# A Transient Nuclear Magnetic Resonance Study of the Kinetics of Methyl *N*-Acetyl-D-glucosaminide Inhibition of Lysozyme*

Brian D. Sykes†

ABSTRACT: The kinetics of lysozyme inhibition by methyl *N*-acetyl-$\alpha$- and -$\beta$-D-glucosaminide has been studied by transient nuclear magnetic resonance methods. The rates of formation and lifetimes of the lysozyme inhibitor complexes have been measured at 33° for the $\alpha$ anomer, and over the temperature range 11–61° for the $\beta$ anomer. The rate constants at 33° are ($k_{-1}$ sec$^{-1}$, $k_1 m^{-1}$ sec$^{-1}$) for $\alpha$, $5.5 \times 10^3$, $1.4 \times 10^5$; for $\beta$, $4.5 \times 10^3$, $1.6 \times 10^5$. The activation parameters for the $\beta$ anomer are ($\Delta H^{\pm}$ kcal, $\Delta S^{\pm}$ eu) for $k_{-1}$, +4.4, −27; for $k_1$, −2.9, −44. These results suggest that the binding of monosaccharides to lysozyme is not a simple biomolecular process. The resonance frequency shift of the acetyl protons of the inhibitor upon binding to lysozyme at 33° is 0.65 ppm for the $\alpha$ anomer and 0.69 ppm for the $\beta$ anomer. The equality of the shifts suggests that the anomers bind at the same site and in a similar configuration.

The inhibition of lysozyme by derivatives of D-glucosamine has been studied recently by several authors (Chipman *et al.*, 1967; Chipman and Schimmel, 1968; Dahlquist *et al.*, 1966; Dahlquist and Raftery, 1968a,b; Neuberger and Wilson, 1967a,b; Raftery *et al.*, 1968; Rupley *et al.*, 1967) and has been successfully interpreted in terms of the known X-ray structure of lysozyme (Blake *et al.*, 1965, 1967; Phillips, 1967; Phillips and Sarma, 1967). However, the binding of the monosaccharide *N*-acetyl-D-glucosamine, a monomer of cell wall oligosaccharide, is complicated by the interconversion and simultaneous binding of $\alpha$ and $\beta$ anomers, and recourse is often made to the alkyl glucosides (see Figure 1) to study the binding of the $\alpha$ and $\beta$ anomers separately. While the relative inhibition caused by the $\alpha$ and $\beta$ anomers has been measured (Neuberger and Wilson, 1967a,b; Raftery *et al.*, 1968) the rates of formation and lifetimes of the lysozyme–monosaccharide inhibitor complexes have not previously been measured.

Recently a transient nuclear magnetic resonance method has been proposed as a direct and simple method for the measurement of the rate constants of such biological exchange reactions (B. D. Sykes, 1968, submitted for publication). It has also been shown that the single requirement of the method, that of a resonance frequency shift upon binding, is satisfied for the acetyl protons of methyl *N*-acetyl-$\alpha$- and -$\beta$-D-glucosaminide upon binding to lysozyme (Thomas, 1966, 1967). In this paper the measurement of the rate constants for the lysozyme–methyl *N*-acetyl-$\alpha$- and -$\beta$-D-glucosaminide complexes by nuclear magnetic resonance is presented. In addition, the activation parameters for the forward and reverse rate constants for the lysozyme–methyl *N*-acetyl-$\beta$-D-glucosaminide complex have been measured.

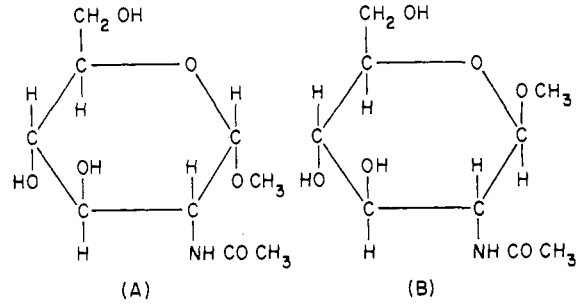


FIGURE 1: Methyl *N*-acetyl-$\alpha$-D-glucosaminide (A) and methyl *N*-acetyl-$\beta$-D-glucosaminide (B).

## Theory

The exchange of a nucleus, or group of equivalent nuclei, between sites of different local magnetic environment, characterized by different resonance frequencies, is a relaxation mechanism for the nuclear spin system. Hence the exchange of an inhibitor molecule between free solution and the active site of an enzyme can cause relaxation of nuclei on the exchanging inhibitor molecule if these nuclei experience a resonance frequency shift upon binding. If the rate of exchange is less than the resonance frequency of the nuclei involved, the exchange will shorten the transverse relaxation time, $T_2$, but will not affect the longitudinal relaxation time, $T_1$.

