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Substituent Effect on Lysozyme-Catalysed Hydrolysis of Some β-Aryl Di-*N*-acetylchitobiosides

BY C. S. TSAI, J. Y. TANG AND S. C. SUBBARAO
Department of Chemistry, Carleton University, Ottawa, Ont., Canada

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Measurements are reported on the kinetics of the lysozyme-catalysed hydrolysis of several β-aryl di-*N*-acetylchitobiosides, some of which have been synthesized for the first time. The catalytic rate constants ($k_{cat.}$) at 45° yield a curved Hammett plot (concave up) and the plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$ has a sharp break. Substrates with electron-withdrawing groups exhibit a kinetic deuterium isotope effect ($k^{H}_{cat.}/k^{D}_{cat.}$), whereas those with electron-donating groups show no such isotope effect. The results suggest the operation of different mechanisms for the two types of substrates.

The recent elucidation of the three-dimensional structure of hen's-egg-white lysozyme (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17) (Blake *et al.* 1965) and the parallel studies of the interaction between the enzyme and various inhibitors have stimulated much interest in the mechanistic studies of reactions catalysed by lysozyme. On the basis of the known non-productive interactions between lysozyme and chitobiose and further fitting of sugar rings as extensions of the observed complex, it has been suggested (Blake *et al.* 1967) that the catalytic activity of lysozyme is due to two carboxyl groups, those of aspartic acid residue 52 (Asp-52) and glutamic acid residue 35 (Glu-35), functioning as a base and an acid respectively to effect the hydrolysis of susceptible glycosidic bonds. From crystallographic investigation and the established mechanism for the acid-catalysed hydrolysis of glycosides, it has been proposed (Blake *et al.* 1967; Vernon, 1967) that the lysozyme catalysis proceeds by general acid catalysis with intermediate carbonium ion formation.

Attempts have been made to synthesize simple substrates for lysozyme (Osawa, 1966; Osawa & Nakazawa, 1966) to facilitate the mechanistic studies of the catalysis. Lowe, Sheppard, Sinnott & Williams (1967) have investigated the lysozyme-catalysed hydrolysis of some β-aryl di-*N*-acetylchitobiosides and observed a linear Hammett plot with a reaction constant of 1·21. The results were interpreted in terms of a rate-determining step involving concerted acid–base or acid–nucleophile catalysis. Recently a mechanism involving general acid catalysis assisted anchimerically by the acetamido group has been proposed (Lowe & Sheppard, 1968). After rejecting several mechanisms Raftery & Rand-Meir (1968) proposed the following mechanism as most likely: (*a*) formation of a carbonium ion, which gives rise stereospecifically to β-anomeric product; (*b*) a double displacement mechanism, which also results in retention of configuration.

Since the detailed mechanism of lysozyme catalysis is not known completely, there is a need for further study, especially on simple systems. In the present work some β-aryl di-*N*-acetylchitobiosides with both electron-withdrawing and electron-donating substituents were synthesized. The hydrolysis of these compounds was studied over a temperature range by using (*a*) lysozyme as a catalyst in H_2O and (*b*) deuterated lysozyme in D_2O. No study of this nature covering a wide range of substituents has been done previously, although the kinetics of the lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides has already been established (Lowe *et al.* 1967).

MATERIALS AND METHODS

Chemicals. Twice-crystallized salt-free lysozyme from hen's-egg white was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Chitin was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Phenol, *p*-methylphenol, *p*-ethylphenol, *p*-methoxyphenol and *p*-chlorophenol were purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., and purified by distillation under reduced pressure. *p*-Nitrophenol was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and purified by crystallization from water. Chloroform ('spectrograde') was obtained from Fisher Scientific Co., Ottawa, Ont., Canada. Citrate buffer, pH 5·1 (0·1 M), was prepared from citric acid and sodium citrate (Gomori, 1955).

Synthesis of substrates. All substrates were synthesized as follows.

