Glycoside Hydrolysis. I. Intramolecular Acetamido and Hydroxyl Group Catalysis in Glycoside Hydrolysis

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Abstract: The hydrolysis of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxyglucopyranosides and *o*- and *p*-nitrophenyl glucosides have been studied at 78.2° (μ = 0.3) between pH 0.75 and 11.72. For the β anomers in which the acetamido and hydroxyl groups are in a *trans*-1,2 disposition to the nitrophenoxy group spontaneous hydrolysis occurs. In contrast, for α anomers in which the acetamido or hydroxyl groups are in a *cis*-1,2 disposition to the nitrophenoxy group only specific acid and specific base catalyzed hydrolysis is found. The spontaneous rate constants for the β anomers are attributed to stereospecific anchimeric participation by the acetamido and hydroxyl groups, for which the former is almost 10^3 times more effective than the latter. For acetamido group participation the spontaneous rate of hydrolysis is insensitive to the concentrations of buffers employed and does not exhibit a significant deuterium solvent kinetic isotope effect. Two kinetically equivalent mechanisms are offered: (a) intramolecular nucleophilic attack by the neutral acetamido group, and (b) intramolecular nucleophilic attack of the ionized acetamido group on the protonated glycoside. Mechanism a is the most reasonable on the basis of the calculated magnitude of the rate constant for b. The possible pertinence of the findings of these studies as related to the mechanism of lysozyme action is discussed.

Lysozyme is the first enzyme to have its tertiary structure determined by X-ray crystallographic methods.[2] It functions catalytically by hydrolyzing β-(1–4)-N-acetylmuramic acid–N-acetylglucosamine glycoside linkages of a polymer of alternating N-acetylmuramic acid–N-acetylglucosamine structure of bacterial cell wall.[3] Lysozyme has also been shown to hydrolyze β-(1–4)-linked oligomers of N-acetylglucosamine,[4] and to be inhibited by N-acetylglucosamine[5] and di-N-acetylchitobiose.[6] From chemical studies[4] and X-ray diffraction studies of lysozyme–inhibitor complexes[2b,7] it is possible to infer that carboxyl groups are the only side-chain functional groups of the enzyme which are both present at the active site and likely to be involved in the bond-breaking steps. A possible mechanism involving these carboxyl groups has been considered,[2b] and model enzyme systems involving neighboring carboxyl group effects have been examined and discussed in detail.[8]

The purpose of the present investigation was to determine if the 2-acetamido substituent of the enzyme substrate could provide anchimeric assistance in the hydrolytic cleavage of the β-glycosidyl bond and thus facilitate the enzymatic catalysis. Neighboring amide group participation has been observed previously in the hydrolysis of esters, amides, and alkyl halides.[9] In addition, Inch and Fletcher[10] have suggested neighboring acetamido participation to explain reactivities of 1-O-acyl-2-acetamido-2-deoxy-β-D-glucopyranoses in methanol and aqueous dioxane, though no detailed kinetic investigation was performed. In approaching the question of neighboring acetamide participation in glycoside hydrolysis we chose to study as model substrates *o*- and *p*-nitrophenyl glucopyranosides and *o*- and *p*-nitrophenyl 2-acetamido-2-deoxyglucopyranosides. These compounds may legitimately be considered as models since chemically and structurally similar *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides have shown activity as substrates for lysozyme.[11]

Experimental Section

Materials. *o*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (I), *o*-nitrophenyl β-D-glucopyranoside (V), and *p*-nitrophenyl α-D-glucopyranoside (VII) were purchased from Pierce Chemical Co. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (III) and *p*-nitrophenyl β-D-glucopyranoside (VI) were purchased from Sigma Chemical Co. *o*-Nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside (II) and *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside (IV) were prepared by the method of Weissmann,[12] and were generously provided by Professor Weissmann.

Kinetics. All kinetic measurements were done at 78.2 ± 0.3° in aqueous buffers at μ = 0.3 with KCl. Buffers employed were hydrochloric acid (pH 0.75 to 1.58), potassium formate (pH 2.50 to 3.40), potassium acetate (pH 3.96 to 6.08), potassium phosphate (pH 7.02 to 8.30), imidazole (pH 7.58), potassium borate (pH 8.85), glycine (pH 8.64 to 8.82), and potassium hydroxide (pH 10.28 to 11.72). With the exception of compounds II and IV for which cases both acid- and base-catalyzed rate constants were very small, at least one buffer dilution experiment using at least three buffer concentrations at constant μ over a tenfold range was performed for each buffer with each glycoside studied. In no instance was a buffer catalytic term detected. In addition the spontaneous rates of hydrolysis of compounds I, III, and V were found to be insensitive to buffer concentration over the entire pH range of the plateau rate. Spectrophotometric rates were followed by observing *o*-nitrophenol formation at 372.6 mμ or *p*-nitrophenol formation at

(1) Predoctoral Fellow, National Institutes of Health. A portion of the material to be submitted by D. P. for the Ph.D. in Chemistry, University of California at Santa Barbara.

(2) (a) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature*, **206**, 757 (1965); (b) D. C. Phillips, *Proc. Natl. Acad. Sci. U. S.*, **57**, 484 (1967).

(3) N. Sharon, T. Osawa, H. M. Flowers, and R. W. Jeanloz, *J. Biol. Chem.*, **241**, 223 (1966).

(4) J. A. Rupley and V. Gates, *Proc. Natl. Acad. Sci. U. S.*, **57**, 496 (1967).

(5) M. Wenzel, H. P. Lenk, and E. Schutte, *Z. Physiol. Chem.*, **327**, 13 (1962).