The effect of exchange upon the spin-lattice relax-

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ation time in the rotating frame $T_1\rho(H_1)$ (Abragam, 1961; Meiboom, 1961; Solomon, 1959a,b; B. D. Sykes, 1968, submitted for publication) depends upon the magnitude of the irradiating radiofrequency field, $H_1$. $T_1\rho(H_1)$ is measured in the following fashion. The nuclear magnetization is aligned along a strong irradiating field $H_1$. The magnetization can then be considered as resulting from a quantization of the nuclear spin states along the field $H_1$ in the interaction representation. This magnetization then relaxes along $H_1$ toward a nearly zero equilibrium magnetization appropriate to a resonance experiment at a frequency $\omega_1 = \gamma H_1$. The decay is characterized by $T_1\rho(H_1)$ which is not, in general, equal to $T_2$ (measured in the absence of $H_1$). In the presence of exchange, $T_1\rho(H_1)$ is equal to $T_1$ for values of $H_1$ such that $\omega_1\tau >> 1$, and equal to $T_2$ for values of $H_1$ such that $\omega_1\tau << 1$, where $\tau$ is the correlation time or lifetime of the exchange process.

In the fast exchange limit, where the rate of exchange is larger than the difference in resonance frequencies between sites, the appropriate expression for $T_1\rho(H_1)$ is

$$\frac{1}{T_1\rho(H_1)} = \sum_{i=A,B} \frac{P_i}{T_1(i)} + \frac{x\tau_A(\delta_A - \delta_B)^2}{(1+x)^3 + (1+x)\tau^2_A\omega^2_1} \quad (1)$$

for the reaction

$$HA + B \rightleftharpoons A + HB \quad (2)$$

where A and B are two sites of different local magnetic environment; H is the nucleus, or group of equivalent nuclei, exchanged; $P_i$ is the probability that H will be in the site $i$ = A,B; $T_1(i)$ is the relaxation time of H in site $i$ in the absence of exchange; $\delta_A - \delta_B$ is the resonance frequency difference between sites A and B; $x$ = [HA]/[HB]; $\omega_1 = \gamma H_1$; and $\tau_A$ is the lifetime of H in site A.

Making the identification

$$\begin{matrix} HA \longrightarrow EI \\ HB \longrightarrow I \text{ (free solution)} \end{matrix} \quad (3)$$

the appropriate expression for the reaction

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \quad (4)$$

where

$$K_D = \frac{[E][I]}{[EI]} = \frac{k_{-1}}{k_1} \quad (5)$$

and

$$x = \frac{[EI]}{[I]} \ll 1, \; [I] \simeq [I^0] \quad (6)$$

is

$$\frac{1}{T_1\rho(H_1)} = \sum_{i=EI,I} \frac{P_i}{T_1(i)} + \frac{[EI]}{[I^0]} \frac{\tau_{EI}(\delta_{EI} - \delta_I)}{(1 + \tau_{EI}^2\omega_1^2)} \quad (7)$$

Setting

$$\delta_{EI} - \delta_I = \Delta, \; \omega_1 = 0 \quad (8)$$

and

$$\frac{1}{\tau_{EI}} = \frac{1}{[EI]} \frac{d[EI]}{dt} = k_{-1} \quad (9)$$

the expression becomes

$$\frac{1}{T_2} - \frac{1}{T_1} = \frac{[E^0]}{K_D + [I^0]} \left(\frac{1}{k_{-1}}\right) \Delta^2 \quad (10)$$

$K_D$ and $\Delta$ are obtained from the resonance frequency shift of the fast exchange averaged resonance, $\delta_{obsd}$, as a function of initial inhibitor concentration at constant initial enzyme concentration. In the fast exchange limit

$$\delta_{obsd} = \frac{[EI]}{[I^0]} \Delta + \delta_I \quad (11)$$

where

$$[EI] = \frac{([E^0] + [I^0] + K_D) \pm \sqrt{([E^0] + [I^0] + K_D)^2 - 4[E^0][I^0]}}{2} \quad (12)$$

For a series of initial inhibitor concentrations, a computer program (Groves *et al.*, 1967; B. D. Sykes, 1968, submitted for publication) is used to calculate the concentrations [EI] according to eq 12, for an assumed value of $K_D$ and with the criterion.

$$0 < [EI]/[E^0] < 1 \quad (13)$$

The program is then used to calculate the slope, $\Delta$, and intercept, $\delta_I$, of a least-mean-square straight line through a plot of $\delta_{obsd}$ *vs.* [EI]/[I$^0$], and the root-mean-square deviation of the points from the straight line. The value of $K_D$ giving the minimum deviation is then chosen. This solution gives $K_D$, $\Delta$, and $\delta_I$. The error limits in Table 1 and Figure 7 indicate the values of $K_D$ (and hence $\Delta$) for which the fit of the experimental points to eq 11–13 differs by one standard deviation of the mean from the best fit.