Chitin (260g.) was treated with a mixture of acetic anhydride (1·5l.) and conc. H_2SO_4 (196ml.) by the procedure of Barker, Foster, Stacey & Webber (1958). This method gave a syrupy mixture of acetylated chito-oligoses (Barker *et al.* 1958). Differential recrystallization from methanol was employed at this stage to obtain 25·5g. of 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranose (chitobiose octa-acetate) in the form of white needles, m.p. 309–310° (decomp.), $[\alpha]_D^{24}+55{\cdot}2°$ (*c*5·2 in acetic acid) [lit. m.p. 301–303° (decomp.), $[\alpha]_D^{30}+56°$ (*c*0·52 in acetic acid)]. Chitobiose octa-acetate was converted into 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl) chloride (α-acetochlorochitobiose) by the method of Osawa (1966). Recrystallization from acetone gave white needles, m.p. 200–202° (decomp.) (lit. m.p. 208–209°).

α-Acetochlorochitobiose was condensed with *p*-nitrophenol (Osawa, 1966) to give β-*p*-nitrophenyl di-*N*-acetylhepta-acetylchitobioside, which was *O*-deacetylated with 1M-sodium methoxide to give the final product. Recrystallization from aqueous methanol gave *p*-nitrophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-*p*-nitrophenyl di-*N*-acetylchitobioside) as white needles, m.p. 227–228° (decomp.), $[\alpha]_D^{24}-23{\cdot}1°$ (*c*0·33 in water) [lit. m.p. 226–227° (decomp.), $[\alpha]_D^{27}-21°$ (*c*0·24 in aq. 70% methanol)]. The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1630, 1550 (NH·CO·CH_3), 1580 (aryl C=C), 850 (aromatic 1,4-disubstituted), 1520, 1340, 860 (NO_2) and 900 (β-glucoside) (Found: C, 46·3; H, 5·6; N, 7·0. $C_{32}H_{31}N_3O_{13},H_2O$ requires C, 46·9; H, 5·9; N, 7·5%).

Phenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-phenyl di-*N*-acetylchitobioside) was recrystallized from water and aqueous methanol to give white needles, m.p. 302–304° (decomp.), $[\alpha]_D^{24}-13{\cdot}6$ (*c*0·36 in water) [lit. m.p. 304°, $[\alpha]_D^{20}-15{\cdot}1$ (in water)]. The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1650, 1550 (NH·CO·CH_3), 1600, 1490 (aryl C=C), 690 (aromatic monosubstituted) and 900 (β-glucoside) (Found: C, 48·7; H, 6·5; N, 5·2. $C_{22}H_{32}N_2O_{11},2H_2O$ requires C, 49·2; H, 6·8; N, 5·2%).

Recrystallization of *p*-methylphenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-*p*-methylphenyl di-*N*-acetylchitobioside) from water gave white needles, m.p. 315–316°, $[\alpha]_D^{24}-9{\cdot}4°$ (*c*2·0 in aq. 70% methanol). The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1650, 1550 (NH·CO·CH_3), 1500 (aryl C=C), 815 (aromatic 1,4-disubstituted) and 900 (β-glucoside) (Found: C, 50·4; H, 6·5; N, 4·7. $C_{23}H_{34}N_2O_{11},2H_2O$ requires C, 50·2; H, 7·0; N, 5·1%).

p-Ethylphenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-*p*-ethylphenyl di-*N*-acetylchitobioside) was prepared by an identical procedure. Recrystallization from aqueous methanol and water gave an amorphous powder, m.p. 306–308° (decomp.), $[\alpha]_D^{24}-8{\cdot}0°$ (*c*2·0 in aq. 70% methanol). The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1650, 1550 (NH·CO·CH_3), 1500 (aryl C=C), 822 (aromatic 1,4-disubstituted) and 900 (β-glucoside) (Found: C, 52·8; H, 6·6; N, 4·9. $C_{24}H_{36}N_2O_{11},H_2O$ requires C, 52·7; H, 7·0; N, 5·1%).

Recrystallization of *p*-methoxyphenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-*p*-methoxyphenyl di-*N*-acetylchitobioside) from water and aqueous methanol gave fine needles, m.p. 296–297° (decomp.), $[\alpha]_D^{24}-8{\cdot}2°$ (*c*2·0 in water). The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1650, 1550 (NH·CO·CH_3), 1500 (aryl C=C), 822 (aromatic 1,4-disubstituted) and 900 (β-glucoside) (Found: C, 48·4; H, 6·5; N, 4·8. $C_{23}H_{34}N_2O_{12},2H_2O$ requires C, 48·8; H, 6·8; N, 4·9%).