(6) J. A. Rupley, *Biochim. Biophys. Acta*, **83**, 245 (1964).

(7) L. N. Johnson and D. C. Phillips, *Nature*, **206**, 761 (1965).

(8) T. C. Bruice and D. Piszkiewicz, *J. Am. Chem. Soc.*, **89**, 3568 (1967).

(9) For a relevant review and discussion see T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1966, p 187.

(10) T. D. Inch and H. G. Fletcher, Jr., *J. Org. Chem.*, **31**, 1810 (1966).

(11) (a) T. Osawa, *Carbohydrate Res.*, **1**, 435 (1966); (b) T. Osawa and Y. Nakazawa, *Biochim. Biophys. Acta*, **130**, 56 (1966).

(12) B. Weissmann, *J. Org. Chem.*, **31**, 2505 (1966).

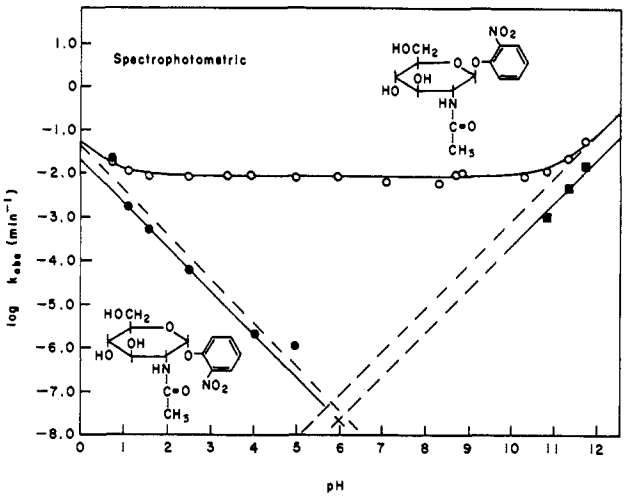


Figure 1. Spectrophotometrically determined pH–log k_{obsd} profiles for the hydrolyses of *o*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (I) and *o*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside (II). Points are experimental and the curves are calculated from eq 1 and the values of k_H, k_{OH}, and k_0 provided in Table I.

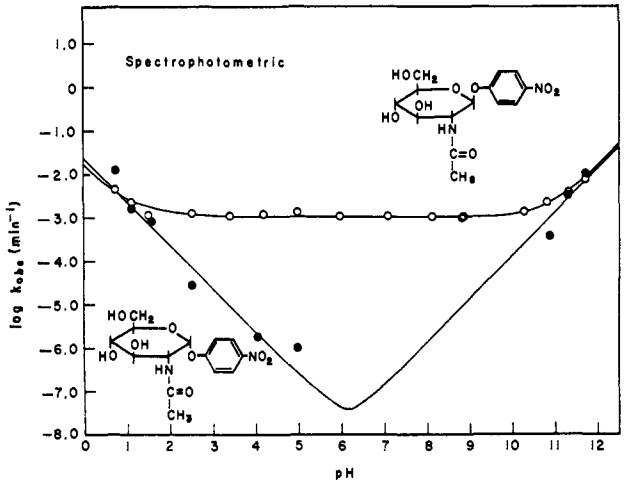


Figure 2. Spectrophotometrically determined pH–log k_{obsd} profiles for the hydrolyses of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (III) and *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-glycopyranoside (IV). Points are experimental and the curves are calculated from eq 1 and the values of k_H, k_{OH}, and k_0 provided in Table I.

348.6 mμ, the isosbestic points of dissociated and undissociated forms, and *p*-nitrophenolate formation at 400 mμ, its approximate λ_{max}. The ultraviolet absorbance spectra of the product solutions of all glycosides hydrolyzed were determined for acid-catalyzed, base-catalyzed, and spontaneous reactions (with the exception of VI where the spontaneous reaction was followed to less than 10% completion). Product solutions of all glycosides hydrolyzed had absorbance spectra identical with *o*- or *p*-nitrophenol at a similar pH, with the exception of II, which in base yielded an unidentified compound having λ_{max} 280 mμ. Polarimetric rates were determined at 78.2°, and α values were read at 589, 578, and 546 mμ at 30°. Polarimetric rate constants determined at all three wavelengths generally agreed within ±10% and were therefore averaged. The pseudo-first-order rate constants (k_{obsd}) were obtained by multiplying the slope of plots of log $[(OD_\infty - OD_0)/(OD_\infty - OD_t)]$ *vs.* time (t) by 2.303, or by the method of Guggenheim.[13]

Apparatus. The pH's of the buffer solutions employed in this study were determined at 78.2 ± 0.1° (the boiling point of 95% ethanol) by means of a Metrohm Ⓔ Type H high-temperature glass electrode, a Radiometer Type TTT 1b autotitrator pH meter, and a thermostated cell previously described.[14] Spectrophotometric rates were generally determined using a Gilford 2000 recording spectrophotometer which was equipped with a cell holder thermostated at 78.2 ± 0.3° by a Haake Type NBe constant-temperature bath. In the case of very slow rates and polarimetric rates, reacting solutions were kept in sealed ampoules in a thermostated aluminum block from which samples were periodically withdrawn and analyzed. Polarimetric rates were allowed to react at 78.2 ± 0.3° and followed using a Perkin-Elmer Model 141 polarimeter with a cell thermostated at 30 ± 0.1°.

(13) E. A. Guggenheim, *Phil. Mag.*, 2, 538 (1926).