The advantages of the computer analysis are twofold. Firstly, the value of the intercept $\delta_I$ need not be measured and a double-reciprocal plot (Spotswood *et al.*, 1967)

$$\frac{1}{\delta_{obsd} - \delta_I} = \frac{1}{\Delta}\left(\frac{[I^0]}{[E^0]}\right) + \frac{K_D}{\Delta[E^0]}, \; [I] \simeq [I^0] \quad (14)$$

can be avoided, since this plot is very sensitive to the choice of $\delta_I$ (Kuntz and Johnston, 1967). Even internal standards are not completely adequate in compensating for the change in solution properties upon changing enzyme concentration (Laszlo *et al.*, 1968; B. D. Sykes and P. G. Schmidt, unpublished results). Secondly, since all concentrations can be calculated exactly, the restriction [I] $\simeq$ [I$^0$] does not need to be imposed and a wider range of concentrations can be used.

## Materials and Methods

The relaxation times $T_1\rho(H_1)$ of the acetyl protons of $\alpha$- and $\beta$-methyl *N*-acetyl-D-glucosaminide were measured on Varian HR-60 and HR-100 spectrometers as previously described (B. D. Sykes, 1968, submitted for publication). $T_2$ was taken as the value of $T_1\rho(H_1)$ at a value of $H_1$ for which further reduction of $H_1$ produced no reduction of $T_1\rho(H_1)$. $T_1$ was measured by plotting ln $[M_0 \exp(-t_{\pi/2}/T_R) - M_X]$ *vs.* $t_{OR}$, where $t_{OR}$ is the time spent off resonance before passage into the center of the resonance, $M_0 \exp(-t_{\pi/2}/T_R)$ is the amplitude of the magnetization initially aligned along the field $H_1$ upon rapid passage into the center of resonance for times $t_{OR}$ >>

TABLE I: Dissociations Constants, Chemical Shifts, and Forward and Reverse Rate Constants for Lysozyme–Inhibitor Complexes.

| Inhibitor | $K_D$ ($m$) | $\Delta$ (ppm) | $k_{-1}$ ($sec^{-1}$) | $k_1$ ($m^{-1}$ $sec^{-1}$) |
|---|---|---|---|---|
| Methyl *N*-acetyl-α-D-glucosaminide | $3.9 \pm 0.5 \times 10^{-2}$ | $0.65 \pm 0.08$ | $5.5 \pm 2.1 \times 10^{3}$ | $1.4 \pm 0.7 \times 10^{5}$ |
| Methyl *N*-acetyl-β-D-glucosaminide | $2.9 \pm 0.4 \times 10^{-2}$ | $0.69 \pm 0.10$ | $4.5 \pm 1.7 \times 10^{3}$ | $1.6 \pm 0.8 \times 10^{5}$ |

$T_1$, and $M_X$ is the amplitude of the magnetization for times $t_{OR} \sim T_1$.

The chemical shifts were measured on a Varian HA-100 spectrometer with respect to internal *t*-butyl alcohol (approximately 0.1% in $D_2O$). A capillary of hexamethyldisilazane, held concentric with the nuclear magnetic resonance tube, was used as the lock signal. The shifts of the acetyl protons of methyl *N*-acetyl-D-glucosaminide and the methyl protons of *t*-butyl alcohol were measured by interpolation between markers calibrated in terms of the difference in frequency between the sweep and lock oscillators. The sample temperature was regulated using a Varian V-6040 temperature controller. The sample temperature was determined from the chemical shifts of a methanol or ethylene glycol sample placed in the probe before each run.

Methyl *N*-acetyl-α-D-glucosaminide (mp 184–185° uncor, $[\alpha]_D^{25}$ +132°) and methyl *N*-acetyl-β-D-glucosaminide (mp 194–195° uncor, $[\alpha]_D^{25}$ −43°) were prepared by the method of Zilliken (Zilliken *et al.*, 1955). P. L. Biochemicals two-times-crystallized, dialyzed, and lyophilized lysozyme (Lot No. LY-1) was used without further purification. All samples were made up in Stohler Isotope Chemicals 99.8% $D_2O$. For relaxation time measurements, a stock inhibitor solution was prepared ([methyl *N*-acetyl-D-glucosaminide] $\approx 8 \times 10^{-2}$ *m* in $D_2O$), added to weighed amounts of lysozyme ([lysozyme] $\approx 0$–$5 \times 10^{-3}$ *m*), and the pH (meter reading) of the final solutions adjusted to $5.3 \pm 0.1$ by adding very small amounts of 0.1 M HCl. For chemical shift measurements a stock lysozyme solution was prepared ([lysozyme] $\approx 2 \times 10^{-3}$ *m* in $D_2O$), brought to pH (meter reading) = $5.3 \pm 0.1$, and added to weighed amounts of methyl *N*-acetyl-D-glucosaminide ([methyl *N*-acetyl-D-glucosaminide] $\approx 4$–$100 \times 10^{-3}$ *m*). *t*-Butyl alcohol (1 μl) was then added to each sample (≈0.7 ml). All room temperature pH measurements were made with a Beckman expandomatic pH meter standardized with Beckman pH 7.00 buffer. pH measurements at other temperatures were thermostated and standardized against 0.05 M KHP, whose pH *vs.* temperature dependence is known (Hodgman, 1965). No buffers were used because of the specific interactions of buffers with lysozyme (B. D. Sykes and C. Parravano, unpublished results). For example, the dissociation constant for *N*-acetyl-D-glucosamine binding to lysozyme was found to be a function of acetate concentration when acetate buffer was used.

