Glycoside Hydrolysis. II. Intramolecular Carboxyl and Acetamido Group Catalysis in β-Glycoside Hydrolysis

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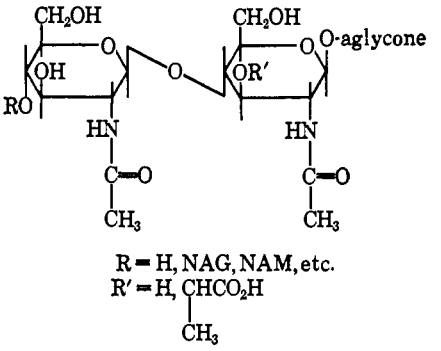
Abstract: The hydrolyses of *o*-carboxyphenyl β-D-glucopyranoside (*o*-CP-Gl) and *o*-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*o*-CP-NAG) have been studied at 78.2° (μ = 0.3) between pH 0.75 and 11.80. The pH–log k_{obsd} profiles for these compounds are characterized by a plateau rate in the acid region, followed by a descending leg of approximate slope of −1.0 above approximately pH 4. The plateau and descending leg are found to have kinetically equivalent interpretations as intramolecular carboxyl group participation in the hydrolysis, or specific acid catalyzed hydrolysis of the carboxylate anion form of the glycosides. The former interpretation has been shown to be correct on the basis that the log k_{rate} values for the calculated constants for specific acid catalyzed hydrolysis of the dissociated glycosides exhibit significant positive deviations from Hammett (σ) plots constructed from the rate constants for specific acid catalysis of the hydrolysis of glucosides not containing carboxyl groups. In addition, *o*-CP-NAG is found to have a plateau rate (k_c) significantly greater than *o*-CP-Gl. To account for this result, intramolecular 2-acetamido group participation as well as intramolecular carboxyl group participation is deduced. A mechanism for the hydrolysis of *o*-CP-NAG involving concerted intramolecular carboxyl group general acid and intramolecular acetamido group nucleophilic catalysis is proposed. By analogy, the spontaneous hydrolysis of *o*-CP-Gl is concluded to occur by intramolecular carboxyl group general acid catalysis. A previous study of the intramolecular acetamido group catalyzed hydrolysis of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides is extended to show that the rate of this reaction is dependent upon aglycone leaving group tendencies (*i.e.*, the Hammett ρ^- value is found to be +2.6). The activation parameters for the spontaneous hydrolyses of *o*-CP-Gl, *o*-CP-NAG, and *o*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside are determined. A value of $-T\Delta S^{\ddagger}$ for the hydrolysis of *o*-CP-NAG which is 2 kcal/mol larger than $-T\Delta S^{\ddagger}$ for the hydrolysis of *o*-CP-Gl is attributed to the necessity of properly orienting an additional catalytic group, the acetamido group, in the rate-determining transition state. The spontaneous rate of hydrolysis (k_c) of the model *o*-CP-NAG is found to be of similar magnitude to k_{cat} for the enzyme-catalyzed hydrolysis of a similar glycoside under comparable conditions. The relationship of these results to the mechanism of lysozyme is discussed.

The enzyme lysozyme, first recognized by Fleming[3] as being present in many tissues and secretions, is the first enzyme to have its tertiary structure determined by X-ray crystallographic methods.[4] Lysozyme has the ability to dissolve certain bacteria by catalytically hydrolyzing β-(1–4)-*N*-acetylmuramic acid–*N*-acetylglucosamine glycoside linkages in the carbohydrate polymer of bacterial cell walls.[5] Lysozyme has also been shown to hydrolyze β-(1–4)-linked oligomers of *N*-acetylglucosamine,[6] β-aryl glycosides of di-*N*-acetylchitobiose[7a,b] and tri-*N*-acetylchitotriose,[7c] and a β-linked benzyl glycoside of di-*N*-acetylchitobiose.[8] Thus, a generalized structure of all lysozyme substrates known to date is shown below (1). From chemical studies[6a] and X-ray diffraction studies of complexes of lysozme with several inhibitors having structures



(1)

analogous to the substrates,[4a,9] 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.

In a previous study on model substrates for lysozyme (*o*- and *p*-nitrophenyl 2-acetamido-2-deoxyglucopyranosides) we have shown that the 2-acetamido substituent of the substrate could provide stereospecific anchimeric assistance in the hydrolysis of the β-glycosidyl bond when nitrophenoxy and acetamido groups are in a *trans*-1,2 disposition, but not in the α-anomer where the configration is *cis*-1,2.[10] The model enzyme study reported here considers (a) the possibility of neighboring carboxyl group participation in the hydrolyses of car-

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(2) To whom inquiries should be directed.

(3) A. Fleming, *Proc. Roy. Soc.* (London), B93, 306 (1922).

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

(5) For a summary of relevant data and lead references see N. Sharon, *ibid.*, B167, 402 (1967).

