Some Properties of Contiguous Binding Subsites on Lysozyme as Determined by Proton Magnetic Resonance Spectroscopy*

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ABSTRACT: The association of chitobiose, chitotriose, and chitotetraose as well as methyl β-chitobioside and methyl β-chitotrioside with lysozyme has been studied using proton magnetic resonance methods. Selective broadening of resonances in the inhibitors due to macromolecular association has been shown to be due to slow exchange rates since chemical shift differences exist for these nuclei between free and enzyme-bound environments. Quantitation of these chemical shifts, under conditions of rapid exchange, has allowed assignment of specific magnetic parameters to three contiguous binding subsites on the enzyme to which acetamidopyranoside residues bind. From these assignments the relative ways in which all of the inhibitors occupy the three subsites have been delineated. It has been shown that the reducing-end pyranose rings of chitobiose, chitotriose, and chitotetraose all bind to one of the three sites (subsite C) with the other pyranose rings of the di-, tri-, and tetrasaccharides occupying subsites B; B and A; B, A, and solution, respectively. 2-Acetamido-2-deoxy-D-glucopyranose has also been shown to occupy subsite C, with its α- and β-anomeric forms oriented differently in that subsite. The α- and β-anomeric forms of chitobiose and chitotriose have been shown to bind identically. It has been shown that methyl β-chitobioside and methyl β-chitotrioside bind to the enzyme with the acetamidopyranose rings at their methyl glycosidic ends in subsite C and the other rings in subsites B, and B plus A, respectively. Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside have also been shown to bind in subsite C. The results obtained agree in general with results obtained elsewhere regarding the binding of some of these saccharides to crystalline lysozyme.

Recent use of proton magnetic resonance techniques as a probe of the association between lysozyme and some of its inhibitors (Raftery *et al.*, 1968b) had demonstrated the potential of the method for studies of enzyme–inhibitor or enzyme–substrate interactions. Based on quantitation of chemical shifts induced in selected nuclei of the inhibitors, as a result of macromolecular association, it has been possible to gain information regarding the environment experienced by these nuclei in the complexed state. One stringent requirement is that the exchange rate between free and bound species of the inhibitor be rapid. It has been shown (Dahlquist and Raftery, 1968a) that the proton magnetic resonance method can be used to determine whether the enzyme-bound orientations of competitive inhibitors are identical or not through a comparison of the magnetic environment(s) experienced by certain nuclei in the inhibitors when bound to the enzyme. In this manner it was shown that although α-2-acetamido-2-deoxy-D-glucopyranose and β-2-acetamido-2-deoxy-D-glucopyranose bind competitively to lysozyme with almost the same binding constant (K_s α-2-acetamido-2-deoxy-D-glucopyranose = 1.6×10^{-2} M; K_s β-2-acetamido-2-deoxy-D-glucopyranose = 3.3×10^{-2} M), their average orientations in the complexed state are different. This conclusion was arrived at through determination of the chemical shift induced in the acetamido methyl protons of the two anomeric forms upon association with the enzyme. Thus the availability of a second parameter, the chemical shift, as an index of association allows additional information to be gleaned regarding the nature of a macromolecular binding site.

Previous studies, employing ultraviolet spectroscopic methods, on the association of β-(1–4)-linked oligosaccharides of 2-acetamido-2-deoxy-D-glucopyranose (Dahlquist *et al.*, 1966) have allowed calculation of the dissociation constants for the monosaccharide through the hexasaccharide in association with the enzyme. In both studies it was shown that binding strength increased with increasing chain length up to the trisaccharide but that the tetra-, penta-, and hexasaccharides did not appear to bind any more strongly than did the trisaccharide. These results indicated that lysozyme contains three contiguous subsites to which acetamidopyranose rings bind strongly. This interpretation is in agreement with findings (Johnson and Phillips, 1965; Blake *et al.*, 1967) employing X-ray analysis techniques to study the association of crystalline lysozyme with 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, and chitotriose. It should be noted that dissociation constants obtained by the ultraviolet spectroscopic method used in this laboratory (Dahlquist *et al.*, 1966) and by others (Rupley *et al.*, 1967), as well as fluorescent spectroscopic techniques further used (Lehrer and Fasman, 1966, 1967; Chipman *et al.*, 1967) to study association of various inhibitors with lysozyme are composed of

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mean values for both anomeric forms of the saccharides under investigation. It is not possible by these techniques to determine whether both anomeric forms bind or to estimate individual binding constants for the α and β forms.

The use of proton magnetic resonance techniques can, however, allow distinction between the different anomeric forms in association with the enzyme. Although it could be shown by such an approach (Raftery *et al.*, 1968b; Dahlquist and Raftery, 1968a,b) that 1:1 complexes were formed between the enzyme and the various monosaccharide inhibitors, it was not possible to relate this to association with only one of the three contiguous subsites rather than to multiple equilibria with all three sites. If this latter possibility were the case the dissociation constants and chemical shifts obtained would represent complex entities. The present study was undertaken in an attempt to resolve this question as well as that of the relative ways in which the mono-, di-, and trisaccharides of 2-acetamido-2-deoxy-D-glucopyranose and their methyl glycosides bind to the enzyme.

Experimental Section

Materials

2-Acetamido-2-deoxy-D-glucopyranose was obtained from the California Corp. for Biochemical Research. The oligosaccharides chitobiose, chitotriose, and chitotetraose were prepared from partial acid hydrolysates of chitin by a gel filtration method (Raftery *et al.*, 1968a) and were shown to be homogeneous by chromatography on paper. The staining procedure used has been described by Powning and Irzykiewicz (1965). Crystallization of chitobiose prepared in this manner was effected from methanol–water and yielded α-chitobiose (mp 262–264° dec).

Synthesis of Chitobiose-N_1-d_3. This material was prepared from chitobiose by the following procedure. Treatment of chitobiose (1.9 g) with 80 ml of pyridine–acetic anhydride (1:2, v/v) for 16 hr at room temperature followed by dilution with chloroform (50 ml) and extraction with cold water (three 100-ml portions), cold dilute sodium bicarbonate (three 100-ml portions), and cold water (two 50-ml portions) gave a solution which on drying over sodium sulfate, evaporation to a syrup, and trituration with diethyl ether yielded a crystalline product. This was characterized as octaacetylchitobiose (mp 300–305°).

Treatment of octaacetylchitobiose (5.4 g) with acetic anhydride (100 ml), which had been saturated with dry HCl at 0°, for 2 days at room temperature followed by dilution with chloroform (100 ml), neutralization by extraction with saturated sodium bicarbonate solution at 0–5°, washing the chloroform extract with cold water, drying over anhydrous sodium sulfate, evaporation to a syrup at 30°, and trituration with diethyl ether yielded a semicrystalline product. Following recrystallization from acetone–petroleum ether (bp 30–60°) this material, characterized by nuclear magnetic resonance and infrared spectra as acetochlorochitobiose, had a melting point of 185–187°; yield 2.4 g.

To obtain heptaacetylchitobiose hydrochloride a solution of acetochlorochitobiose (1.0 g) in dry nitromethane (30 ml) was left at room temperature for 1 day after addition of 50 μg of 0.1 N HCl and 50 μg of water. The crystallized hydrochloride was collected, washed with ether, and air dried. The supernatant was again treated with 50 μg of 0.1 N HCl and 50 μg of water and left overnight. Four such treatments gave 0.7 g of the hydrochloride, mp 218° dec.

Octaacetylchitobiose-N_1-d_3 was obtained by acetylation of 0.7 g of heptaacetylchitobiose hydrochloride with a mixture of pyridine (5 g) and acetic anhydride-d_6 (1.0 g) for 4 hr at room temperature. The product was isolated by the procedures already outlined: yield 0.5 g, mp 300–305°. Deacetylation of this material (0.4 g) in methanol containing sodium methoxide (0.1 M) at 40° for 3 hr followed by evaporation of the solvent, dissolution in water, neutralization with dilute H_2SO_4, and gel filtration on a column (2.5 × 90 cm) of Bio-Gel P-2 gave chitobiose-N_1-d_3; yield 0.15 g, mp 260–262° dec.