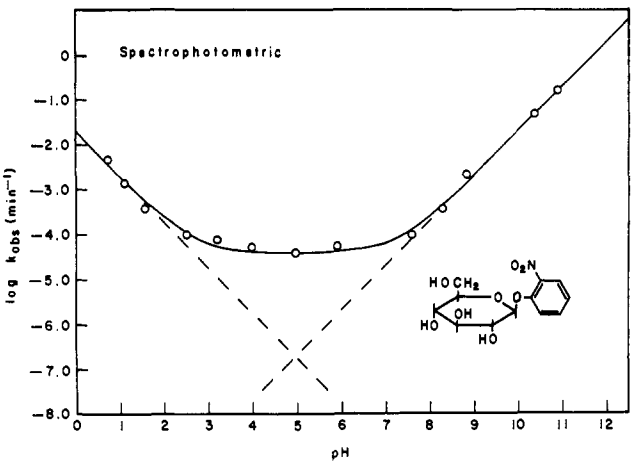


Figure 3. Spectrophotometrically determined pH–log k_{obsd} profile for the hydrolysis of *o*-nitrophenyl β-D-glucopyranoside (V). Points are experimental and the curve is calculated from eq 1 and the values of k_H, k_{OH}, and k_0 provided in Table I. Plateau rates (at pH 2.52 to 7.60) for V were followed to approximately 10% completion, and yielded a product solution with spectrum identical with *o*-nitrophenol (or *o*-nitrophenolate). When the hydrolysis of V in this region was followed to greater than 25% completion, the initial increase in OD was followed by a decrease in OD yielding a product solution of complex spectrum. The apparent secondary reaction was not observed for any other glycoside hydrolyzed, and it was not investigated further.

Results

The spectrophotometrically determined pH–log rate profiles for the hydrolyses of the nitrophenyl 2-acetamido-2-deoxy-D-glucopyranosides (I, II, III, and IV) and the nitrophenyl D-glucopyranosides (V, VI, and VII) are shown, with structural anomers on the same plot, in Figures 1 (I and II), 2 (III and IV), 3 (V), and 4 (VI and VII). The profiles of the *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (I and III) and *o*- and *p*-nitrophenyl β-D-glucopyranosides (V and VI) follow the rate expression

$$k_{obsd} = k_H a_H + k_{OH} K_w / a_H + k_0 \qquad (1)$$

where k_H is the second-order rate constant for specific acid catalyzed hydrolysis, k_{OH} is the second-order rate constant for specific base catalyzed hydrolysis, K_w is the dissociation constant of water (2.34×10^{-13} at 78.2° [15]), and k_0 is the first-order rate constant for spontaneous hydrolysis. The profiles for the hydrolysis of the α anomers (II, IV, and VII) show only specific acid (k_H) and specific base catalyzed (k_{OH}) hydrolyses. (Compound II showed a base-catalyzed reaction yielding a compound, which was not identified, having λ_{max} 280

(14) T. C. Bruice and F. H. Marquardt, *J. Am. Chem. Soc.*, 84, 365 (1962).

(15) Interpolated from values given in A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," John Wiley and Sons, Inc., New York, N. Y., 1962, p 171.

Table I. Kinetic Constants for the Hydrolyses of Nitrophenyl Glycopyranosides (78.2°; Solvent H_2O; 0.3 μ with KCl)[a]

Compound		k_H, M^{-1} min^{-1}	k_{OH}, M^{-1} min^{-1}	k_0, min^{-1}	$k_0^{H_2O}/k_0^{D_2O}$
I	*o*-NP-β-D-NAG	4.47×10^{-2}	3.80×10^{-1}	8.72×10^{-3}	1.06
II	*o*-NP-α-D-NAG	2.24×10^{-2}	9.55×10^{-2} [b]		
III	*p*-NP-β-D-NAG	1.78×10^{-2}	6.60×10^{-2}	1.03×10^{-3}	1.36
IV	*p*-NP-α-D-NAG	2.40×10^{-2}	5.62×10^{-2}		
V	*o*-NP-β-D-gluco	2.00×10^{-2}	9.33	4×10^{-5} [c]	0.83
VI	*p*-NP-β-D-gluco	8.32×10^{-3}	1.99	3×10^{-6} [c]	
VII	*p*-NP-α-D-gluco	3.72×10^{-2}	2.09×10^{1}		

[a] $k_{obsd} = k_H a_H + k_{OH} K_w / a_H + k_0$. [b] Product formation (not *o*-nitrophenolate) followed at 280 mμ. [c] Not known with great accuracy.

Table II. Values for α Infinity for the Hydrolysis of IV and α for Equilibrated N-Acetylglucosamine

pH	λ 589 mμ $[\alpha]_\infty^{30}$[a]	λ 589 mμ $[\alpha]_{eq}^{30}$[b]	λ 578 mμ $[\alpha]_\infty^{30}$[a]	λ 578 mμ $[\alpha]_{eq}^{30}$[b]	λ 546 mμ $[\alpha]_\infty^{30}$[a]	λ 546 mμ $[\alpha]_{eq}^{30}$[b]
0.75	55.6	52.1	63.3	55.4	74.1	62.4
2.52	39.8	37.4	42.4	39.4	45.0	44.4
5.96	33.0	34.6	37.7	36.8	42.4	41.4
8.30	17.0	28.4	24.7	30.4	27.8	34.0
8.77	−3.9	1.4	−1.3	6.2	−5.1	4.8
10.82	−2.6	0[c]	−1.3	0[c]	−2.6	0[c]

[a] Uncertainty in $[\alpha]_\infty^{30}$ is ±3.0°. [b] N-Acetylglucosamine in solution was equilibrated at 78.2° for 8 hr (about seven half-lives in k_0 for IV). [c] Solutions of N-acetylglucosamine turned dark brown after 4 hr and would not transmit light. Value of $[\alpha]_{eq}^{30} = 0$ is from literature.[16] See Results section of text.

mμ. After approximately 1 month at 78.2° this unidentified compound yielded *o*-nitrophenol, which was identified by its spectrum.) None of the α anomers had a spontaneous rate of hydrolysis (k_0). A summary of determined values of k_H, k_{OH}, and k_0 is presented in Table I.

