acid (HCl) is shown in Fig. 5 for a chitosan with F_A of 0.62, showing that the degradation rate increase in direct proportion to the acid concentration ($0.01\ M < [HCl] < 0.8\ M$), indicating that acid hydrolysis is the mechanism by which this chitosan is degraded. The viscosity assay is quite sensitive, and can be used to determine very low degrees of chain scission (α), and relative numbers of bonds broken less than 0.005 can be measured (see Figs. 7–9). Since the change in the F_A-value due to deacetylation in dilute acid will be equal to the relative numbers of bonds broken (see Table 2), it follows that the change in F_A-value as a result of de-*N*-acetylation during the degradation experiment for all chitosans used in this study will be negligible, except for the highly de-*N*-acetylated chitosans, to be discussed later.

The degradation constants were also measured as a function of the chemical composition of the chitosans (F_A), and the results are presented in Fig. 6. It is seen that the degradation constants increase in direct proportion to F_A. In order to compare the acid degradation data with the specificity results from NMR, we need to express the rate of acid degradation as a function of F_A. The rate constants can be related to the rate of cleavage of the four different glycosidic linkages as follows:

$$k = k_{AA}F_{AA} + k_{AD}F_{AD} + k_{DA}F_{DA} + k_{DD}F_{DD} \quad (1)$$

where k_{AA}, k_{AD}, k_{DA}, and k_{DD} are the rate constants at the given acid concentration for cleavage of the four different glycosidic linkages **A–A**, **A–D**, **D–A** and **D–D**, respectively, and F_{AA}, F_{AD}, F_{DA} and F_{DD} are the fractional content of the diads **AA**, **AD**, **DA** and **DD**, respectively. The results from the specificity of NMR studies indicated that the rates of acid hydrolysis of the **D–D** and **D–A** glycosidic linkages can be neglected relative to the **A–A** and **A–D** linkages, implying that $k_{AA} = k_{AD} \gg k_{DA} = k_{DD}$, and Eq. (1) can be simplified to

$$k \approx k_{AA}F_{AA} + k_{AA}F_{AD} \quad (2)$$

The chitosans have a Bernoullian (random) distribution of **A**- and **D**-units (Vårum et al., 1991a,b) meaning that