Synthesis of Methyl β-Chitobioside. Acetochlorochitobiose was prepared as already described. This material (0.4 g) was reacted in anhydrous methanol (25 ml) containing 1.0 g of silver carbonate and 1.0 g of anhydrous calcium sulfate for 16 hr at room temperature. It was then filtered, evaporated to a syrup, dissolved in chloroform (50 ml), extracted twice with 50 ml of dilute aqueous ammonia and with dilute bicarbonate, dried over anhydrous sodium sulfate, and evaporated to dryness. Deacetylation was effected in 0.1 M sodium methoxide in methanol (30 ml) at 40° for 3 hr. After evaporation of the solvent the residue was dissolved in water (50 ml) and quickly neutralized. The inorganic salts and sugars which contained free reducing termini were quantitatively removed by stirring the aqueous solution with 5 g of Amberlite MB-1 (mixed-bed ion exchangers) for 1 hr. The supernatant was lyophilized; yield 0.120 g. Final purification was effected by dissolving this material in water (2 ml) and subjecting it to gel filtration on a column (1.5 × 90 cm) of Bio-Gel P-2 using water as the eluting solvent. The glycoside was detected by reading the absorbance of the eluted fractions at 225 mμ. The requisite tubes were pooled and lyophilized to give 100 mg of methyl β-chitobioside, mp 287–288°.

Synthesis of Methyl β-Chitotrioside. Chitotriose (4.5 g) was converted into its peracetyl derivative by heating under reflux in acetic anhydride (180 ml) containing 3.1 g of anhydrous sodium acetate for 1 hr with vigorous stirring (Osawa, 1966). The product was isolated by methods described for the isolation of octaacetylchitobiose: yield 5.0 g, mp 315°. Reaction in acetic anhydride–dry HCl for 48 hr as described for the preparation of acetochlorochitobiose yielded, after working up, 0.7 g of acetochlorochitotriose. This material (0.5 g) was treated with dry methanol (25 ml) in the presence of silver carbonate (1 g) and anhydrous calcium sulfate (1.0 g) for 24 hr with stirring in the dark. The reaction mixture was filtered, evaporated to a syrup, and dissolved in chloroform (100 ml). The chloroform solution was extracted twice with an equal volume of 5% ammonia solution and once with saturated NaCl solution, and dried over anhydrous sodium sulfate. The chloro-

form was evaporated and the resulting amorphous solid was dried *in vacuo*. This material was deacylated in 0.1 N sodium methoxide at 40° for 3 hr and left overnight at room temperature. The sodium methoxide was neutralized with acetic acid and the solution was then evaporated to dryness. The residue was dissolved in water (100 ml) and deionized by treatment with the mixed-bed ion exchanger Amberlite MB-1. This treatment also removed any reducing sugars present. The residual solution was lyophilized. Fractionation on a column (1.5 × 70 cm) of Bio-Gel P-2 using water as eluting solvent gave two compounds, which were characterized as methyl β-chitobioside (50 mg, mp 287–288°) and methyl β-chitotrioside (20 mg, mp 306–310° dec) by virtue of their nuclear magnetic resonance spectra. They were found to be homogeneous compounds on paper chromatography in pyridine–water–2 pentanol (1:1:1).

Lysozyme (Lot No. 96B-8572) was purchased from Sigma Chemical Co. Amberlite MB-1 mixed-bed ion exchanger was obtained from Mallinckrodt.

Methods

Enzyme solutions contained approximately 3×10^{-3} M lysozyme (Sigma Chemical Co., Lot No. 96B-8572) and 0.5% each of methanol and acetone as internal proton magnetic resonance standards. The buffers were made by mixing 0.1 M citric acid with either 0.1 M sodium citrate, 0.1 M disodium phosphate, or 0.1 M trisodium phosphate. The exact concentration of the enzyme was determined by removing 25 μl, diluting to 5.00 ml with 0.1 M citrate buffer (pH 5.5), and measuring the optical density of the solution at 280 mμ with a Cary Model 14 spectrophotometer. The known extinction coefficient was used to estimate lysozyme concentrations (Sophianopoulos *et al.*, 1962).

All spectra were obtained with a Varian HA-100 nuclear magnetic resonance spectrometer, operating in frequency sweep mode. The water resonance was used as a lock signal, and each sample was allowed to come to thermal equilibrium before measurements were taken. For measurements at temperatures other than 31°, the operating temperature of the probe, a Varian V-4341 variable-temperature accessory was employed. The difference in chemical shift of methanol or ethylene glycol as a function of temperature was used to determine the probe temperature.

Chemical shifts were determined by electronic counting of the difference between the sweep frequency and manual oscillator frequency using a Hewlett-Packard counter.

The chemical shift of the glycosidic methyl group in the bound state was determined by measurement of the ratio of the glycosidic methyl group shift to the acetamido methyl group shift. This ratio was then multiplied by the value of the chemical shift, Δ, of the bound acetamido group which was determined separately from a concentration study of the observed chemical shift of the acetamido group resonance. This method was used because the glycosidic methyl group displayed only a small observed shift, and the error in a concentration study of its resonance would have been very large.

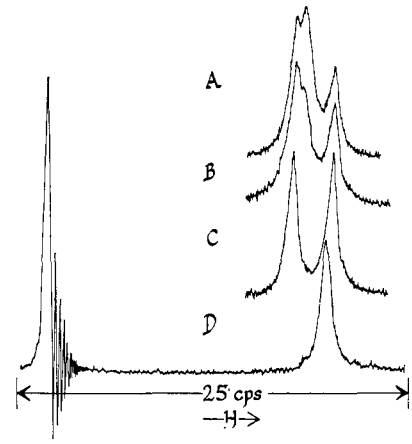

FIGURE 1: Proton magnetic resonance spectra of the acetamido methyl groups of: (A) chitotetraose, (B) chitotriose, (C) chitobiose, and (D) 2-acetamido-2-deoxy-D-glucopyranose. The sharp and intense resonance to lowest field is that for the methyl protons of an acetone internal standard.

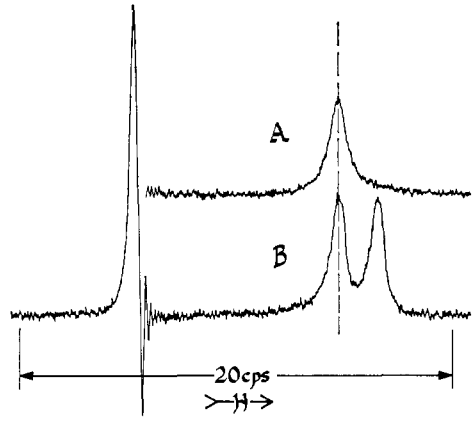

FIGURE 2: Proton magnetic resonance spectra of the acetamido methyl protons of: (A) chitobiose-N_1-d_3 and (B) chitobiose, relative to an acetone standard (to lowest field).

All data were analyzed by least-squares methods. The error limits quoted are standard deviations, σ, from the mean.

Mutarotation Studies. A weighted amount of crystalline α-chitobiose was thermally equilibrated in a 1.00-ml volumetric flask in a water bath at 31°. This material was dissolved in a thermally equilibrated solution which contained enzyme, buffer, and acetone. After the sample dissolved, it was transferred to a nuclear magnetic resonance tube, equilibrated in the water bath for 1 min, and placed in the probe. The spectrometer was locked on the water resonance, and the spectrum recorded. A spectrum could be produced in this manner in 3–4 min from the time the crystalline inhibitor was dissolved.

Results

Assignment of Methyl Group Proton Resonances in Chitin Oligosaccharides. The various chitin oligosaccharides have magnetically nonequivalent acetamido

methyl group resonances. The proton magnetic resonance spectra, due to these methyl groups, of the mono-, di-, tri-, and tetrasaccharides are shown in Figure 1. The resonances for chitobiose cannot be unambiguously assigned to its two acetamido methyl groups without specific deuteration of one of them. This was accomplished by use of an N→O acyl shift previously employed in this laboratory for preparation of specifically labeled chitotriose (Dahlquist and Raftery, 1967). The proton magnetic resonance spectra of chitobiose and chitobiose-N_1-d_3 are compared in Figure 2. The deuterated compound lacks the acetamido resonance to higher field and therefore this resonance in chitobiose can be attributed to the acetamido group on the pyranose ring at the reducing end of the disaccharide.