Recrystallization of *p*-chlorophenyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (β-*p*-chlorophenyl di-*N*-acetylchitobioside) from water gave white needles, m.p. 293–294° (decomp.), $[\alpha]_D^{24}-8{\cdot}2°$ (*c*2·0 in aq. 70% methanol). The i.r. spectrum (Nujol) showed absorptions (cm.$^{-1}$) at 3200 (OH, NH), 1640, 1545 (NH·CO·CH_3), 1475 (aryl C=C), 822 (aromatic 1,4-disubstituted) and 900 (β-glucoside) (Found: C, 46·1; H, 5·9; Cl, 6·3; N, 4·7. $C_{22}H_{31}ClN_2O_{11},2H_2O$ requires C, 46·3; H, 6·2; Cl, 6·2; N, 4·9%).

Ionization constants of phenols. Ionization constants of purified *p*-substituted phenols (0·01M) in 0·1M-KCl solution were determined potentiometrically with a titration assembly (Radiometer TTT1c, SBRI and SBUI) at 35°. Measured *pK* values were plotted against Hammett σ^- values (Biggs & Robinson, 1961). The unknown σ^- value of ethyl group for phenol series was interpolated from this plot.

Deuterated lysozyme and buffer solutions. Deuterated lysozyme was prepared by dissolving lysozyme (150mg.) in D_2O (75ml.) in an ice bath. The solution was kept in a cold-room (4°) for 24hr. and freeze-dried. The deuterated lysozyme was dissolved in D_2O (7·5ml.) and the whole process was repeated to obtain fully deuterated lysozyme. Praissman & Rupley (1968) have reported that a complete exchange of hydrogen atoms in lysozyme by tritium takes place in 24hr. at 0°. $CH_3·CO_2D$ was prepared by allowing acetyl chloride to react with D_2O (Murry & Williams, 1958). NaOD was prepared by the action of sodium on D_2O. Aqueous 0·1M-sodium acetate buffer was prepared by partially neutralizing (pH5·1) acetic acid with NaOH, and deuterated 0·1M-acetate buffer was prepared similarly from $CH_3·CO_2D$ and NaOD in D_2O. In the latter case the formula of Fife & Bruice (1961) was used to correct the pH reading.

Kinetic studies. Kinetic studies were carried out by measuring the formation of phenols from the reaction mixture. Standard curves of extinction versus concentration of phenol (in chloroform) were used in the conversion of spectrophotometric readings into concentrations. Substrate solution of known concentration was prepared in 0·1M-citrate buffer, pH5·1. Lysozyme solution (0·25mM) was prepared in the same buffer. These solutions were placed in a water bath at 45±0·05°. To the substrate solution (4ml.) was added 1ml. of lysozyme solution to give a final enzyme concentration of 0·05mM and substrate concentrations ranging from 1mM to 20mM. The first sample (1ml.) was withdrawn immediately after the addition of the enzyme solution and other samples were withdrawn at suitable time-intervals. Each sample withdrawn from the reaction mixture was extracted with

Table 1. *Data used in the analysis of reaction mixtures*

Phenol formed	Wavelength used (nm.)	σ^-
p-Methylphenol	279	−0·152
p-Ethylphenol	278	−0·132
p-Methoxyphenol	292	−0·130
Phenol	271	0
p-Chlorophenol	282	+0·223
p-Nitrophenol	308	+1·243

'spectrograde' chloroform (4ml.) by shaking for 1·5min. The mixtures were then centrifuged at 4500 rev./min. for 8min. The phenols in the chloroform layer were determined spectrophotometrically by using the wavelengths shown in Table 1. The rate of spontaneous solvolysis of substrates under the experimental conditions was low compared with the rate in the presence of lysozyme, and was neglected. The kinetic parameters, $k_{cat.}$ and K'_m, were calculated from initial velocities according to the equation:

$$v = \frac{k_{cat.}[S]_0[E]_0}{K'_m + [S]_0}$$

The molecular weight of lysozyme was taken as 14500 (Jollès, 1964) for the calculations.