## Results and Discussion

*Methyl N-Acetyl-α- and -β-D-glucosaminide at 33°.* The chemical shifts of the fast exchange averaged line,

TABLE II: Activation Enthalpies and Entropies of Methyl *N*-Acetyl-β-D-glucosaminide Binding to Lysozyme.

| Rate Constant | $\Delta H^{\ddagger}$ (kcal) | $\Delta S^{\ddagger}$ (eu) |
|---|---|---|
| $k_{-1}$ | $+4.4 \pm 0.7$ | $-27 \pm 6$ |
| $k_1$ | $-2.9 \pm 0.5$ | $-44 \pm 9$ |

$\delta_{obsd}$, as a function of initial inhibitor concentration for methyl *N*-acetyl-α-D-glucosaminide with lysozyme and methyl *N*-acetyl-β-D-glucosaminide with lysozyme at 33° are presented in Figure 2. The results of the computer fit of the data of Figure 2 to equations 11 and 12 are presented in Table I. A check on the computer fit is provided by the fact that the same intercept, $\delta_I$, was predicted for methyl *N*-acetyl-α-D-glucosaminide and methyl *N*-acetyl-β-D-glucosaminide which is, of course, the observed result. This parameter is very sensitive to deviations of the experimental data from eq 11 and 12. There was no deviation of either anomer from a simple 1:1 binding over the concentration range used.

The trend of the dissociation constants is in agreement with the results of other workers (Neuberger and Wilson, 1967a,b; Raftery *et al.*, 1968). The equality of the shifts of both anomers upon binding to lysozyme suggests that both anomers bind in essentially the same fashion with respect to the position of the acetyl protons in relation to the cause of the shifts. The shifts are within the range of those expected from proximity to an aromatic residue (Johnson and Bovey, 1958), which presumably is tryptophan 108 near site C in lysozyme (Neuberger and Wilson, 1967a; Rupley *et al.*, 1967). The larger dissociation constant of the α anomer can then be explained in terms of a stearic interaction between the methyl group on the inhibitor (see Figure 1) and residues 52 (Asp) and 109 (Val) in the active site of lysozyme (Neuberger and Wilson, 1967a).

The relaxation time measurements at 33° for the α and β anomers are presented in Figures 3 and 4, respectively. The difference in relaxation times $(1/T_2) - (1/T_1)$ is directly proportional to the initial enzyme concentration, as predicted by eq 10, thereby ruling out processes such as active site to active site dimerization at these conditions of temperature, concentration, and pH (Bruzzesi *et al.*, 1965; Sophianopoulous and Van Holde, 1964). The slope of $1/T_1$ in both plots could be dues 52 (Asp) and 109 (Val) in the active site of lysoacetyl protons of the inhibitor while bound to the lysozyme, a magnetic interaction of the acetyl protons with nuclei in the active site of lysozyme, or a viscosity effect.

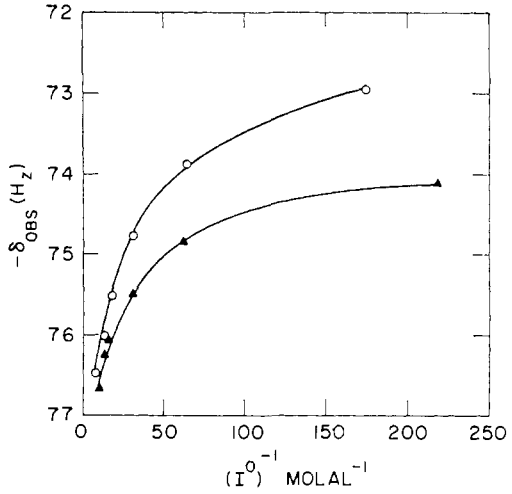


FIGURE 2: The chemical shifts of the acetyl protons of methyl *N*-acetyl-D-glucosaminide with respect to internal *t*-butyl alcohol at 100 MHz as a function of methyl *N*-acetyl-D-glucosaminide concentration; [lysozyme] = 2.55 × $10^{-3}$ *m*; $T$ = 33°; α-methyl *N*-acetyl-D-glucosaminide (▲); β-methyl *N*-acetyl-D-glucosaminide (●).

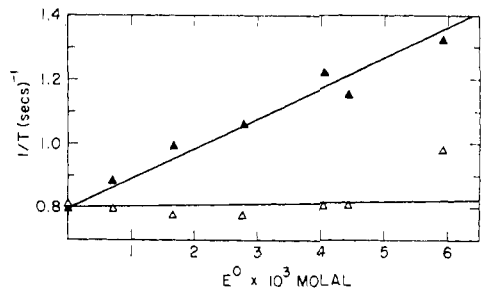


FIGURE 3: The relaxation times $T_1$ and $T_2$ at 60 MHz for the acetyl protons of α-methyl *N*-acetyl-D-glucosaminide as a function of lysozyme concentration; $T$ = 33°; [α-methyl *N*-acetyl-D-glucosaminide] = 8.03 × $10^{-2}$ *m*; $1/T_1$ (△); $1/T_2$ (▲).