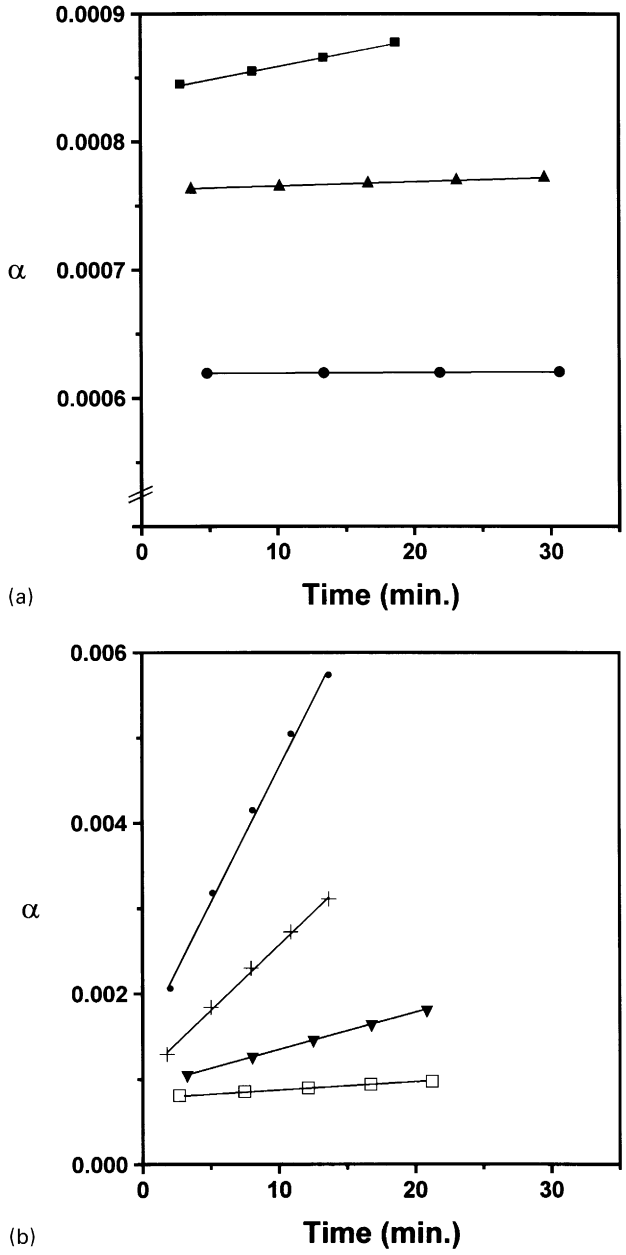

Fig. 7. Time course of degradation of the chitosan with F_A of 0.62 (see Table 1) in 0.4 M HCl (chitosan concentration: 1.5 mg/ml) at different temperatures (20.0–77.0°C), where α is plotted versus time. Note different α-axis in (a) and (b). (a) ● 20.0°C; ▲ 29.3°C; ■ 39.5°C. (b) □ 49.3°C; ▼ 59.9°C; × 71.0°C; ● 77.0°C.

$F_{AA} = F_A^2$ and $F_{AD} = F_A F_D$, and we get

$$k \approx k_{AA}F_A(F_A + F_D) \quad (3)$$

As $F_A + F_D = 1$, k should increase in direct proportion to F_A, with the above assumptions, which is in agreement with the experimental data shown in Fig. 6. Thus, the viscosity results are in line with the proton NMR results with respect to the specificity in the acid hydrolysis reaction of partially *N*-acetylated chitosans.

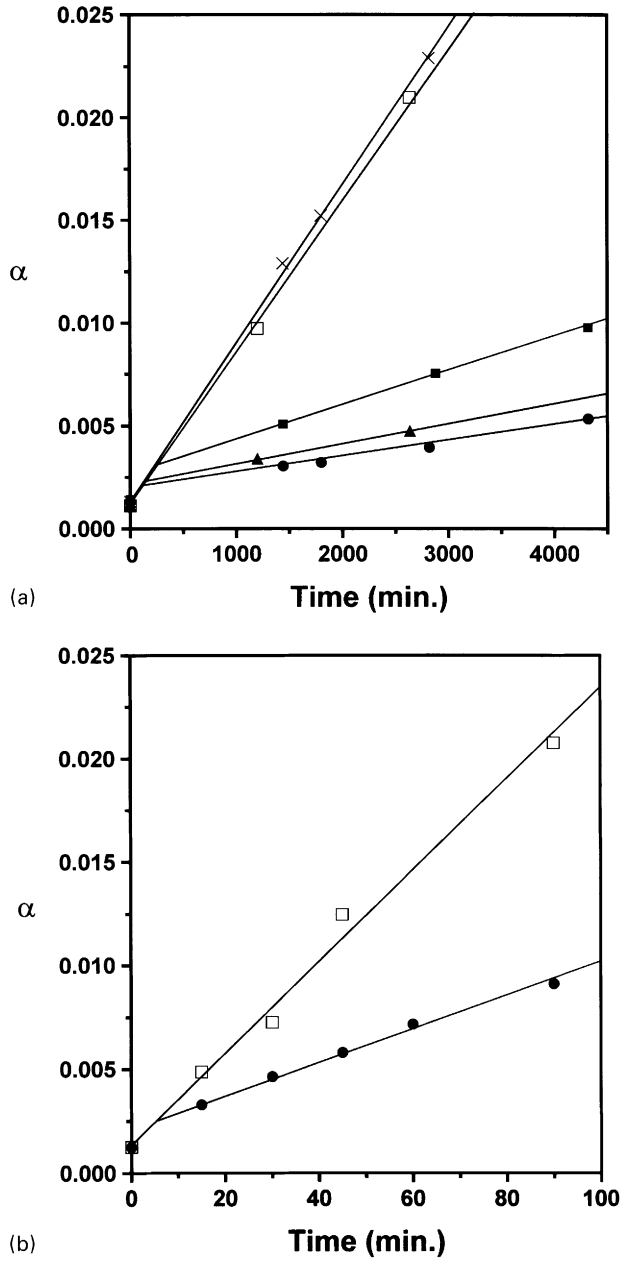

Fig. 8. Time course of degradation of the chitosan with $F_A = 0.002$ (see Table 1) in 0.4 M HCl (chitosan concentration: 2.0 mg/ml) at different temperatures, where α is plotted versus time. Note different time-axis in (a) and (b). (a) ● 75°C; ▲ 80°C; ■ 84°C; □ 90°C; × 95°C. (b) ● 110°C; □ 120°C.