For studies of isotope effect, kinetic runs were conducted with lysozyme and deuterated lysozyme in acetate (pD 5·1) and deuterated acetate (pD 5·1) buffer solutions respectively.

RESULTS

Michaelis–Menten kinetics. Initial velocities of enzymic reactions were estimated from the rate of liberation of phenols, which correlated linearly with time during the early stage of the reaction at higher temperatures, although an induction period (Maksimov, Kaverzneva & Kravchenko, 1965) was observed at lower reaction temperatures.

The linear double-reciprocal plots (Lineweaver & Burk, 1934) exemplified in Fig. 1 indicate that the lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides follows Michaelis–Menten kinetics. Kinetic constants calculated from these plots are presented in Table 2.

Variation in rates. The rates of lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides are influenced by the insertion of electron-donating and electron-withdrawing groups in the benzene ring of the substrates. Both electron-donating and electron-withdrawing substituents increase the rate, the unsubstituted phenyl compound showing the minimum $k_{cat.}$ value (Table 2). The Hammett equation, which expresses reaction rates in terms of substituent constants, has been very useful in the elucidation of the mechanisms of organic reactions (Hammett, 1940; Jaffé, 1953). Its use in enzymic reactions has been reported (Nath & Rydon, 1954; Hall, Hollingshead & Rydon, 1962; Deitrich, Hellerman & Wein, 1962; Radda, 1964; Lowe *et al.* 1967). The conclusions to be drawn from existing experimental evidence can be summarized briefly as follows. The Hammett plot is (*a*) a straight line if the rate-limiting step and the reaction mechanism remain the same, (*b*) a concave-down curve if the rate-limiting step changes, or (*c*) a concave-up curve if the reaction mechanism alters (Leffler & Grunwald, 1963). When $\log k_{cat.}$ is plotted against substituent constants (σ^-), a concave-up curve is obtained in the present case (Fig. 2). This might be due to an abrupt change in reaction mechanism as the substituents change from electron-donating to electron-attracting. To the authors' knowledge this is the first instance of a concave-up Hammett plot observed for enzymic reactions. However, several cases of concave-up Hammett plots are known for organic reactions; in these cases, the curves have also been explained as due to a change in mechanism (Ceccon, Papa &

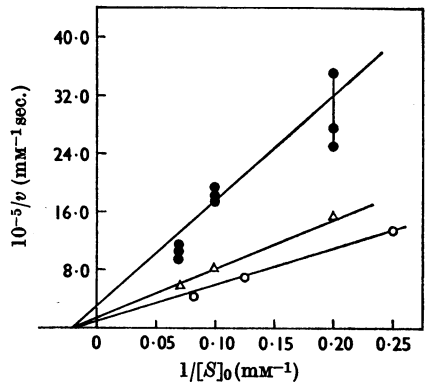

Fig. 1. Double-reciprocal (Lineweaver–Burk) plot for the hydrolysis of β-*p*-ethylphenyl di-*N*-acetylchitobioside at 35° (●), 45° (△) and 50° (○).

Table 2. *Kinetic parameters for the lysozyme-catalysed hydrolysis of* β-para-*substituted aryl di-*N*-acetylchitobiosides at* 45°

The β-*para*-substituted aryl di-*N*-acetylchitobiosides are indicated as β-R-ADAC, where R is the substituent in the *para* position.

Substrate	$10^5 k_{cat.}$ (sec.$^{-1}$)	K_m (mM)
β-C_2H_5–ADAC	15·00	50
β-CH_3–ADAC	8·30	45
β-CH_3·O–ADAC	7·10	33
β-H–ADAC	2·90	27
β-Cl–ADAC	4·00	10
β-NO_2–ADAC	13·30	10

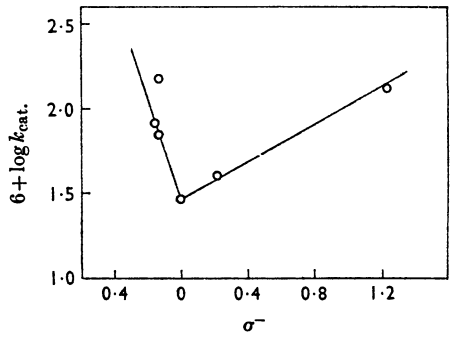

Fig. 2. Hammett plot for the lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides at 45° with $\rho = +0{\cdot}55$ and $\rho = -2{\cdot}96$.