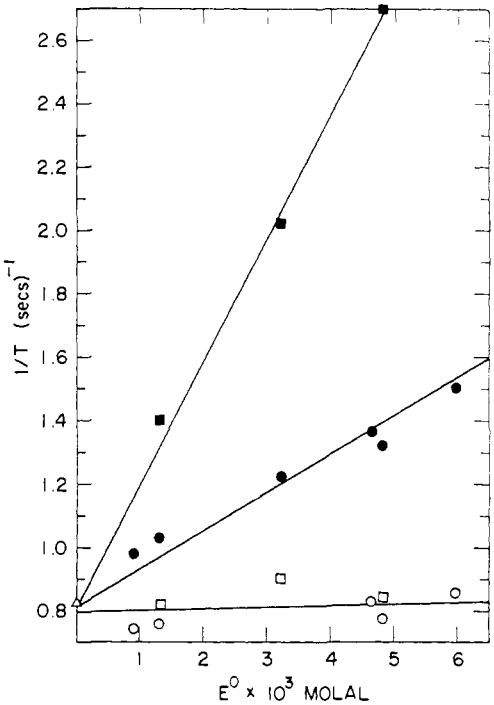


FIGURE 4: The relaxation times $T_1$ and $T_2$ for the acetyl protons of β-methyl *N*-acetyl-D-glucosaminide as a function of lysozyme concentration; $T$ = 33°; [β-methyl *N*-acetyl-D-glucosaminide] = 8.36 × $10^{-2}$ *m*; $1/T_1$ at 60 MHz (○); $1/T_2$ at 60 MHz (●); $1/T_1$ at 100 MHz (□); $1/T_2$ at 100 MHz (■); average of all $1/T_2$'s and $1/T_2$'s for $E^\circ$ = 0 (△).

Whatever the cause, the effect is the same for both anomers, and independent of resonance frequency. This implies that the correlation time of the relaxation mechanisms other than exchange is shorter than $10^{-9}$ sec ($1/\omega_0$). It is obvious, however, that the largest contribution to the line width is caused by exchange, contrary to the proposal of Thomas (1966, 1967). The exchange effect is different for the two anomers. The values of $k_{-1}$ and $k_1$ obtained from these results and eq 10 are presented in Table I. The largest portion of the difference in the $K_D$'s appears as a difference in $k_{-1}$ consistent with the proposal of an additional steric interaction for the α anomer (see previous discussion). The value of $k_1$ is smaller than the diffusion-controlled limit approached by some enzyme substrate reactions (Mahler and Cordes 1966). However, the rate constant, $k_1$, is an apparent constant whose derivation is dependent upon the total enzyme concentration and the total binding process. As such, the fact that it is smaller than that predicted by Chipman and Schimmel (1968) may reflect the fact that lysozyme exists in several ionization states, only one of which is capable of binding inhibitor, or that the binding process involves several steps, only one of which is diffusional.

The ratio of the contribution of exchange to relaxation at 100 MHz to the contribution at 60 MHz is equal to 3.2 compared with a value of 2.8 expected from eq 10. The difference presumably reflects experimental error and the approximations involved in the derivation of eq 10. The error limits for the rate constants in Table I represent the probable errors accumulated from the measured relaxation times, chemical shifts, and dissociation constants. The difference in $k_{-1}$ for methyl *N*-acetyl-α- and -β-D-glucosaminide is significant, however, since all of the sources of error considered (*i.e.*, systematic errors in the measurement of $T_1$ and $T_2$) would affect both rate constants equally.

*Methyl N-Acetyl-β-D-glucosaminide as a Function of Temperature.* The observed chemical shift at 100 MHz of the fast exchange averaged acetyl proton resonance for methyl *N*-acetyl-β-D-glucosaminide binding to lysozyme is presented in Figures 2 and 5 as a function of initial inhibitor concentration for various temperatures over the range 7–61°. A temperature of 61° is well below the transition temperature for lysozyme denaturation, which is 75° over the pH range 5.0–5.5 (Hamacuchi and Sakai, 1965). In addition, the 220-MHz spectrum of lysozyme (McDonald and Phillips, 1967) has been shown not to undergo any gross changes with temperature for temperatures below 75–80°. The values of the association constant, $K_A$, and the chemical shift of the bound inhibitor, Δ, obtained by a computer fit of the results