The proton magnetic resonance spectrum for the acetamido methyl resonances of chitotriose is shown in Figure 1. The chemical shift of the resonance to higher field corresponds closely to that due to the acetamido methyl group at the reducing end of chitobiose (see Table I) and has therefore been assigned to the methyl group at the reducing end of chitotriose. The acetamido methyl resonances to lower field consist of a poorly resolved doublet of unequal line width with the broader resonance occurring about 0.45 Hz upfield from the sharper one. It was not possible to directly assign these two resonances specifically to each of the acetamido methyl groups on the nonreducing pyranose rings of the trisaccharide. Such an assignment was possible, however, based on a comparison with the proton magnetic resonance spectrum of the tetrasaccharide, chitotetraose. Examination of the acetamido methyl group proton magnetic resonance spectrum of chitotetraose revealed a resonance to higher field which could again be assigned to the methyl group on the pyranose ring at the reducing end of the molecule. In addition a poorly resolved doublet was observed as in the case of the trisaccharide. However, the peak heights of the components of the doublet were nearly equal indicating that the broad resonance was due to two methyl groups and the sharp resonance due to a single methyl group. On this basis the broad component of the doublet (occurring to higher field) was assigned in one case to the middle acetamido methyl group of chitotriose and in the other case to the two interior acetamido methyl groups of chitotetraose. The sharp resonance in the doublet, occurring to lower field, was in each case assigned to the acetamido methyl group on the nonreducing end of chitotriose and chitotetraose. The chemical shift of this resonance also corresponds closely to that for the acetamido methyl group on the nonreducing end of chitobiose (see Table I). It would appear from the present results that the interior acetamido methyl groups of chitotriose and chitotetraose are somewhat restricted in their environment and that as a result their resonances have greater intrinsic line widths.

We have also studied the association of the methyl glycosides of chitobiose and chitotriose with lysozyme. Previously we have shown (Raftery *et al.*, 1968b,c) that methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside are well suited for such studies since their binding to the enzyme is not complicated by attendant mutarotation as in the case of the free saccharides. The proton magnetic resonance spectrum (at 60 MHz) of methyl β-chitobioside revealed three methyl groups as singlets. The one furthest downfield (τ 6.49) was, by analogy with methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, due to the glycosidic methyl group. The other two methyl resonances occurred very close to the positions occupied by the acetamido methyl resonances of 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and were on this basis assigned to the two acetamido methyl groups of methyl β-chitobioside. The proton magnetic resonance spectrum at 100 MHz of these two groups is shown in Figure 3 and compared with the acetamido methyl group spectra of chitobiose, 2-acetamido-2-deoxy-D-glucopyranose, and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside. The acetamido methyl resonance to lower field corresponds closely to that which has already been assigned to the nonreducing end of chitobiose and

TABLE I: Chemical Shift Data (Apparent Maxima) for Methyl Groups in Chitin Oligosaccharides and Their Methyl Glycosides.

Inhibitor	Resonance[a]				
	CH_3–N_1[b]	CH_3–N_2[b]	CH_3–N_3[b]	CH_3–N_4[b]	OCH_3[c]
2-Acetamido-2-deoxy-D-glucopyranose	17.71				
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	18.79				14.72
Chitobiose	18.35	15.63			
Methyl β-chitobioside	19.51	15.33			13.94
Chitotriose	18.32	16.28	15.83		
Methyl β-chitotrioside	19.53	16.28	15.90		13.33
Chitotetraose	18.35	16.54	16.54	15.98	

[a] The acetamido methyl groups are numbered 1, 2, 3, and 4 beginning at the reducing or glycosidic termini of the molecules. All chemical shifts are in Hz at 100 MHz. [b] Values relative to acetone; all chemical shifts to higher field. [c] Values relative to methanol; all chemical shifts to lower field.

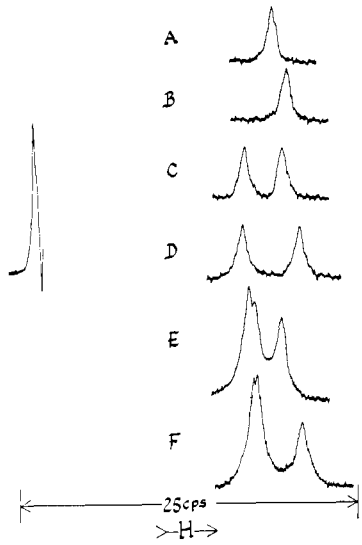

FIGURE 3: Proton magnetic resonance spectra of the acetamido methyl protons of: (A) 2-acetamido-2-deoxy-D-glucopyranose, (B) methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, (C) chitobiose, (D) methyl β-chitobioside, (E) chitotriose, and (F) methyl β-chitotrioside. All spectra were measured relative to an internal acetone standard, shown to lower field.

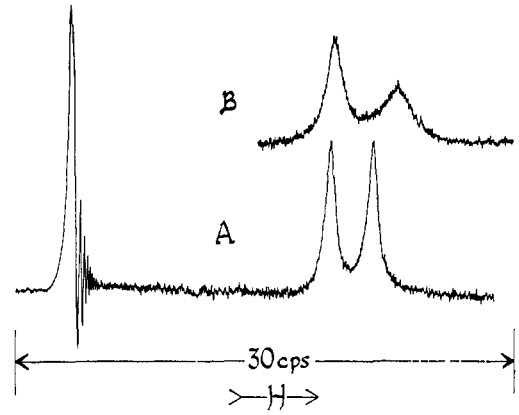

FIGURE 4: Proton magnetic resonance spectra of the acetamido methyl protons of: (A) chitobiose and (B) chitobiose ($\sim 5 \times 10^{-2}$ M) in the presence of lysozyme (3×10^{-3} M) in 0.1 M citrate buffer (pH 5.5) at 31°. Spectra were measured relative to an internal acetone standard which is shown to lower field of the acetamido methyl resonances.

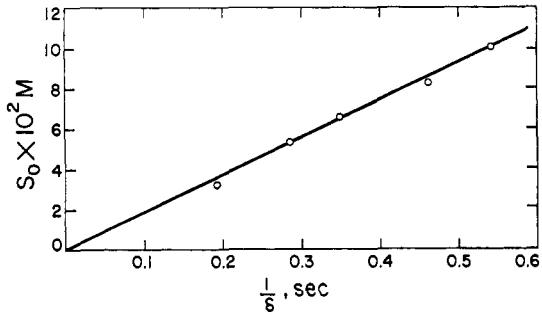

FIGURE 5: Plot of the reciprocal of the observed chemical shift, δ, for the reducing end acetamido methyl group resonance of chitobiose *vs.* varying chitobiose concentrations, S_0, in the presence of a constant concentration of lysozyme (3×10^{-3} M).

on this basis can be assigned to the sugar residue distal from the glycosidic methyl group of methyl β-chitobioside. Table I shows the chemical shifts of these resonances relative to acetone. That the resonance to higher field (19.51 Hz relative to acetone) represents the acetamido methyl group at the glycosidic end of methyl β-chitobioside may be inferred from the comparison with the acetamido methyl resonance of 2-acetamido-2-deoxy-D-glucopyranose, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and chitobiose, as shown in Figure 3. The upfield shift caused in the acetamido methyl resonance of 2-acetamido-2-deoxy-D-glucopyranose on glycosidation to form methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (1.08 Hz) was also seen on glycosidation of chitobiose. The change in chemical shift in this case was 0.96 Hz.