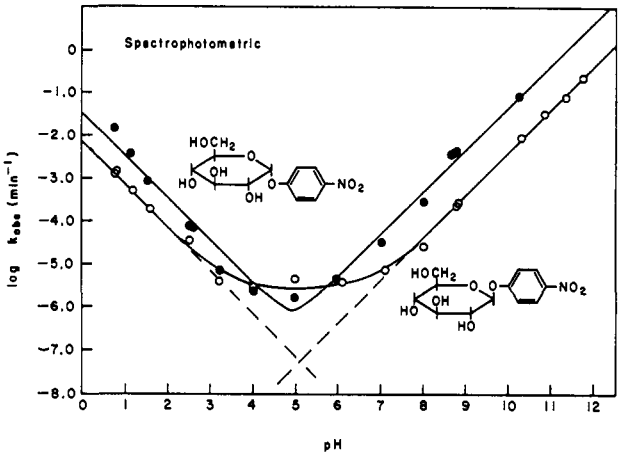


Figure 4. Spectrophotometrically determined pH–log k_{obsd} profiles for the hydrolyses of *p*-nitrophenyl β-D-glucopyranoside (VI) and *p*-nitrophenyl α-D-glucopyranoside (VII). Points are experimental and the curves are calculated from eq 1 and the values of k_H, k_{OH}, and k_0 provided in Table I.

The hydrolysis of I was also followed polarimetrically at six pH's across the entire plateau region (pH 0.75–10.82), and, as would be expected, polarimetric rates were equal to spectrophotometric rates as seen in Figure 5. The α infinity values were determined at approximately 7 and 15 half-lives and remained constant, giving no indication of a second reaction. The α infinity values varied somewhat with pH, but agreed with the α values for N-acetylglucosamine which had been equilibrated at 78.2° for 8 hr (approximately seven half-lives in the hydrolysis of I (Table II)). Both α infinity and α values for equilibrated N-acetylglucosamine were found to tend toward zero as pH increased. This result is in accord with the observation of Kuhn and Krüger[16] that N-acetylglucosamine reacts rapidly in base to yield the optically inactive 3-acetamidofuran. We believe it is reasonable to conclude, therefore, that N-acetylglucosamine and *o*-nitrophenol are liberated simultaneously, and any intermediate, if formed, would be less stable than I.

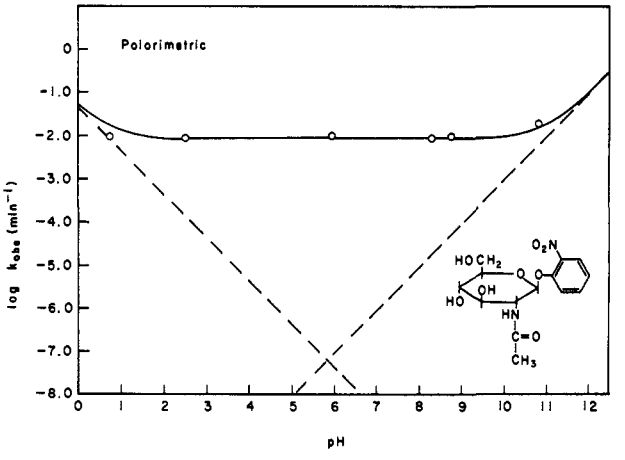


Figure 5. Polarimetrically determined pH–log k_{obsd} profile for the hydrolysis of *o*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (I). Points are experimental and the curve is identical with that of the spectrophotometrically determined pH–log k_{obsd} profile given in Figure 1.

The kinetic deuterium solvent isotope effects on the hydrolyses of the β anomers I, III, and V were determined at pH = pD values in the plateau region (pH = pD[17] = 6.08 for I and III, pH = pD[17] = 5.12 for V). The rates of spontaneous hydrolysis (k_0) of I, III, and V

(16) R. Kuhn and G. Krüger, *Chem. Ber.*, **89**, 1473 (1956).

(17) The pH meter reading in D_2O solutions was converted to pD by employing the formula of T. H. Fife and T. C. Bruice, *J. Phys. Chem.*, **65**, 1079 (1961).

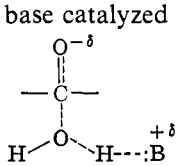
were found to be relatively insensitive to solvent isotope effects with $k_0^{H_2O}/k_0^{D_2O}$ = 1.06, 1.36, and 0.83, respectively. This suggests first that a proton transfer is not involved in the rate-determining transition state, and, second, that water itself is not involved.

Buffer dilution experiments on the hydrolyses of I, III, and V were performed covering the entire plateau region. No buffer catalytic effect was observed for formate, acetate, phosphate, borate, imidazole, or glycine buffers in as much as a tenfold buffer dilution. Therefore, buffers may not act mechanistically as a general acid, general base, or nucleophilic catalyst in the spontaneous hydrolyses of I, III, or V.