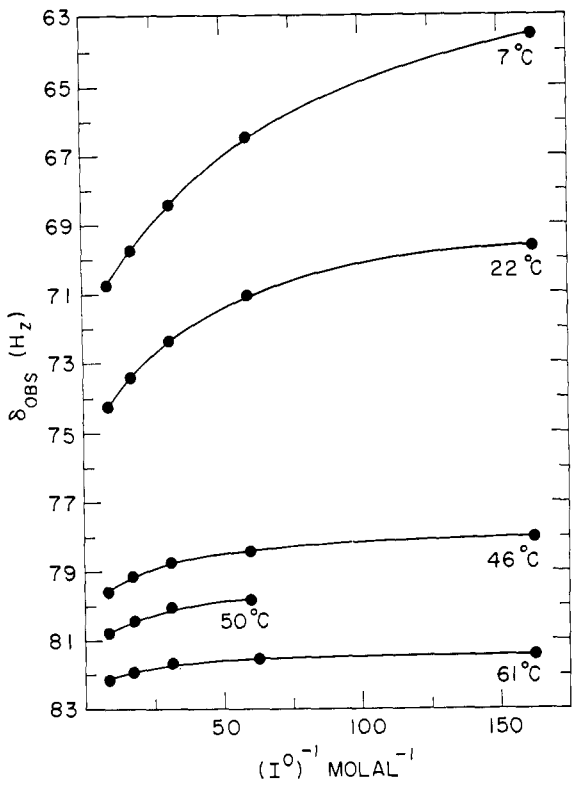


FIGURE 5: The chemical shifts of the acetyl protons of β-methyl *N*-acetyl-D-glucosaminide with respect to internal *t*-butyl alcohol at 100 MHz as a function of α-methyl *N*-acetyl-D-glucosaminide concentration and temperature; [lysozyme] = $2.52 \times 10^{-3}$ *m;* absolute shifts have been displaced for a clearer presentation.

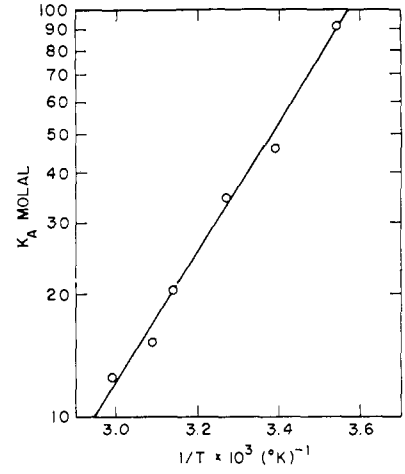


FIGURE 6: Association constant for β-methyl *N*-acetyl-D-glucosamide binding to lysozyme as a function of temperature; $\Delta G^\circ = -2.2$ kcal, $\Delta H^\circ = -7.3$ kcal, and $\Delta S^\circ = -17$ eu.

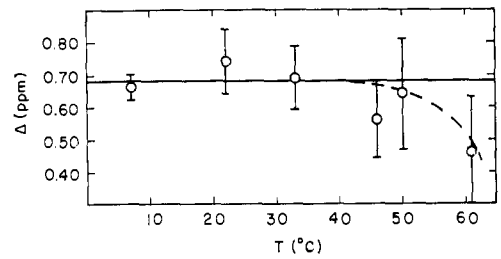


FIGURE 7: The chemical shift of β-methyl *N*-acetyl-D-glucosaminide bound to lysozyme as a function of temperature. Both the solid and dashed lines were considered in the calculation of rate constants (see Discussion).

of Figures 2 and 5 to eq 11 and 12, are presented in Figures 6 and 7, respectively. The thermodynamic parameters, $\Delta H^\circ = -7.34$ kcal, $\Delta S^\circ = -17.1$ eu, and $\Delta G^\circ = -2.24$ kcal, obtained from Figure 6 are in good agreement with the results of Chipman *et al.* (1967) for *N*-acetyl-D-glucosamine binding to lysozyme. The chemical shift of the bound inhibitor appears to decrease at high temperatures. However, since the range of observed shifts decreases with increasing temperature (see Figure 5) and the errors in the measurements remain constant, the errors in $K_A$ and Δ are largest at the higher temperatures.

The relaxation times $1/T_1$ and $1/T_2$ at 60 MHz for methyl *N*-acetyl-β-D-glucosaminide exchanging with lysozyme are presented in Figure 8 for various temperatures over the range 11–64°. The forward and reverse rate constants obtained from eq 10 and the results of Figures 6–8 are presented in Figure 9. The form of the rate constants as a function of temperature is assumed to be

$$k_r = \frac{kT}{h} \exp(\Delta S^{\ddagger}/R) \exp(-\Delta H^{\ddagger}/RT)$$

As with $K_A$ and Δ, the relative errors in the measurement of $(1/T_2) - (1/T_1)$ are the largest for the higher temperatures. For this reason, Δ was taken as 0.68 ppm and independent of temperature in the calculation of rate constants. If this assumption is not made the rate constants appear to drop off for temperatures above 50°. Both results are shown in Figure 9. The low-temperature points were preferentially weighted in the determination of the activation parameters of the rate constants from Figure 9. The activation parameters are presented in Table III.