The proton magnetic resonance spectra due to the acetamido methyl groups of chitotriose and methyl β-chitotriose are shown in Figures 3E–F, respectively. It is evident that the resonance to higher field in Figure 3F corresponds to the methyl group at the glycosidic end of the molecule. It has been shifted to higher field upon glycosidation when compared with the resonance due to the acetamido methyl group at the reducing end of chitotriose. The increase in chemical shift to higher field on glycosidation was 1.21 Hz. The acetamido methyl resonances to lower field in Figure 3F are barely resolved. Their chemical shifts relative to acetone are given in Table I. It can be seen that the component to higher field has the same chemical shift as that which we have previously ascribed to the internal acetamido methyl group of chitotriose. Thus we also assign this resonance to the internal acetamido group of methyl β-chitotrioside and the resonance to lower field to the acetamido methyl group at the nonglycosidic end of the trisaccharide glycoside.

The Association of Chitobiose with Lysozyme. The effect of binding to lysozyme on the acetamido methyl resonances of chitobiose at pH 5.5 and 31° is shown in Figure 4. The most pronounced effect observed was that the reducing end acetamido methyl resonance was shifted to higher field and was at the same time broadened considerably when compared with the spectrum obtained in the absence of enzyme. The nonreducing end acetamido methyl resonance, on the other hand, was not broadened nearly so much and displayed no chemical shift.

We have previously shown (Dahlquist and Raftery, 1968a) that for a single binding site the observed shift (δ) is related to the total substrate or inhibitor concentration (S_0) by $S_0 = (\Delta E_0/\delta) - K_s - (E_0 - ES)$, where Δ is the chemical shift associated with the binding site environment, the term E_0 represents the total enzyme concentration, and K_s is the dissociation constant of the enzyme–substrate or enzyme–inhibitor complex. This equation can be simplified to $S_0 = (\Delta E_0/\delta) - K_s$ (Raft-

ery *et al.*, 1968c). From a plot of S_0 *vs.* $1/\delta$ values for K_s and Δ can be obtained. Figure 5 shows such a plot for chitobiose in association with lysozyme at pH 5.0 and 45°. The value of K_s under these conditions was found to be equal to zero, within the error limits. This is in accord with the value of $K_s = 1.8 \times 10^{-4}$ M determined by ultraviolet spectroscopic methods (Dahlquist *et al.*, 1966) since this value is essentially zero relative to the concentration range of chitobiose used ($2\text{–}10 \times 10^{-2}$ M) for the proton magnetic resonance measurements. The value of Δ calculated from this plot was 0.57 ± 0.04 ppm to higher field. Since the α- and β-anomeric forms of the disaccharide were present at mutarotation equilibrium in the sample of chitobiose used, this value of Δ is not a true measure of the binding environment but is equal to

$$\frac{\Delta_\alpha}{\frac{[\alpha]}{S_0} + \frac{K_\alpha}{K_\beta} \times \frac{[\beta]}{S_0}} \text{ or } \frac{\Delta_\beta}{\frac{[\beta]}{S_0} + \frac{K_\beta}{K_\alpha} \times \frac{[\alpha]}{S_0}}$$

where S_0 refers to the total inhibitor or substrate concentration, $[\alpha]$ and $[\beta]$ to the concentrations of the α- and β-anomeric forms at mutarotation equilibrium, K_α and K_β to the dissociation constants of the enzyme–α and enzyme–β complexes, and Δ_α and Δ_β to the chemical shifts associated with the binding sites of the α- and β-anomeric forms.[1]

We have shown earlier (Dahlquist and Raftery, 1968a) that the value K_α/K_β can be determined when the α and β forms of the associating saccharide show resolved resonances in the presence of the enzyme. However, the anomeric forms of the disaccharide did not resolve over the pH range 2–10, at both 31 and 45°. This strongly suggests that the enzyme does not differentiate between the two anomeric forms. Furthermore, at 31° the spectrum of chitobiose in the presence of lysozyme did not appear to undergo any change in shape or chemical shift when a sample of the disaccharide which was predominantly α-chitobiose was dissolved in enzyme solution and the acetamido methyl resonances observed as a function of time. At 45° and pH 5.5, conditions under which the reducing end acetamido methyl resonances are sharper because of increased exchange rates, a freshly dissolved sample of α-chitobiose showed the same shift as a sample which had attained mutarotation equilibrium. Unfortunately, it was not possible to perform experiments where the resonances of the freshly dissolved disaccharide were observed directly as a function of time at the higher temperature because sufficient hydrolysis and transglycosylation occurred in the 30–45 min required for the experiment. Thus the disaccharide concentration was changed and sufficient trisaccharide was produced to compete effectively for the disaccharide binding site. The results obtained clearly demonstrate that both anomeric forms of the saccharide occupy magnetically equivalent positions on the enzyme surface with the same affinity, and that these equivalent positions are competitive for both anomers. These results can be most reasonably accommodated if the equivalent positions are actually the same binding site, with both anomers having the same average orientation in that binding site.

The Association of Methyl β-Chitobioside with Lysozyme. Our previous results (Raftery *et al.*, 1968b; Dahlquist and Raftery, 1968a,b) on the association with lysozyme of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and the α and β forms of 2-acetamido-2-deoxy-D-glucopyranose, using proton magnetic resonance methods, have shown that all these monosaccharides bind with the same average orientation on the enzyme with the exception of α-2-acetamido-2-deoxy-D-glucopyranose. This last saccharide binds competitively with the β anomer but does not enter precisely the same environment as judged from the chemical shift undergone by its acetamido methyl group. In addition, it was shown that the glycosidic methyl group of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside undergoes a chemical shift to lower field due to association with the enzyme whereas the glycosidic methyl group of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside did not appear to undergo any change in chemical shift due to binding. In the work presented here the association with lysozyme of methyl β-chitobioside at pH 5.5, 31°, was studied using both the two acetamido methyl resonances and the glycosidic methyl resonance to probe the magnetic environment of the site on the enzyme to which it binds. Figure 6 shows the effects of binding to the enzyme on the acetamido methyl resonances. The most pronounced effects observed were that the acetamido methyl resonance of the methyl glycosidic end was shifted to higher field and also was broadened considerably. The resonance due to the acetamido methyl group distal from the glycosidic methyl group did not appear to undergo any change in chemical shift. This resonance was broadened but by an amount less than that observed for the acetamido methyl resonance at the glycosidic end of the molecule. The glycosidic methyl group was also found to undergo a chemical shift due to association with the enzyme as shown in Figure 7. As in the case of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside this shift was to lower field. Qualitatively, these observed shifts are in agreement with the behavior of the monosaccharide, the monosaccharide β-methyl glycoside, and the disaccharide chitobiose. Calculation of the chemical shift, Δ, for the acetamido methyl protons proximal to the glycosidic end of the disaccharide glycoside yielded a value of 0.60 ± 0.05 ppm to higher field. Similarly a value of 0.20 ± 0.03 ppm to lower field was obtained for the glycosidic methyl group. Table II lists these values and compares them with the chemical shifts observed for the various monosaccharides and monosaccharide glycosides previously studied.

A study of the association of methyl β-chitobioside with lysozyme at pH 9.7 and 55° was also conducted. The effect of adding varying amounts of the glycoside to a constant amount of enzyme on the acetamido methyl group resonances of the small molecule is shown in Figure 8. It was seen that the resonance due to the

[1] For a complete discussion of this question relating to determination of values of K_s and Δ for such anomeric mixtures see Dahlquist and Raftery (1968a).

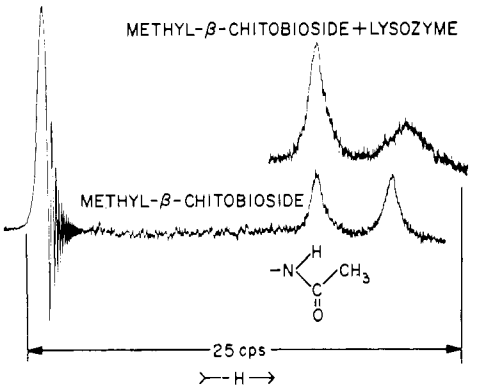

FIGURE 6: Proton magnetic resonance spectra of the acetamido methyl resonances of methyl β-chitobioside in the absence and presence of lysozyme in 0.1 M citrate buffer (pH 5.5), 31°. An acetone internal standard is shown at lower field.