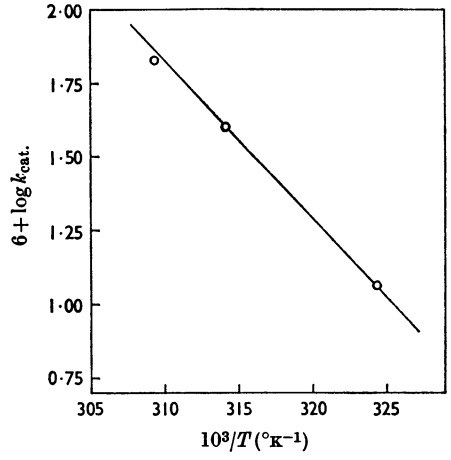

Fig. 3. Arrhenius plot of log $k_{cat.}$ (catalytic rate constant) against $1/T$ for the lysozyme-catalysed hydrolysis of β-*p*-chlorophenyl di-*N*-acetylchitobioside.

Fava, 1966). For substrates with electron-donating substituents the reaction constant is highly negative (−2·96), which may indicate that these reactions proceed via polar transition states.

Temperature effects. In enzymic reactions increasing temperature may cause denaturation of the enzyme as well as increasing the reaction rate. The effect of temperature on lysozyme has been investigated by Hayashi, Kaugimiya & Funatsu (1968). No loss in the enzymic activity was observed when the temperature was raised to 50°, and therefore the active structure of lysozyme was presumably unaltered. Since the present investigation was carried out in the temperature range 35–50° and the Arrhenius plots are linear (Fig. 3) the inactivation of the enzyme was neglected.

Table 3. *Thermodynamic parameters for the lysozyme-catalysed hydrolysis of* β-para-*substituted aryl di-*N*-acetylchitobiosides*

The β-*para*-substituted aryl di-*N*-acetylchitobiosides are indicated as β-R–ADAC, where R is the substituent in the *para* position.

Substrate	$\Delta H^{\ddagger}$ ($k_{cal.}$/mole)	$\Delta S^{\ddagger}$ (cal./mole-deg.)
β-C_2H_5–ADAC	16·9	−24·8
β-CH_3–ADAC	21·8	−11·3
β-CH_3·O–ADAC	22·0	−10·8
β-H–ADAC	24·8	−1·4
β-Cl–ADAC	24·8	0·0
β-NO_2–ADAC	25·2	+2·6

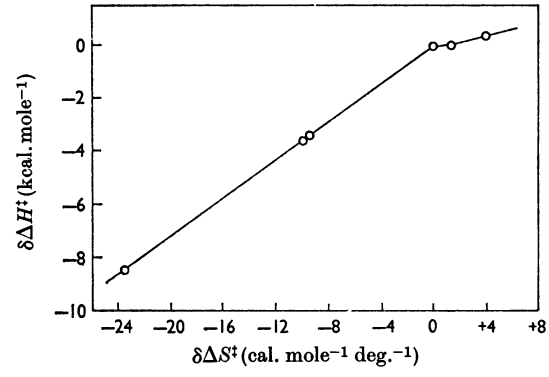

Fig. 4. Plot of enthalpy–entropy relationship. Differences in enthalpies of activation ($\Delta H^{\ddagger}$) between β-*para*-substituted aryl di-*N*-acetylchitobiosides were plotted against corresponding differences in entropies of activation ($\Delta S^{\ddagger}$).