Discussion

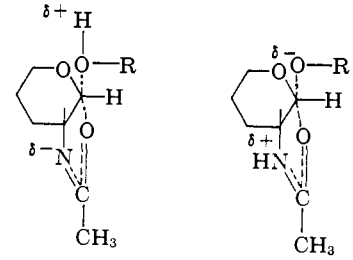
The observations of greatest interest, in the present study, are that the *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides hydrolyze spontaneously across the extended pH range of *ca.* 1.5 to 10.5 (Figures 1 and 2). Furthermore, the mechanism associated with the spontaneous hydrolysis increases the observed hydrolytic rate constant at neutrality by a factor of about 10^5 over that for specific base and specific acid catalyzed hydrolysis. By inspection of Figures 1 and 2 it is seen that the mechanism for this most efficient spontaneous mechanism is stereospecific since the log k_{obsd} *vs.* pH profiles for the corresponding α anomers do not exhibit a plateau. Thus, the spontaneous hydrolytic mechanism requires a *trans*-1,2 disposition of acetamido and nitrophenoxy groups. Smaller but still detectable spontaneous rates of hydrolysis were observed for *o*- and *p*-nitrophenyl β-D-glucopyranoside (Figures 3 and 4) where nitrophenoxy and 2-hydroxyl groups are also in a *trans*-1,2 disposition. No spontaneous rate of solvolysis is detectable if the nitrophenoxy and 2-hydroxyl groups are in a *cis*-1,2 configuration. These results support anchimeric assistance by the carbonyl oxygen and the 2-hydroxyl group in the hydrolysis of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and *o*- and *p*-nitrophenyl β-D-glucopyranoside, respectively. The *cis*-1,2 configuration precludes on a steric basis intramolecular nucleophilic attack by any group at the C-2 position of the glucopyranose ring. Participation by the acetamido group is seen to be almost 10^3 times more effective than that by the 2-hydroxyl group.

In the hydrolysis of acid anhydrides, electron-deficient esters, lactones, etc., the spontaneous rates of solvolysis are found to be associated with sizable kinetic deuterium solvent isotope effects. In addition the solvolysis of these substrates is subject to catalysis by buffer species and the catalytic coefficient for buffer catalysis is found to be considerably smaller in D_2O than in H_2O.[18] These experimental results are interpretable on the basis that the addition of water to the substrate is general base catalyzed as in (2). In (2), B:



(2)

represents either a water molecule (spontaneous hydrolysis) or the basic species of the buffer (buffer catalysis) and the kinetic deuterium solvent isotope effect is attributed to the partial breaking of the H–O bond in the transition state. In contrast to this usual state of affairs the spontaneous hydrolyses of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides do not exhibit a kinetic deuterium solvent isotope effect, and the values of the observed pseudo-first-order rates of hydrolysis at any pH are not subject to alteration with the concentration of the various buffers employed. These findings establish that proton transfer has not occurred partially in the transition state. Two kinetically equivalent mechanisms, one in which proton transfer is complete (3a) at the transition state and one in which proton transfer occurs after the transition state (3b), are in accord with the experimental results.



(3)

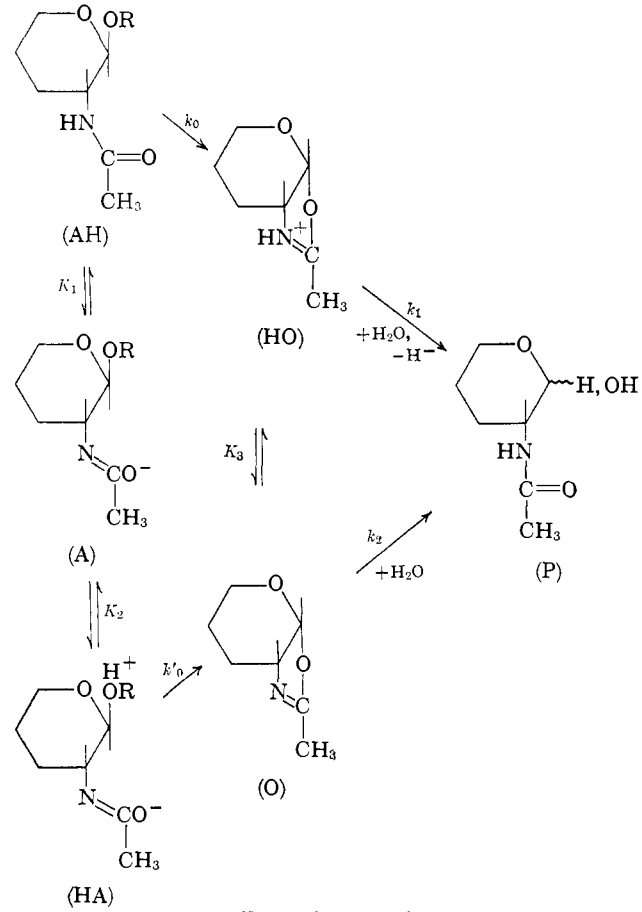
a b

R = *o*-nitrophenyl, *p*-nitrophenyl

Analogous mechanisms may be considered for the spontaneous hydrolysis of the *o*- and *p*-nitrophenyl β-D-glucopyranosides which also exhibit no buffer catalysis or deuterium solvent kinetic isotope effect.

The kinetic schemes for intramolecular participation of the acetamido group are provided in Chart I. The

Chart I



R = *o*-nitrophenyl, *p*-nitrophenyl

(18) Reference 9, p 28.

two possible reacting species (AH and HA of Chart I) are related by K_1, the dissociation constant of the proton on the acetamido nitrogen, and K_2, the dissociation constant of the protonated glycoside. The protonated and unprotonated oxazoline intermediates (HO and O) would also be related by the dissociation constant K_3. After the rate-determining nucleophilic attack, both protonated and unprotonated oxazoline might rapidly hydrolyze to the product, N-acetylglucosamine (P).