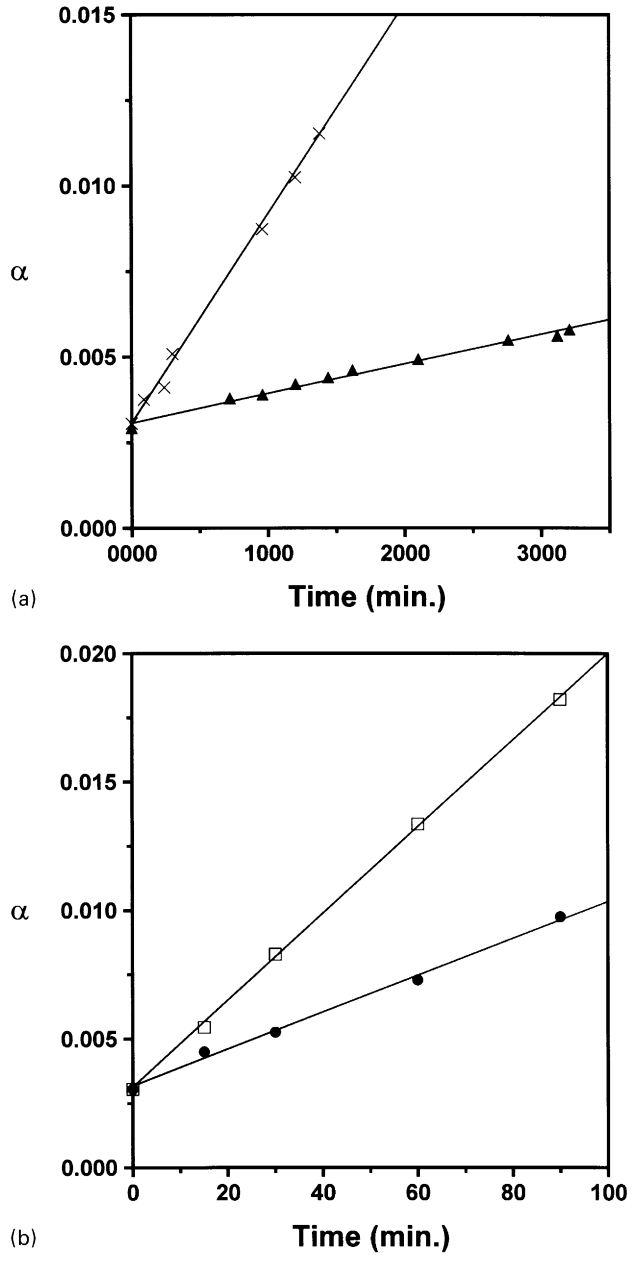

Fig. 9. Time course of degradation of the chitosan with $F_A < 0.0003$ (see Table 1) in 0.4 M HCl (chitosan concentration: 2.0 mg/ml) at different temperatures, where α is plotted versus time. Note different time-axis in (a) and (b). (a) ▲ 80°C; × 95°C. (b) ● 110°C; □ 120°C.

From Fig. 6 a ratio of more than 1000 between the extrapolated value of the rate constants of a fully *N*-acetylated chitin ($F_A = 1$; $k = 7 \times 10^{-5}$ min^{-1}) and a fully de-*N*-acetylated chitosan ($F_A = 0$; $k = 5 \times 10^{-8}$ min^{-1}) is estimated (0.4 M HCl and 60°C).

3.5. Degradation rates as a function of temperature

The acid hydrolysis rate constants of four chitosans, two extensively de-*N*-acetylated chitosans ($F_A = 0.002$ and $F_A < 0.0003$) and two chitosans with F_A of 0.47 and 0.62, were determined as a function of temperature by the viscosity assay in 0.4 M HCl. The time course of degradation of the chitosan with $F_A = 0.62$ at different temperatures is presented in Fig. 7a and b as plots of degree of scission (α) versus time, showing a linear increase in α with time. Similar plots were obtained for the chitosan with $F_A = 0.47$ (data not shown). The more extensively de-*N*-acetylated chitosans were hydrolysed much more slowly by acid compared to the more acetylated chitosans. The time course of the degradation of the chitosan with $F_A = 0.002$ is shown