The fact that the rate constant, $k_1$, decreases with increasing temperature, and that $\Delta H$ for $k_{-1}$ is less than $|\Delta H^\circ|$ suggests that the binding process is not simply

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (15)$$

but rather involves a more complex reaction mechanism. One possible choice is that lysozyme exists as an equilibrium mixture of several isomers (reactive and unreactive). Such an isomerization has been observed for ribonuclease (French and Hammes, 1965), and is suggested for lysozyme by the fact that the dependence of the binding constant for the trisaccharide upon pH is "bell shaped" (Lehrer and Fasman, 1966; Rupley *et al.*, 1967). Temperature-jump studies with the di- and trisaccharide (Chipman and Schimmel, 1968) have shown, however, that there is no slowly equilibrating isomerization of lysozyme at pH 6.0 and that the bimolecular

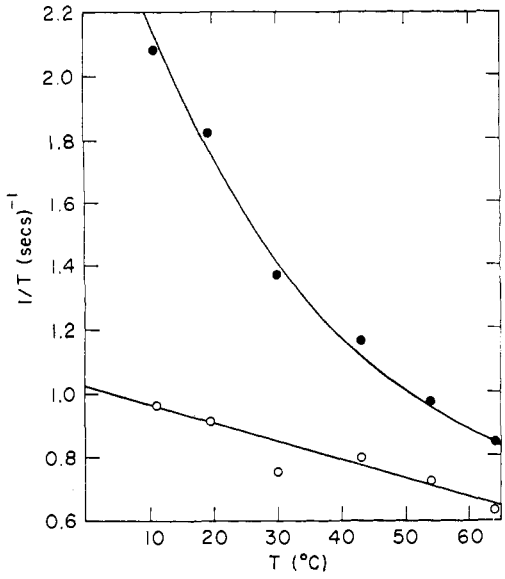


FIGURE 8: Relaxation times at 60 MHz for acetyl protons of $\beta$-methyl *N*-acetyl-D-glucosaminide exchanging with lysozyme as a function of temperature, [lysozyme] = $4.44 \times 10^{-3}$ *m*; [$\beta$-methyl *N*-acetyl-D-glucosaminide] = $7.94 \times 10^{-2}$ *m*; $1/T_1$ (○); $1/T_2$ (●).

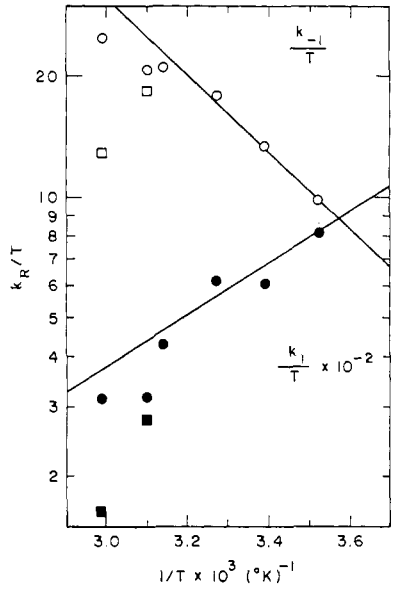


FIGURE 9: Rate constants, $k_1$ and $k_{-1}$, for $\beta$-methyl *N*-acetyl-D-glucosaminide binding to lysozyme as a function of temperature, pH 5.3 ± 0.1; $k_{-1}/T$ (○ and □); $k_1/T \times 10^{-2}$ (● and ■). The rate constants represented by circles were calculated assuming $\Delta$ = 0.68 ppm and independent of temperature (see Discussion); this assumption was not made in calculating the rate constants represented by the squares (see Figure 7).

reaction is solely responsible for the relaxation kinetics after a temperature jump for the di- and trisaccharide. In addition, all pH measurements were thermostated and there was no change in the pH of the lysozyme–inhibitor solutions as a function of temperature. A slowly equilibrating isomerization of lysozyme at pH $\neq$ 6.0 is not ruled out, however, nor is it impossible for the state of ionization of an important group to change with temperature without a change in the pH of the sample.

A second possible mechanism is that the monosaccharide inhibitor binds to lysozyme by forming an initial complex at a rate near diffusional, followed by a conformational change to produce a second complex. Such a mechanism has been proposed for proteolytic enzymes, especially for $\alpha$-chymotrypsin (Haynes and Feeney, 1968; Moon *et al.*, 1965). Let us consider the effect of this choice of mechanism

$$E + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EI)_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} (EI)_2$$

$$K_2 = \frac{[(EI)_1]}{[E][I]} = \frac{k_2}{k_{-2}}; \quad K_3 = \frac{[(EI)_2]}{[(EI)_1]} = \frac{k_3}{k_{-3}} \tag{16}$$

upon the nuclear magnetic resonance results. With the following assumptions

$$k_3 \gg k_{-3} \text{ and } \Delta_1 \ll \Delta_2 \tag{17}$$

the lifetime of the bound inhibitor becomes

$$\frac{1}{\tau} = \frac{1}{[(EI)_2]} \frac{d[(EI)_2]}{dt} = k_{-3} \tag{18}$$

rather than $k_{-1}$ and the apparent association constant becomes

$$\frac{[(EI)_1] + [(EI)_2]}{[E][I]} = K_2(1 + K_3) \simeq K_2K_3 \tag{19}$$

rather than $K_A$. At 33°, these rates are

$$k_{-3} = 4.8 \times 10^3 \text{ sec}^{-1}$$
$$K_2K_3 = 34.5 \ m^{-1} \tag{20}$$