The kinetically equivalent rate-determining mechanisms k_0 and k_0' are related as follows.

$$K_1 = \frac{(A)(H)}{(AH)} \qquad K_2 = \frac{(H)(A)}{(HA)}$$

$$v = k_0(AH) \qquad v = k_0'(HA)$$

$$= k_0\frac{(A)(H)}{K_1} \qquad = k_0'\frac{(H)(A)}{K_2} \qquad (4)$$

$$k_0\frac{(A)(H)}{K_1} = k_0'\frac{(H)(A)}{K_2}$$

$$k_0' = k_0\frac{K_2}{K_1}$$

A reasonable estimate of the lower limit of K_1 based on determined dissociation constants of amides is $K_1 = 10^{-13}$ based on a value of 9.5 for trifluoroacetanilide.[19] Similarly an upper limit for the value of K_2 may be estimated to be $K_2 = 10^7$ based on a value of $pK_a = -3.59$ for diethyl ether[20] and $pK_a \leq -6$ for several phenolic ethers.[21] Consequently an approximate value of k_0' may be calculated to be $k_0' = 10^{18}$ min^{-1}. This value is at least two orders of magnitude greater than the vibrational frequency of the hydrogen molecule (7.9×10^{15} min^{-1} by the simple harmonic oscillator approximation),[22] the fastest molecular vibration. On this basis the estimated value of k_0' is impossibly high, and therefore the mechanism defined by k_0' would be of no consequence. The mechanism described by k_0 then accounts for the spontaneous hydrolyses observed for *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (I and III). This conclusion is reasonable since it is in agreement with the general observation that when giving anchimeric assistance in the solvolyses of esters, amides, and alkyl halides the nucleophilic center of the neutral amide group is generally the carbonyl oxygen, and the nucleophilic center of the amide anion is usually the nitrogen.[9]

The postulated steady-state protonated oxazoline intermediate (HO) and its conjugate base (O) have a precedent in a recently synthesized analogous compound, 3′,5′,6′-tri-O-acetyl-2-methyl-α-D-glucofurano-[2′,1′:4,5]-2-oxazoline.[23] A dissociation equilibrium between the protonated and unprotonated forms of oxazoline (K_3) may be of significance since the analogous compound 2-methyl-Δ^2-oxazoline has a dissociation constant with $pK = 5.5$.[24] Thus, pathways for the decomposition of oxazoline intermediate *via* k_1 or k_2 or both may lead to the product N-acetylglucosamine (P).

The specific acid catalyzed hydrolysis of simple glucosides has been investigated and shown to proceed with fission of the hexose-protonated oxygen bond of the glycoside linkage and involve pyranosyl carbonium ions as intermediates.[25] In light of the intramolecular acetamido participation in the spontaneous hydrolyses of I and III, the question may be raised if the 2-acetamido group may also assist in the acid-catalyzed hydrolysis by displacing the protonated nitrophenyl group. Since both *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (I and III) have second-order acid-catalyzed rates (k_H) of similar magnitudes as their α anomers (II and IV), this does not seem likely.

The mechanisms of specific base catalyzed hydrolysis of glycosides have been reviewed.[26] Studies on the basic hydrolyses of phenyl D-glucosides have shown that the β anomers, which are in the *trans*-1,2 configuration, generally react at a much faster rate than their α anomers by a mechanism which involves neighboring C-2 oxyanion participation.[26] However, when a strongly electron-withdrawing group, such as the nitro group, is present on the phenyl ring, both α and β anomers react readily, thus suggesting some other mechanism to be operative.[27] A bimolecular nucleophilic aromatic substitution mechanism at the C-1 position of the nitrophenyl ring has been suggested for both α[28] and β anomers.[29] This mechanism is supported by the observation that when solvolyzed in methanolic sodium methoxide *p*-nitrophenyl β-D-galactoside, *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl 2-O-methyl-β-D-galactoside, and *p*-nitrophenyl 2-O-methyl-α-D-mannoside yield substantial amounts of *p*-nitroanisole as product.[30] Our experiments show that *p*-nitrophenyl α-D-glucopyranoside (VII) has a second-order rate constant for its base-catalyzed hydrolysis (k_{OH}) which is substantially greater than for its β anomer (VI) (Table I). This observation is in accord with the interpretation that C-2 oxyanion participation is not involved in the alkaline hydrolysis mechanism.

Again, in light of the intramolecular acetamido group catalysis in the spontaneous hydrolysis of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (I and III), the question might be asked if acetamido group participation might be of significance in their base-catalyzed hydrolysis. A possible mechanism analogous to C-2 oxyanion participation might be envisioned in which a proton is abstracted from the 2-acetamido nitrogen of the β anomers (to form A, Chart I) followed by intramolecular nucleophilic displacement of *p*-nitrophenolate ion. For *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (III) this mechanism may be ruled out since both III and its α anomer (VI) have second-order rates in base (k_{OH}) (Table I) which are of comparable magnitude. This same conclusion probably holds true for *o*-nitrophenyl 2-acetamido-

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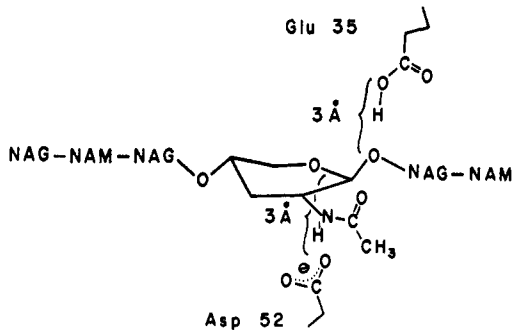


Figure 6. Approximate spatial arrangement at the active site of lysozyme–substrate complex.[2b] The bond broken is believed to be between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) rings. To fit the active site, the NAM ring is distorted into the half-chair conformation.