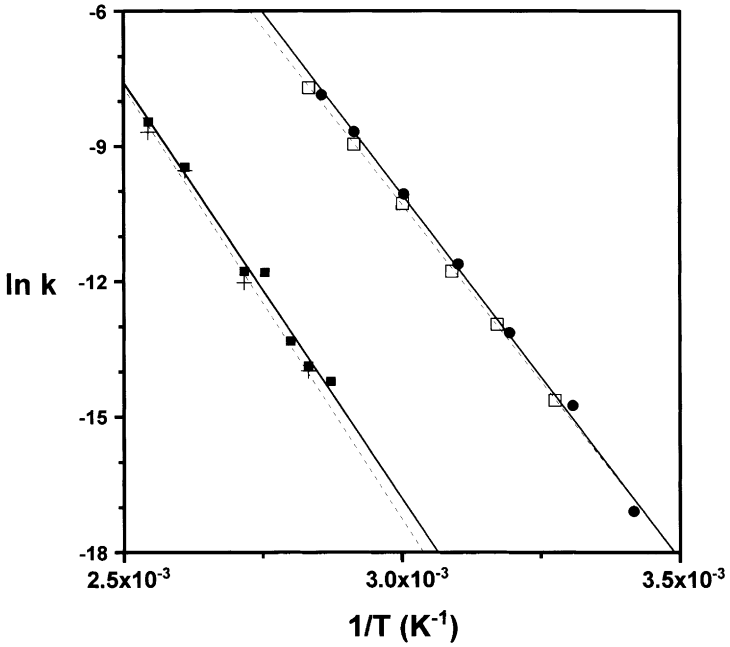

Fig. 10. Degradation rate constants (logarithmic scale), determined from the slopes of the time course of degradation in Figs. 7–9 as a function of the inverse of the absolute temperature of chitosans with different chemical composition. - - × - $F_A < 0.0003$; –■– $F_A = 0.002$; - - ■ - - $F_A = 0.47$; –●– $F_A = 0.62$.

in Fig. 8a and b, as plots of α versus time. It should be noted that at lower temperatures where relative α-values lower than 0.002 are determined, an initial non-linearity in the curves can be seen (e.g. Fig. 8a) with a faster initial degradation rate, which then decreases and becomes linear when relative α-values increase above 0.002. This can be explained by the presence of the very low fraction of acetylated units ($F_A = 0.002$) which are hydrolysed three orders of magnitude faster than the deacetylated units and the use of the viscometric assay where relative α-values in the same range as the F_A-value of the chitosan that is degraded. When the relative α-values exceed the F_A-value of the chitosan, linear time curves should be obtained, which is indeed observed in Fig. 8, and the slope of the line at relative α-values above 0.002 should then represent the rate constant for the acid hydrolysis of the glycosidic linkage between two deacetylated units (k_{DD}). In order to test this explanation, the chitosan with $F_A = 0.002$ was further de-*N*-acetylated to F_A less than 0.0003, and the time course of the degradation at different temperatures of this more extensively de-*N*-acetylated chitosan is presented in Fig. 9a and b, showing a linear increase in α with time even at the lowest relative α-values.

The results from the degradation experiments of the four chitosans at the different temperatures are presented in Fig. 10 as a plot of the natural logarithm of the rate constants as a function of the inverse of the absolute temperature (Arrhenius plot). The activation energies calculated from the slopes of the lines (see Fig. 10) for the two almost fully de-*N*-acetylated chitosans ($F_A = 0.002$ and $F_A < 0.0003$) were 152.2 ± 8.1 and 158.1 ± 9.8 kJ mol^{-1}, respectively. The values are the same within the experimental error of the determination, and this activation energy should then represent the activation energy for hydrolysis of the **D–D** glycosidic linkage in the chitosans. The activation energies for acid hydrolysis of two partially *N*-acetylated chitosans ($F_A = 0.47$ and 0.62) were determined to 130.4 ± 2.5 and 134.3 ± 3.1 kJ mol^{-1}, respectively, representing the activation energy for hydrolysis of the **A–A** and **A–D** glycosidic linkage in chitosans.

The activation energies of the two chitosans with the higher F_A-values of 0.47 and 0.62 are similar to the value of activation energy of the model compound methyl-2-acetamido-2-deoxy-β-D-glucopyranose of 118.4 kJ mol^{-1} (Moggridge & Neuberger, 1938) and to the activation energy of 123.8 kJ mol^{-1} of chitin reported by Meyer and Wehrli (1937), but higher than the activation energies for hydrolysis of chitin in 11 M HCl of 94.1 kJ mol^{-1} reported by Rupley (1964). Holme, Foros, Pettersen, Dornish and Smidsrød (2000) have studied the thermal degradation of chitosan chlorides in the solid state, and suggested that the primary degradation mechanism is by acid hydrolysis. They determined activation energies around 110 kJ mol^{-1}.

The activation energy of the highly de-*N*-acetylated chitosans is significantly higher than the activation energy of the chitosans with the higher F_A-values. Our results are consistent with the activation energy of 150.6 kJ mol^{-1} of the model compound methyl-2-amino-2-deoxy-β-D-glucopyranose, which was also found to be much higher compared to an activation energy of 118.4 kJ mol^{-1} for the *N*-acetylated methyl-glucosaminide (Moggridge & Neuberger, 1938).

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