From the experimental activation energies, enthalpies ($\Delta H^{\ddagger}$) and entropies ($\Delta S^{\ddagger}$) of activation for the reactions are calculated from equations 1 and 2 (Laidler, 1965) and presented in Table 3.

$$\Delta H^{\ddagger} = E - \boldsymbol{R}T \qquad (1)$$

and

$$k_{cat.} = (kT/h)\exp(\Delta S^{\ddagger}/\boldsymbol{R})\exp(-\Delta H^{\ddagger}/\boldsymbol{R}T) \qquad (2)$$

Fig. 4 shows a plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$ in which a sharp break is observed. Linear relationships between entropies and enthalpies of activation have been commonly observed in the kinetic studies of groups of similar organic reactions and has been discussed in great detail by Leffler & Grunwald (1963). Leffler (1955, 1966) has also pointed out that the enthalpy–entropy plot can be a straight line only if there is a single interaction mechanism; with two or more independent interaction mechanisms, two different slopes, scattered diagrams or any other pattern can be expected. The isokinetic

Table 4. *Isotope effect* ($k^H_{cat.}/k^D_{cat.}$) *values at* 45° *for the lysozyme-catalysed hydrolysis of* β-para-*substituted di-*N*-acetylchitobiosides*

The β-*para*-substituted aryl di-*N*-acetylchitobiosides are indicated as β-R–ADAC, where R is the substituent in the *para* position.

Substrate	$10^5 k^H_{cat.}$ (sec.$^{-1}$)	$10^5 k^D_{cat.}$ (sec.$^{-1}$)	$k^H_{cat.}/k^D_{cat.}$
β-C_2H_5–ADAC	14·36	12·60	1·14
β-CH_3–ADAC	8·62	7·29	1·18
β-CH_3·O–ADAC	6·90	6·27	1·10
β-H–ADAC	2·63	2·33	1·13
β-Cl–ADAC	4·56	2·62	1·74
β-NO_2–ADAC	12·19	6·06	2·01

temperature for substrates with electron-donating substituents is 358°K (β_1) and for the substrates with electron-withdrawing substituents is 81·3°K (β_2). Since β_1 and β_2 are different from each other and from the average experimental temperature (316·6°K), they imply the operation of different mechanisms for the two groups of substrates containing electron-donating and electron-withdrawing substituents. The large difference in $\Delta S^\ddagger$ of more than 20 cal./mole-deg. between the substrates with nitro and ethyl substituents further lends support to the above view (Long, Pritchard & Stafford, 1957; Schaleger & Long, 1963).

Isotope effects. The ratio of $k^H_{cat.}$ to $k^D_{cat.}$ represents the isotope effect. The results are presented in Table 4. The kinetics of enzyme reactions in D_2O are difficult to interpret because (1) changes in the conformation of the protein or the active site or both, (2) a change in the activity of nucleophiles and (3) differences in the solvation of the transition state may occur. Praissman & Rupley (1968) studied the tritium–hydrogen exchange of lysozyme and lysozyme–saccharide complex and found that no profound conformational changes took place in the tritiated lysozyme. Bender, Pollock & Neveu (1962) investigated the effect of H_2O and D_2O as a solvent on general base catalysis by imidazole, as well as on nucleophilic catalysis by imidazole. They found that an isotope effect of 2–3 is observed in general base catalysis, which involves as an important step a proton transfer between water and imidazole. In nucleophilic catalysis they observed no isotope effect. The calculated $\Delta S^\ddagger$ values (Table 3) indicate that the transition states for the reactions with substrates containing electron-withdrawing substituents are less solvated than the initial states, whereas for reactions with substrates containing electron-donating substituents, the transition states are considerably more solvated; hence the isotope effect due to transition-state solvation should be more significant in the latter reactions than the former. The observed isotope effects for these reactions are very small (1·1–1·2), so that the contribution of the solvation isotope effect can be neglected. The observed isotope effects for electron-withdrawing groups (Table 4) can only be explained by assuming a rate-determining step that involves a proton transfer. It is noteworthy that the isotope effect increases from 1·74 for substrate with chloro substituent to 2·01 for substrate with nitro substituent. It is well known that in a series of similar reactions the kinetic isotope effects will be a maximum when the proton is symmetrically situated in the transition state (Westheimer, 1961). A plausible way of explaining the observed changes in the isotope effect is to assume that the proton is in different positions in the two transition states.