2-deoxy-β-D-glucopyranoside (I) also. However, direct comparison of base-catalyzed rates of hydrolysis (k_{OH}) of I and its α anomer (II) cannot be made since II reacts in base to give an unidentified product which is not *o*-nitrophenolate.

The results of this investigation demonstrate that the 2-acetamido substituent of model lysozyme substrates (I and III) can provide anchimeric assistance in the hydrolyses of their β-glycosidyl bonds. The possible relevance of this finding to the mechanism of action of lysozyme may be seen by examining a three-dimensional model of the lysozyme–substrate complex.[2b,31] Two carboxylic acid side-chain groups are found at what is considered to be the active site of the enzyme (see Figure 6). Glutamic acid residue 35 is about 3 A above the glycosidic linkage which is to be broken and it has been considered as a possible general acid type catalyst.[2b] Aspartic acid residue 52 has one of its oxygen atoms about 3 A below both the anomeric carbon of the glycosidic bond broken and the adjacent ring oxygen and it has been proposed to be in the carboxylate anion form.[2b] Allowing for some flexiblility in the enzyme–substrate complex, aspartate-52 is positioned so that it could conceivably give nucleophilic assistance in the enzymatic mechanism, thus forming a covalently bound glycosyl–enzyme intermediate.[32] Carboxylate anions have been shown to give nucleophilic assistance in the hydrolysis of esters.[33] Intramolecular carboxyl group catalysis has been reported in the hydrolysis of *o*-carboxyphenyl β-D-glucopyranoside,[34] but a distinction was not made between the kinetically equivalent mechanisms of intramolecular participation of the carboxyl group as a nucleophile on the O-1 protonated glucoside, participation of the carboxyl as an intramolecular general acid catalyst, or a concerted combination of the two. Our results which show the susceptibility of a glycoside to apparent nucleophilic assistance in its hydrolysis suggest that similar assistance by aspartate-52 in the enzymatic mechanism is a possibility.

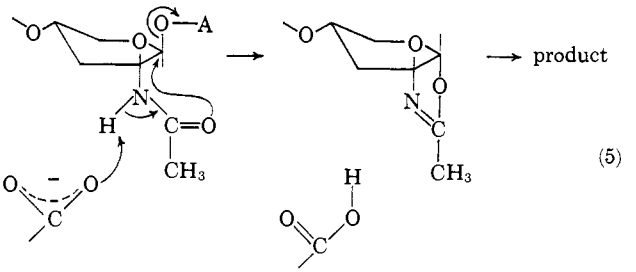
(31) We wish to thank Professor J. A. Rupley for allowing us to examine his space-filling lysozyme model which he constructed from coordinates supplied to him by Professor D. C. Phillips.

(32) After examining a model of the enzyme–substrate complex, Vernon considered this mechanism unlikely on the basis that "the nucleophile (Asp-52) and the leaving group cannot assume the correct geometry for the transition state of a bimolecular substitution." Also, the covalently bound glycosyl–enzyme could not be formed without considerable distortion: C. A. Vernon, *Proc. Roy. Soc.* (London), **B167**, 389 (1967).

(33) Reference 9, p 107.

(34) B. Capon, *Tetrahedron Letters*, 911 (1963); see also ref 8.

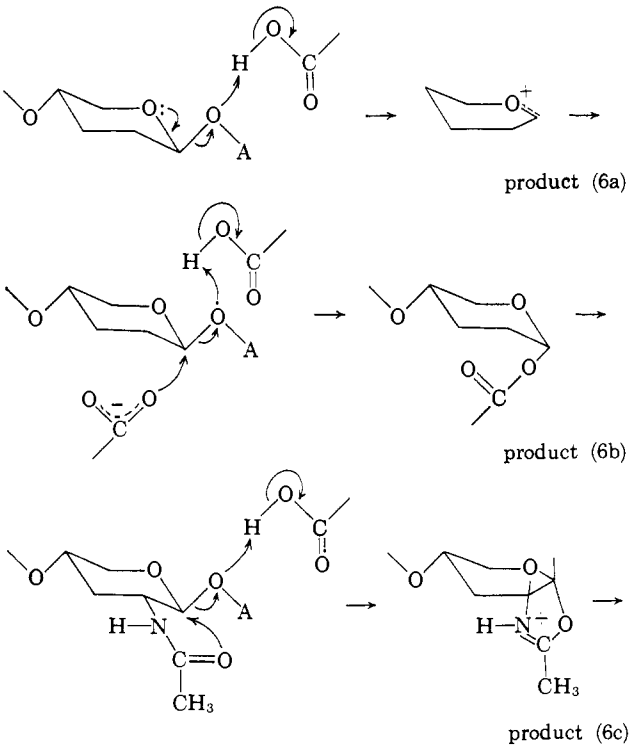
Our results also suggest the interesting possibility that the 2-acetamido group of the substrate may participate in the enzymatic mechanism. Since carboxylate anions have been shown to act as general bases in some hydrolytic reactions of esters and anhydrides,[33] the question arises logically if aspartate-52 might act as a general base for lysozyme by abstracting the proton of the 2-acetamido nitrogen with a simultaneous displacement of aglycone (eq 5). Examination



(5)

of a space-filling model of the lysozyme–substrate complex indicates that such general base catalysis by aspartate-52 is not sterically feasible. When the carbonyl oxygen of the 2-acetamido group is positioned below the anomeric carbon to make intramolecular participation possible, the proton on the nitrogen is forced to face away from aspartate-52 and out of contact with any surface of the enzyme. When the 2-acetamido group is rotated around the C-2,N bond to bring the proton into contact with aspartate-52 where it may be abstracted, the carbonyl oxygen has been rotated approximately 180° away from the anomeric carbon and thus cannot give intramolecular participation.