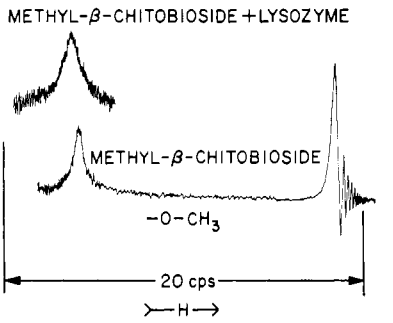

FIGURE 7: Proton magnetic resonance spectra of the glycosidic methyl protons of methyl β-chitobioside in the absence and presence of lysozyme in 0.1 M citrate buffer (pH 5.5), 31°. A methanol internal standard is shown at higher field.

acetamido methyl group proximal to the glycosidic group was shifted to higher field and that this shift increased as the concentration of the glycoside was decreased. The calculated chemical shift of this resonance for the bound form of the associating small molecule was 0.80 ± 0.04 ppm to higher field. The acetamido methyl group distal from the glycosidic end did not undergo any change in chemical shift as a result of association with lysozyme. As expected, a slight broadening of the resonance was observed as a result of macromolecular association. The glycosidic methyl group was found to undergo a chemical shift to lower field due to binding to the enzyme. The value obtained for Δ was 0.16 ± 0.02 ppm to lower field.

The Association of Chitotriose with Lysozyme. At pH 5.5 and 31° the acetamido methyl group proton mag-

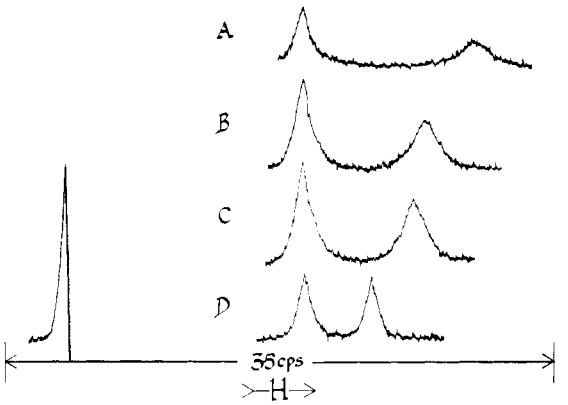

FIGURE 8: Proton magnetic resonance spectra of the acetamido methyl protons of methyl β-chitobioside in the absence and presence of lysozyme (3.12×10^{-3} M), in citrate–phosphate buffer (pH 9.7): (A) (4.18×10^{-2} M) with enzyme, (B) (7.85×10^{-2} M) with enzyme, (C) (9.70×10^{-2} M) with enzyme, and (D) (5×10^{-2} M) without enzyme.

TABLE II: Chemical Shift Data for Inhibitors and Substrates Complexed with Lysozyme at pH 4.9–5.4 and at Various Temperatures.

Compound	Temp (°C)	Δ (ppm)[a]		
		N_1–CH_3[b]	N_2–CH_3[b]	OCH_3[c]
α-2-Acetamido-2-deoxy-D-glucopyranose	31	0.68 ± 0.02		
β-2-Acetamido-2-deoxy-D-glucopyranose	31	0.51 ± 0.03		
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	31	0.55 ± 0.02		0
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	31	0.54 ± 0.04		0.17 ± 0.03
	55	0.51 ± 0.03		0.16 ± 0.05
Chitobiose	45	0.57 ± 0.04	0	
Methyl β-chitobiose	35	0.60 ± 0.05	0	0.20 ± 0.05

[a] The acetamido methyl groups are numbered 1 and 2 beginning at the reducing or glycosidic termini of the inhibitor molecules. [b] Values relative to acetone; all chemical shifts to higher field. [c] Values relative to methanol; all chemical shifts to lower field.

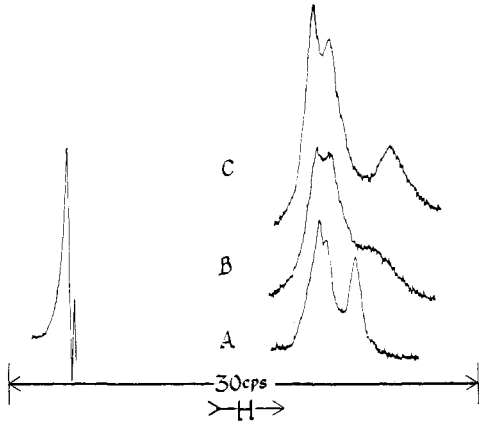

FIGURE 9: Proton magnetic resonance spectra of the acetamido methyl protons of chitotriose in the absence and presence of lysozyme (3.12×10^{-3} M), in citrate–phosphate buffer (pH 9.7): (A) chitotriose, (B) chitotriose (9.62×10^{-2} M) plus lysozyme at 55°; (C) chitotriose (9.62×10^{-2} M) plus lysozyme at 65°. An internal standard of acetone is shown at lowest field.

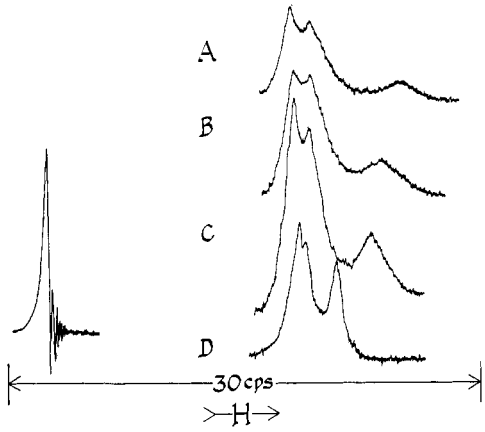

FIGURE 10: Proton magnetic resonance spectra of the acetamido methyl groups of chitotriose in the absence and presence of lysozyme (3.1×10^{-3} M) at pH 9.7, 65°, in citrate–phosphate buffer: (A) chitotriose (5.48×10^{-3} M) plus lysozyme, (B) chitotriose (7.71×10^{-2} M) plus lysozyme, (C) chitotriose (9.62×10^{-2} M) plus lysozyme, and (D) chitotriose without added lysozyme. An internal standard of acetone is included and shown at lower field.

netic resonance spectrum of chitotriose was not affected very much by the presence of lysozyme. A slight broadening of the resonances was observed but no detectable changes in chemical shift were seen. Under the conditions used it was known that the enzyme (3×10^{-3} M) was saturated with the trisaccharide ($2–8 \times 10^{-2}$ M) since the dissociation constant had previously been shown (Dahlquist *et al.*, 1966) to be 6×10^{-6} M. Owing to this strong binding it was possible that the dissociation rate of the lysozyme–chitotriose complex was too slow to satisfy the condition necessary to obtain a spectrum representative of the average of free and enzyme-bound chitotriose species. In an attempt to approach this condition the experiment was repeated but an elevated temperature was employed. At 55°, 20° below the thermal denaturation transition temperature for the enzyme, a more pronounced effect on the proton magnetic resonance spectrum of the acetamido methyl groups was observed. Unfortunately chitotriose was found to be fairly rapidly hydrolyzed by lysozyme under these conditions. To circumvent this complication the high-temperature binding studies were carried out at pH 9.7. A separate study was conducted to show that hydrolysis of the trisaccharide by the enzyme did not occur at any detectable rate at pH 9.7 and 65°. The effect of binding to lysozyme at this pH and 55° on the acetamido methyl resonances of chitotriose is shown in Figure 9B. Under these conditions broadening of the resonances was observed, and it was also evident that a chemical shift to higher field was experienced by the resonance corresponding to the acetamido methyl group at the reducing end of the trisaccharide. However, this resonance appeared to be very broad and it seemed as though the enzyme–substrate complex was still too long lived to satisfy the exchange rate necessary to obtain a spectrum representative of the weighted average of bound and unbound species. Upon raising the temperature to 65°, a profound effect was seen as shown in Figure 9C. The resonance corresponding to the acetamido methyl group at the reducing end of the trisaccharide was clearly resolved and was further shifted to higher field. Thus the increase in temperature to 65° caused an increase in the exchange rate sufficient to cause a sharper resonance for the reducing end acetamido methyl group. It is possible, however, that the observed spectrum still was not the weighted average of the free and bound species. Figure 10 shows the effects of adding varying concentrations of the trisaccharide to a constant amount of enzyme at pH 9.7 and 65°. No separate effects attributable to the α- and β-anomeric forms of the trisaccharide were observed in contrast to those seen for α- and β-2-acetamido-2-deoxy-D-glucopyranose (Raftery *et al.*, 1968b,c; Dahlquist and Raftery, 1968a). This is in agreement with the results obtained for α- and β-chitobiose during the present study. Therefore it is evident that the α- and β-anomeric forms of the trisaccharide both bind to the enzyme and the acetamido methyl protons at the reducing ends of both anomeric forms undergo a chemical shift to higher field due to the magnetic environment experienced in the complexed state. The value of Δ calculated for the shift of these protons was 0.61 ± 0.12 ppm to higher field. However, there is reason to doubt that this determination was made under conditions which allow "averaging" of the observed chemical shift between the free and bound forms. Since the dissociation constant of the trisaccharide–enzyme complex is small under the conditions used for the chemical shift measurements, the observed chemical shift, at constant enzyme concentration, should be linear with respect to substrate concentration. However, the observed shift increased proportionately more than the substrate concentration was decreased. This suggests that at low S_0/E_0 ratios, the exchange rate was more rapid and a more nearly "averaged" spectrum was obtained. Therefore, the measurements taken at the lowest S_0/E_0 ratio should be the most correct. The value of Δ for the acetamido methyl reso-