DISCUSSION

With the evidence available, two plausible mechanisms for lysozyme action on substrates with two different types of substituents can be proposed, as shown in Scheme 1 (see also Raftery & Rand-Meir, 1968). These mechanisms are based on several assumptions. The most important ones are that no gross conformational change occurs to the enzyme molecule during the catalytic process and that the hydrolysis cleaves the glycosylic oxygen linkage with retention of configuration (Rupley, 1967). The extent of neighbouring-group participation is uncertain and has not been considered here.

The lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides with electron-donating substituents may proceed by the first mechanistic pathway (I). This involves a rapid pre-equilibrium protonation of the glycosidic oxygen atom to form the conjugate acid, followed by heterolysis of the exocyclic $O–C_{(1)}$ bond to give a carbonium ion, which is stabilized by the Asp-52 carboxylate. Subsequent hydrolysis completes the reaction. This model predicts a negative reaction constant (ρ) and a decrease in the entropy of activation, which is in agreement with the experimental findings. The hydrolysis of substrates with electron-withdrawing substituents may proceed by the second mechanistic pathway (II). The electron-withdrawing group does not favour the protonation of the glycosidic oxygen, but facilitates the nucleophilic attack by Asp-52 in concert with the abstraction of a proton from Glu-35 to form a glycosyl–enzyme complex, which is subsequently hydrolysed by water. This is a double displacement mechanism, which accounts for the retention of configuration (Koshland, 1953). The reaction constant, the activation parameters and the isotope effects observed in the present work are consistent with the above model.

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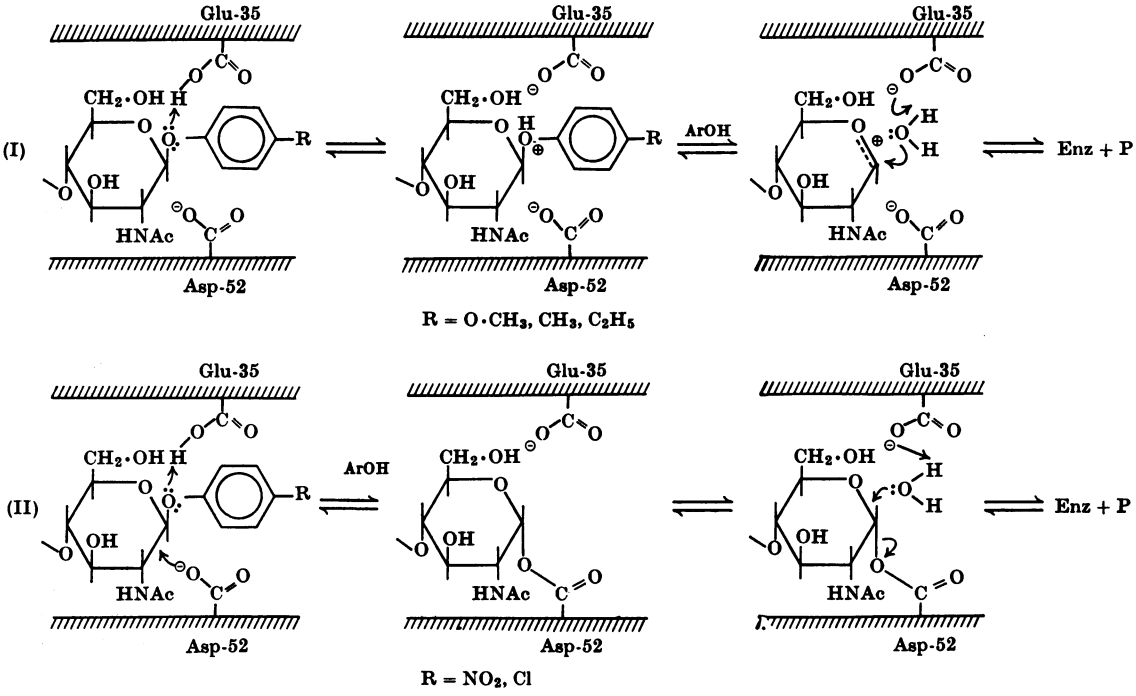

Scheme 1. Plausible mechanisms for the lysozyme-catalysed hydrolysis of β-aryl di-*N*-acetylchitobiosides.

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