and

$$k_2 \approx 4.5 \times 10^6 \ m^{-1} \text{ sec}^{-1}$$

where the value of $k_2$ has been estimated by extrapolation of the temperature-jump results (Chipman and Schimmel, 1968). From eq 20

$$k_{-2} \approx 27k_3 \tag{21}$$

The fact that $k_1$, now $k_3K_2$, decreases with increasing temperature is accounted for by this choice of mechanism by assuming $k_3$ increases with temperature less rapidly than $K_2$ decreases with temperature. Also, $\Delta H$ for $k_{-1}$ becomes the activation enthalpy for $k_{-3}$, which could reasonably be less than $|\Delta H°|$. The large values of $\Delta S^{\ddagger}$ are presumably consistent with the steric requirements of saccharide binding in the active site of lysozyme.

## Conclusion

These results indicate the utility of transient nuclear magnetic resonance methods for the measurement of the rate constants of biological exchange reactions. In particular, temperature studies are facilitated. It is important to realize, however, that the rate constants obtained are apparent constants dependent upon the total binding process and the total concentration of species.

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References

Abragam, A. (1961), The Principles of Nuclear Magnetism, London, Ocford, Chapter XII.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1965), *Nature 206*, 757.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc. (London) B167*, 365.

Bruzzesi, M. R., Chiancone, E., and Antonini, E. (1965), *Biochemistry 4*, 1796.

Chipman, D. M., Grisaro, V., and Sharon, N. (1967), *J. Biol. Chem. 242*, 4388.

Chipman, D. M., and Schimmel, P. R. (1968), *J. Biol. Chem. 243*, 3771.

Dalhquist, F. W., Jao, L., and Raftery, M., (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 26.

Dahlquist, F. W., and Raftery, M. A. (1968a), *Biochemistry 7*, 3269.

Dhalquist, F. W., and Raftery, M. A. (1968b), *Biochemistry 7*, 3277.

French, T. C., and Hammes, G. G. (1965), *J. Am. Chem. Soc. 87*, 4669.

Groves, P. D., Huck, P. J., and Homer, J. (1967), *Chem. Ind. (London)*, 915.

Hamacuchi, K. and Sakai, H. (1965), *J. Biochem. (Tokyo) 57*, 721.

Haynes, R., and Feeney, R. E. (1968), *Biochemistry 7*, 2879.

Hodgman, C. D., Ed. (1965), *in* Handbook of Chemistry and Physics, Vol. 44, 48 ed, Cleveland, Ohio, Chemical Rubber, p 1715.

Johnson, C. E., Jr., and Bovey, F. A. (1958), *J. Chem. Phys. 29*, 1012.

Kuntz, I. D., Jr., and Johnston, M. D., Jr. (1967), *J. Am. Chem. Soc. 89*, 6008.

Laszlo, P., Speert, A., Ottinger, R., and Reisse, J. (1968), *J. Chem. Phys. 48*, 1732.

Lehrer, S. S., and Fasman, C. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 133.

Mahler, H. R., and Cordes, E. H. (1966), Biological Chemistry, New York, N. Y., Harper & Row, p 275.

McDonald, C. C., and Phillips, W. D. (1967), *J. Am. Chem. Soc. 89*, 6332.

Meiboom, S. (1961), *J. Chem. Phys. 34*, 375.

Moon, A. Y., Sturtevant, J. M., Hess, G. P. (1965), *J. Biol. Chem. 240*, 4204.

Neuberger, A., and Wilson, B. M. (1967a), *Biochim. Biohpys. Acta 147*, 473.

Neuberger, A., and Wilson, B. M. (1967b), *Nature 215*, 524.

Phillips, D. C. (1967), *Proc. Natl. Acad. U. S. 57*, 844.

Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc. (London) B167*, 378.

Raftery, M. A., Dalhquist, F. W., Chan, S. I., and Parsons, S. M. (1968), *J. Biol. Chem. 243*, 4175.

Rupley, J. A., Butler, L., Gerring, M., Hartdegen, F. J., and Pecoro, R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1088.

Solomon, I. (1959a), *Compt. Rend. 248*, 92.

Solomon, I. (1959b), *Compt. Rend. 249*, 1631.

Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.

Spotswood, T. M., Evans, J. M., and Richards, J. H. (1967), *J. Am. Chem. Soc. 89*, 5052.

Thomas, E. W. (1966), *Biochem. Biophys. Res. Commun. 24*, 611.

Thomas, E. W. (1967), *Biochem. Biophys. Res. Commun. 29*, 628.

Zilliken, F., Rose, C. S., Braun, G. A., and Grorgy, P. (1955), *Arch. Biochem. Biophys. 54*, 392.