nance of the reducing end acetamido methyl resonance at the lowest S_0/E_0 ratio was 0.70 ppm upfield. Further, in those instances where incomplete exchange was observed, the best theoretical fit to the observed spectra was obtained with a value of 0.77 ppm upfield (Dahlquist and Raftery, 1968c).

The resonance corresponding to the central acetamido group of the trisaccharide did not appear to undergo any chemical shift due to association with lysozyme. A small broadening effect, as expected, was observed.

The resonance corresponding to the acetamido methyl group on the nonreducing end of chitotriose displayed a downfield shift in the presence of lysozyme. This is in contrast to the upfield shifts previously observed for the other acetamido methyl resonances of chitotriose as well as for chitobiose, methyl β-chitobioside, and various derivatives of 2-acetamido-2-deoxy-D-glucopyranose. It allows distinction of the magnetic environment associated with the site on the enzyme occupied by the nonreducing end acetamido methyl group of chitotriose. The magnitude of the downfield shift observed for this resonance was $\Delta = 0.08 \pm 0.01$ ppm.

The Association of Methyl β-Chitotrioside with Lysozyme. The association of the trisaccharide glycoside with the enzyme was studied only at pH 9.7 and at elevated temperatures. Results similar to those obtained for chitotriose binding to lysozyme as a function of temperature were observed. In Figure 11B the result of adding lysozyme to a solution of the glycoside at 55° is shown clearly to be that all methyl group resonances were broadened and that the resonance corresponding to the acetamido methyl group proximal to the glycosidic group appeared also to be shifted to higher field. However, the spectrum obtained was reminiscent of those previously observed for chitobiose, methyl β-chitobioside, and chitotriose when the rate of dissociation of the enzyme–substrate or enzyme–inhibitor complex was too slow to obtain a spectrum representing a weighted average of enzyme-bound and free forms of the small molecule. Raising the temperature to 65° decreased the line width of the reducing end acetamido methyl resonance. Again, it is probable that the observed spectrum is still not representative of the fast exchange limit. The type of spectrum seen is shown in Figure 11C. Clearly the resonance corresponding to the acetamido methyl group proximal to the glycosidic methyl group was shifted to higher field due to association with the enzyme. The calculated chemical shift for this group was $\Delta = 0.63$ ppm to higher field. This value is probably somewhat low because of incomplete exchange.

The resonance due to the middle acetamido methyl group of the glycoside did not appear to undergo any change in chemical shift due to macromolecular association whereas the acetamido methyl group distal from the glycosidic end of the molecule displayed a small downfield shift of its proton magnetic resonance signals upon association with the enzyme. The calculated value of Δ was 0.08 ± 0.02 ppm to lower field. This result agrees well with the downfield shift obtained for the acetamido methyl group at the nonreducing end of chitotriose in association with lysozyme.

The glycosidic methyl group of methyl β-chitotrioside

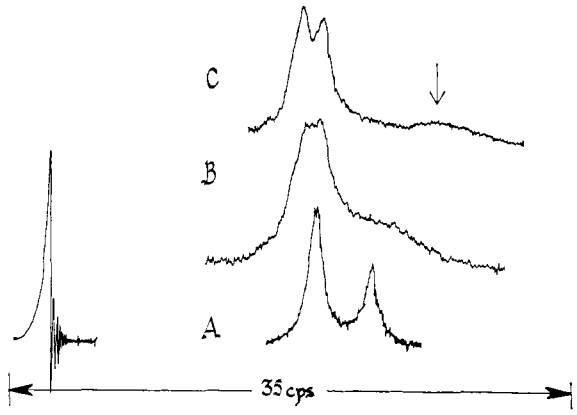

FIGURE 11: Proton magnetic resonance spectra of the acetamido methyl groups of methyl β-chitotrioside at pH 9.7, in the presence and absence of lysozyme (1.56×10^{-3} M): (A) methyl β-chitotrioside, (B) methyl β-chitotrioside (2.36×10^{-2} M) plus lysozyme at 55°; and (C) methyl β-chitotrioside (2.36×10^{-2} M) plus lysozyme at 65°. An internal standard of acetone was included and its resonance is shown at lower field.

was also found to undergo a downfield shift in its proton magnetic resonance spectrum in the presence of lysozyme. The value of Δ obtained was 0.19 ppm to lower field. This value agrees quite well with those obtained for the glycosidic methyl groups of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside and methyl β-chitobioside. The chemical shift results for all of the mono-, di-, and trisaccharides and their glycosides are summarized in Tables II and III.

The Association of Chitotetraose with Lysozyme. A study of the effects of added lysozyme on the proton magnetic resonance spectrum of the acetamido methyl groups of chitotetraose was carried out at pH 9.7 and 65°. It was shown that using these conditions no detectable hydrolysis of the tetrasaccharide occurred over a period of 1 hr. The results obtained are shown in Figure 12. It was observed that in comparison with the spectrum of the free tetrasaccharide the presence of enzyme caused broadening of all the methyl resonances and appeared to shift that resonance corresponding to the acetamido methyl group at the reducing end of the tetrasaccharide to higher field. The spectrum was reminiscent of those obtained for chitotriose and methyl β-chitotrioside in the presence of lysozyme when the rate of dissociation of the enzyme–inhibitor complex was not sufficiently fast to result in sharpening of the observed resonances. Upon raising the temperature to 70° irreversible denaturation of the protein was observed. Therefore it was not possible to obtain more clear-cut results for the tetrasaccharide in association with the enzyme. It was possible, however, from the results seen at 65° to say qualitatively that the acetamido methyl group at the reducing end of chitotetraose can occupy the environment experienced by the acetamido methyl groups at the reducing ends of 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, and chitotriose and proximal to the glycosidic methyl group of methyl 2-acetamido-2-deoxy-

TABLE III: Chemical Shift Data for Inhibitors and Substrates Complexed with Lysozyme at pH 9.7, at Various Temperatures.

Compound	Temp (°C)	Δ (ppm)[a] CH_3–N_1[b]	CH_3–N_2[b]	CH_3–N_3[b]	–OCH_3[c]
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	31	0.36			0.16 ± 0.02
Chitobiose	55	0.77 ± 0.04	0		
Methyl β-chitobioside	55	0.80 ± 0.04	0		0.16 ± 0.02
Chitotriose[d]	65	0.61 ± 0.12	0	0.08	
Methyl β-chitotrioside[d]	65	0.63	0	0.08	0.19

[a] The acetamido methyl groups are numbered 1, 2, and 3 beginning at the reducing or glycosidic termini of the inhibitor molecules. [b] Values relative to acetone; all chemical shifts to higher field. [c] Values relative to methanol; all chemical shifts lower field. [d] Not at fast exchange limit.