The observation from the enzyme–substrate model that general base catalysis by aspartic acid residue 52 is not possible is of particular interest, in view of our findings that for intramolecular participation by the 2-acetamido group general base catalysis is unimportant.



Thus, the enzyme would not be expected to act as a general base catalyst if, as the model studies show, such catalysis would probably be ineffective and unnecessary.

Possible mechanisms of lysozyme catalysis involving three distinct intermediates have been considered: (a) protonated glycosyl bond fission leading to pyranosyl carbonium ion,[2b] (b) nucleophilic displacement by aspartate to give a covalently bound α-glycosyl–enzyme, and (c) intramolecular nucleophilic displacement by the 2-acetamido group to yield a protonated oxazolene. If general acid catalysis by glutamic acid-35 is involved in the enzyme mechanism as has been suggested,[2b] the following possible mechanisms may be written.

Rupley and Gates,[4] in examining transfer reactions catalyzed by lysozyme, have shown that retention of configuration of the β anomeric carbon occurs. The reaction of a possible glycosyl–enzyme intermediate (from eq 6b) or an oxazolene intermediate (from eq 6c) with water or other acceptor would lead of necessity to β product. Thus, the stereochemistry of the product for either eq 6b or 6c would be predetermined to be the β configuration by the double-displacement nature of the over-all reaction.

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The Reaction of Indolenine Salts with Nucleophiles

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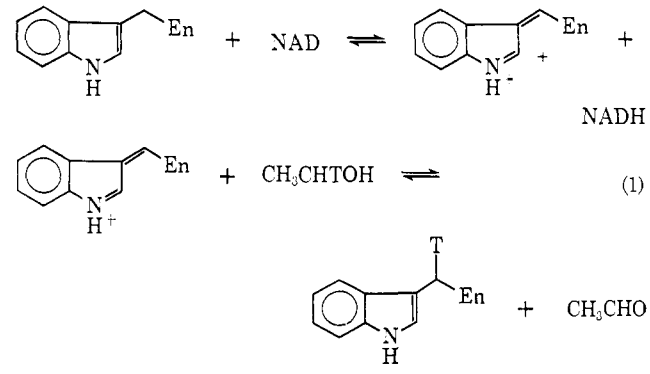
Abstract: The suggestion that an indolenine moiety may be an intermediate in the mechanism of action of the dehydrogenase enzymes has led to a study of the reduction of substituted phenylindolenine hydrosulfates by diethyl 2,6-dimethyl-1,4-dihydropyridyl-3,5-dicarboxylate (Hantzch ester). These reactions affording 3,5-dicarbethoxyl-2,6-dimethylpyridinium hydrosulfate and the corresponding 3-benzylindole as products were found to be first order with respect to each reactant with rate constants 500 times greater in acetonitrile than in ethanol. The extinction coefficient of the visible band of the indolenine salts undergoes a 100-fold parallel change in these solvents. These effects were interpreted as being due to tighter solvation of the indolenine salt by ethanol as compared to acetonitrile. The presence of intermediate charge-transfer complexes could not be determined because of the magnitude of the rate constants. The inclusion of radical inhibitors in the reaction solutions had no effect on the rate of the reaction. The reaction of the phenylindolenine salts with secondary amines was found to yield the corresponding adducts II (R = H), although the nmr spectrum of the imidazole adduct does not seem to be consistent with this structure. Kinetic studies of the reaction of the indolenine salts with secondary amines showed the reactions to be complex (*i.e.*, see Figures 3 and 4). In contrast the reaction of secondary amines with the phenyl-N-methylindolenine salt was simple first order with respect to each reactant with rate constants comparable to reduction by the Hantzch ester. The complexity of the reaction with the phenylindolenine salt is attributed to acid-base equilibria between the protonated indolenine (protonated imine) and the amine. By comparison of the rate constants for the reaction of the unprotonated phenylindolenine salt with aziridine and morpholine (*i.e.*, Scheme I (h)) with the constants for the reaction of the phenyl-N-methyl analog with these amines it is concluded that protonation increases the reaction rate 12000-fold. These phenomena are discussed with references to the dehydrogenase enzymes.

In 1965 Schellenberg[3a] reported that tritium was transferred to a tryptophan residue of yeast alcohol dehydrogenase during the enzymatic conversion of ethanol-1-^{3}H to acetaldehyde. Establishment of the position of the tritium label led to the postulation of (1) for the mechanism of action of yeast alcohol dehydrogenase. Analogous experiments with L-lactate and L-malate dehydrogenases gave similar results.[3b] Recently, Palm has shown that tritium labeling of yeast alcohol dehydrogenase takes place more slowly than does NADH formation and that A-NADT transfers tritium to the enzyme in the absence of acetaldehyde.[4] It



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(2) To whom inquiries should be directed.

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