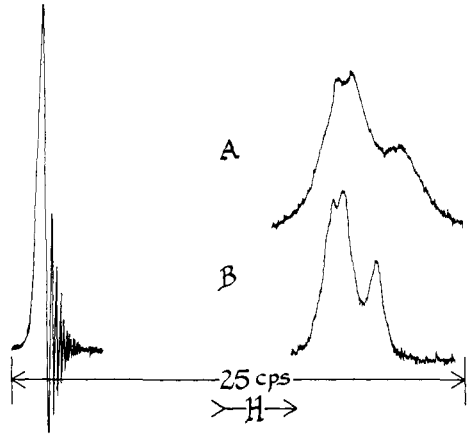

FIGURE 12: Proton magnetic resonance spectra of the acetamido methyl groups of chitotetraose in the absence and presence of lysozyme (3.1×10^{-3} M) at pH 9.7 and 65°: (A) chitotetraose (6.09×10^{-2} M) plus lysozyme and (B) chitotetraose (5.0×10^{-2} M) without lysozyme. An internal standard of acetone was included and its resonance is shown to lower field.

β-D-glucopyranoside, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, methyl β-chitobioside, and methyl β-chitotrioside.

Discussion

In general two types of information may be obtained from a nuclear magnetic resonance experiment of the type described in this investigation: (a) the position or chemical shift of the resonance, which is controlled by the magnetic environment, and (b) the shape or width of the observed resonance, which is controlled by the relaxation times. Earlier work from this laboratory (Raftery *et al.*, 1968b,c; Dahlquist and Raftery, 1968a, b) on inhibitor binding to lysozyme has shown that the chemical shifts of selected nuclei in the inhibitors are very sensitive to small changes in their enzyme-bound environments. The information obtainable from such changes in chemical shifts of selected nuclei in various inhibitor molecules is more valuable, for this reason, than information gleaned from line-width changes due to macromolecular association.

While the work reported here was in progress a brief account of the effect of lysozyme on the nuclear magnetic resonance spectra of chitobiose and methyl β-chitobioside (Thomas, 1968) has appeared. The only effect which was observed was an enzyme-induced broadening of the reducing end acetamido methyl proton resonance in chitobiose and a similar broadening of the resonance due to the acetamido methyl group proximal to the glycosidic end of methyl β-chitobioside. The results reported here on the association of these inhibitors with lysozyme as well as other molecules such as chitotriose, methyl β-chitotrioside, and chitotetraose show this type of "selective" line broadening at low temperatures. Extrapolated line widths for enzyme-bound forms of the inhibitors from such observed "selective" broadening are of the order of 150–250 cps. In view of the line widths normally observed for methyl groups on proteins (McDonald and Phillips, 1967) such values are clearly unrealistic. There are several possible explanations for the broadening effects published by Thomas (1968) and observed in the initial stages of our investigations. One such explanation is the phenomenon of exchange broadening, which occurs when the rate of exchange of a nucleus between two (or more) environments is of the order of $1/2\pi(\Delta\nu)$, where $\Delta\nu$ is the chemical shift difference for the nucleus in one site relative to the other site(s). Another explanation is the presence of paramagnetic material at or near one of the environments.

Jardetzky (1964) has suggested that the cause of differential broadening in the nuclear magnetic resonance spectrum of small molecules in the presence of macromolecules to which they bind is the restricted movement of the small molecule in the binding site. This restricted movement gives rise to large dipolar fields which are efficient in causing relaxation and therefore results in broadened lines. The more strongly a particular portion

of the molecule is held in place on the macromolecule, the more broadened its resonance will appear. This explanation was suggested by Thomas (1968) to account for the large increase in the line width of the reducing end acetamido methyl group resonance of chitobiose and the resonance due to the acetamido methyl group proximal to the glycosidic end of methyl β-chitobioside in the presence of lysozyme.

It is interesting to note that the exceptionally broadened resonances are also those which, in the present investigation, show *measurable chemical shifts* upon association with lysozyme. The line widths are dramatically affected by temperature as are the chemical shifts of the broadened resonances. This is shown in Figures 4 and 8–10. Separate measurements of the binding constants for the various chitin oligomers used showed that there was no significant change in the fraction of the oligomer which was bound to the enzyme as the temperature was varied. These facts strongly suggest that the broadening is caused by slow exchange of the saccharide between the free and bound state. In a separate communication (Dahlquist and Raftery, 1968c) we describe the use of such line-width measurements on the acetamido methyl resonances of chitobiose and chitotriose to measure the exchange rates of the free and bound species. The observed spectra are completely explainable using line widths for the bound state of 8–15 Hz and formation rate constants for the enzyme–substrate complex of 10^6–10^7 l. mole^{-1} sec^{-1}. While it appears that there are slightly different line widths associated with the various subsites in the binding region of lysozyme, these are within the range of 8–15 Hz and do not account for the large differential broadening effects mentioned earlier. The results of curve fitting to match the observed spectra for the exchange of chitobiose and chitotriose are summarized in Table IV.

The data presented in Tables II–IV suggest that the various subsites associated with the binding of chitobiose and chitotriose may be assigned specific magnetic parameters. At pH 9.7, the reducing end acetamido methyl group resonances of chitobiose and chitotriose both displayed chemical shifts of 0.77 ± 0.04 ppm upfield with a half-width of 15 Hz while bound. Thus, subsite C may be assigned these values for the acetamido methyl group resonances of sugar units bound in that subsite at pH 9.7.

The acetamido methyl resonances corresponding to the nonreducing sugar ring of chitobiose and the central sugar residue of chitotriose both displayed no measurable chemical shift and a half-width of 10 Hz while associated with subsite B. The nonreducing end acetamido methyl resonance of chitotriose showed a chemical shift of 0.08 ppm to lower field and a half-width of 8 Hz while bound to the enzyme at subsite A.

The methyl β-glycosides of chitobiose and chitotriose also fit nicely into the general pattern. At pH 9.7 the glycosidic methyl group resonance of each had a chemical shift of 0.18 ± 0.02 ppm to lower field. The acetamido methyl resonances proximal to the glycosidic methyl groups of each glycoside also showed values of Δ which would place that sugar residue in each case in subsite C. Again, the central sugar residue of the trisaccharide and the sugar residue distal from the glycosidic end of the disaccharide showed no measurable chemical shifts in the appropriate acetamido methyl resonances, thereby placing them in subsite B. The acetamido methyl resonance distal from the glycosidic methyl group of methyl β-chitotrioside showed the small downfield shift (0.08 ppm) characteristic of subsite A.

The binding orientation of chitotetraose is more difficult to define quantitatively because the exchange rate of the saccharide–enzyme complex was too slow to allow more than a qualitative measure of chemical shifts. The spectra obtained for chitotetraose were reminiscent of the chitotriose spectra obtained at temperatures about 10° lower. This corresponds to about a factor of two in exchange rate. The reducing end acetamido methyl resonance underwent changes similar to the reducing end resonance of chitotriose. Thus, the reducing end of the tetrasaccharide most probably occupies subsite C, with the other sugar residues being in subsites B and A, and either in solution or in a weakly binding subsite beyond subsite A.

All these results are summarized in Figure 13 which depicts the lysozyme molecule to have three contiguous binding subsites to which acetamido pyranosides bind *strongly*. These subsites are labeled A, B, and C and have been assigned particular magnetic properties on the basis

TABLE IV: Magnetic Parameters and Rate Constants for Formation of Enzyme–Substrate Complex Which Give the Best Theoretical Fit to the Observed Spectra (Dahlquist and Raftery, 1968c).

		Δ (ppm)[a]			Half Width (Hz)			
Inhibitor	Temp Range (°C)	CH_3–N_1[b]	CH_3–N_2[b]	CH_3–N_3[b]	CH_3–N_1[b]	CH_3–N_2[b]	CH_3–N_3[b]	k_f[c] (Range) (l. mole^{-1} sec^{-1})
Chitobiose, pH 5	10–50	0.57	0		15	10		10^6–10^7
Chitobiose, pH 9.7	10–50	0.77	0		15	10		10^6–10^7
Chitotriose, pH 9.7	10–65	0.77	0	−0.09	15	10	8	5×10^6–10^7

[a] The acetamido methyl groups are numbered beginning at the reducing termini of the inhibitor molecules. [b] Values relative to acetone; all chemical shifts are to higher field. [c] Second-order rate constant for formation of the enzyme–inhibitor complex.

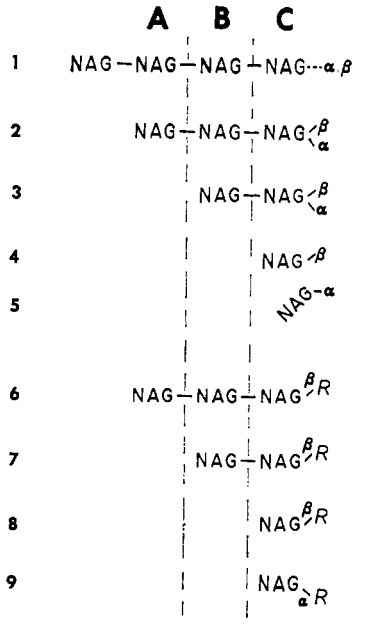

FIGURE 13: Scheme for the relative modes of association with lysozyme of various saccharide inhibitors. Where α- and β-anomeric forms of the free saccharides are shown on a single line (as with chitotetraose) no information on relative binding modes was obtained. Where α and β forms are depicted separately (as with α-2-acetamido-2-deoxy-D-glucopyranose and β-2-acetamido-2-deoxy-D-glucopyranose different binding modes for the anomers were elucidated. Where α and β forms are shown (as with chitotriose and chitobiose) on the same molecule on two levels it was shown that both anomeric forms bind identically. In the glycoside series methyl groups are depicted by R, and the anomeric form of the acetyl glycoside is indicated.

of the effects observed. The various oligomers of 2-acetamido-2-deoxy-D-glucopyranose bind with their reducing ends occupying subsite C. The α- and β-anomeric forms of the di- and trisaccharides bind identically, while insufficient information is available to judge whether both anomeric forms of the tetrasaccharide bind identically. The anomeric forms of the monosaccharide, 2-acetamido-2-deoxy-D-glucopyranose, have been shown previously (Dahlquist and Raftery, 1968a) to bind with different orientations in subsite C. On the other hand, the corresponding α- and β-methyl glycosides of 2-acetamido-2-deoxy-D-glucopyranose bind identically in subsite C (Raftery *et al.*, 1968b), and it has been further shown that the orientations of both glycosides are identical with the orientation of β-2-acetamido-2-deoxy-D-glucopyranose in the same subsite. The present work has shown that methyl β-chitobioside and methyl β-chitotrioside bind with their glycosidic pyranoside rings in subsite C on the basis of the chemical shifts of their glycosidic methyl groups and their acetamido methyl groups.

These observations of the relative binding orientations of the saccharides are in general agreement with X-ray analysis studies of Blake *et al.* (1967) on crystalline lysozyme–saccharide complexes. These crystal studies have shown an extensive binding area on the enzyme's surface containing at least three binding subsites: A, B, and C. It was found that the reducing ends of β-2-acetamido-2-deoxy-D-glucopyranose, and chitotriose at high resolution (2 Å) and chitobiose at low resolution (6 Å) bind in subsite C, while subsite B binds the nonreducing end of chitobiose and the central sugar ring of chitotriose. Subsite A was found to be occupied only by the nonreducing end of chitotriose. The X-ray studies also demonstrated that chitobiose is bound in an anomalous way.

The authors had not, however, determined the conditions which distinguished between the two binding modes of chitobiose to lysozyme. Our results show clearly that lysozyme binds both anomeric forms of the disaccharide equally well and with the same orientation at pH 5. Furthermore, because of the equality of the acetamido methyl group shifts of methyl β-chitobioside and chitobiose, it appears that α- and β-chitobiose and methyl β-chitobioside bind identically with lysozyme at pH 9.7. The fact that there was no resolution of resonances due to the α- and β-anomeric forms also supports this hypothesis. These facts suggest that the anomalous binding of chitobiose observed in the crystalline enzyme is not due to differential binding of the anomeric forms of chitobiose, assuming identical binding properties for the enzyme in solution and in the crystalline state.

While the crystallographic studies did not allow distinction of the anomeric form(s) of chitotriose which bound to the enzyme, we can say from our studies that both anomeric forms of the trisaccharide bind equally well and in an identical manner.

The general agreement, however, on the *relative* modes of association of a homologous series of inhibitors with an enzyme as determined by difference Fourier analysis for crystalline preparations and by nuclear magnetic resonance methods for solutions is encouraging.

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Biochemical Studies of Synapses *in Vitro*. II. Potassium Transport*

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ABSTRACT: Previous studies of rat brain synaptosomes *in vitro* demonstrated modulation of protein synthesis by ionic constituents and endogenous energy availability, and suggested that isolated nerve endings may support active ion transport. Utilizing a rapid filtration technique to measure intrasynaptosomal potassium accumulation, the various parameters influencing uptake of cold potassium and ^{42}K were studied at 23°. Immediately after isolation, synaptosomes contained an average of 0.092 μmole of potassium/mg of protein. At 23° with 100 mM sodium and 10 mM potassium in the external medium, the synaptosomal potassium increased to approximately 0.14–0.25 μmole/mg of protein after 8 min. The optimal potassium accumulation occurred with 50 mM sodium present in the medium. With 50 mM sodium, potassium content rose to 0.20–0.30 μmole/mg of protein after 8 min. At this optimal level of sodium (50 mM), potassium concentrations greater than 10 mM produced minimal increase in potassium accumulation. Neither adenosine triphosphate, adenosine diphosphate, glucose, increased oxygen concentration, nor the substrates α-ketoglutarate, succinate, fumarate, and glutamate influenced potassium accumulation. Ouabain was an effective inhibitor only of the sodium-dependent potassium accumulation. In sodium-free medium ouabain had essentially no effect on potassium ion accumulation. In the presence of 50 mM sodium and ouabain (10^{-4} M), the total potassium content of the synaptosomes declined by 54%.

The extent of inhibition by ouabain was equivalent to the extent of activation by sodium. Both 2,4-dinitrophenol and potassium cyanide inhibited potassium accumulation. Total potassium and ^{42}K uptake by synaptosome decreased as a function of time after isolation with the most marked reduction in the ouabain-inhibitable component. Total synaptosomal volume was measured with the use of *N*-methyl-[^{14}C]antipyrene, and the potassium content per milligram of protein was expressed in synaptosomal potassium concentrations. With 10 mM potassium and 50 mM sodium, there was an increase of intrasynaptosomal potassium concentration from approximately 71 mM following isolation to 103 mM, representing a significant accumulation of potassium against a concentration gradient. The present studies demonstrate that isolated rat brain synaptosomes support active potassium transport.

The key to the organization and function of the nervous system is given by the pattern of synaptic connections between cells and the efficiency of such synapses in interneuronal communication. In chemical synapses ionic fluxes mediate the release of neurotransmitter from the presynaptic terminal and determine the consequence of the neurotransmitter–receptor interaction in the postsynaptic terminal. Thus, in both presynaptic and postsynaptic terminals a detailed understanding of the factors underlying the movement of ions is essential for an understanding of synaptic function.

The usefulness of synaptosomes isolated from rat brain cortex as a model for studying the effects of energy availability and ions upon synaptic metabolism has recently been demonstrated (Appel and Autilio, 1969). Such synaptosomes were demonstrated to synthesize proteins *in vitro*. The protein synthesis was enhanced fourfold by 100 mM sodium and 10 mM potassium and was inhibited by ouabain as well as dinitrophenol, oligomycin, and potassium cyanide. It was not enhanced by an exogenous supply of energy or substrate. In essence,

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