(6) (a) J. A. Rupley and V. Gates, *Proc. Natl. Acad. Sci. U. S.*, 57, 496 (1967); (b) J. A. Rupley, *Proc. Roy. Soc.* (London), B167, 416 (1967).

(7) (a) T. Osawa, *Carbohydrate Res.*, 1, 435 (1966); (b) G. Lowe, G. Sheppard, M. L. Sinnott, and A. Williams, *Biochem. J.*, 504, 893 (1967); (c) T. Osawa and Y. Nakazawa, *Biochim. Biophys. Acta*, 130, 56 (1966).

(8) R. W. Jeanloz, 153rd National Meeting of the American Chemical Society, Miami Beach, Fla., 1967, Abstract C49.

(9) (a) L. N. Johnson and D. C. Phillips, *Nature*, 206, 761 (1965); (b) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc.* (London), B167, 378 (1967).

(10) D. Piszkiewicz and T. C. Bruice, *J. Am. Chem. Soc.*, 89, 6237 (1967).

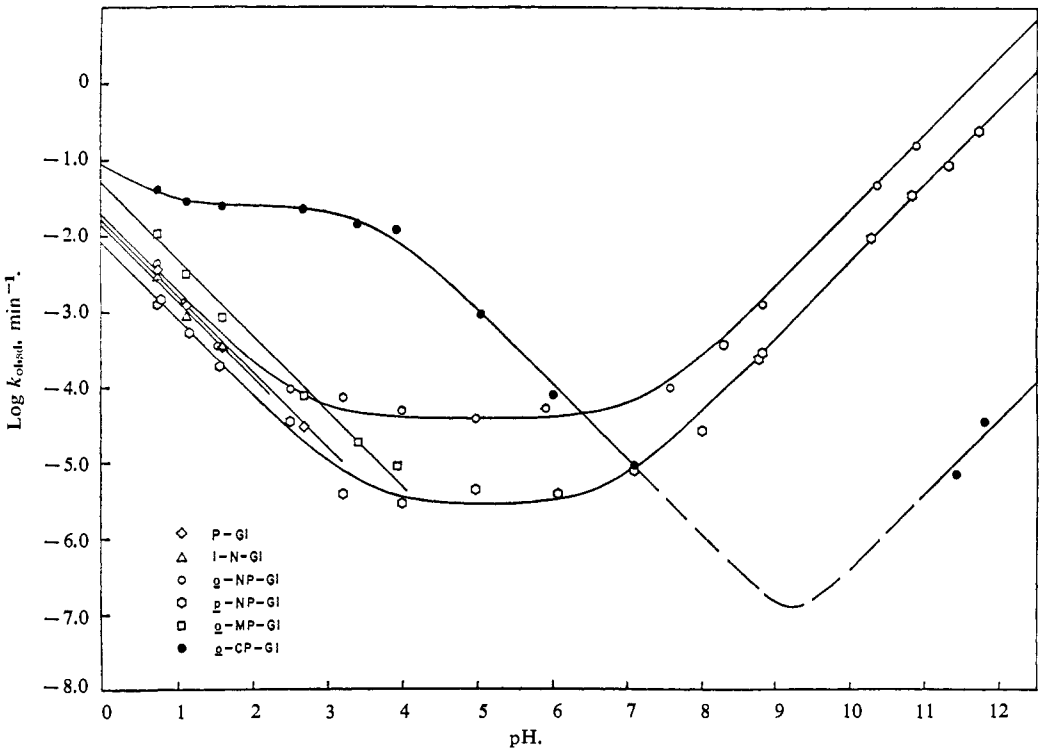


Figure 1. Spectrophotometrically determined pH–log k_{obsd} profiles for the hydrolyses of substituted phenyl β-D-glucopyranosides. Points are experimental, and the curves are calculated from the appropriate over-all rate expression 2 or 3 or 4 and the derived rate constants of Table II. Data for *o*-NP-Gl and *p*-NP-Gl is from ref 10.

boxy-substituted glycosides, and (b) the possibility of concerted intramolecular carboxyl and acetamido group catalysis in glycoside hydrolysis. *o*-Carboxyphenyl β-D-glucopyranoside has been reported to hydrolyze in the acid region with neighboring carboxyl participation,[11] but because of the preliminary nature of the original report[12] and the need to determine the rate constants at the temperature employed in our studies, the hydrolysis of this compound was reinvestigated in detail. In addition, the hydrolysis of *o*-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as a model for the lysozyme substrate complex was studied to determine if carboxyl and acetamido groups could act in a concerted fashion in a hydrolytic mechanism.

Experimental Section

Materials. Of the glycosides used in this study phenyl β-D-glucopyranoside (P-Gl), 1-naphthyl β-D-glucopyranoside (1-N-Gl), and 1-naphthyl 2-acetamido-2-deoxy-β-D-glucopyranoside (1-N-NAG) were purchased from Pierce Chemical Company. *o*-Methoxycarbonylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*o*-MP-NAG) and *o*-carboxyphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*o*-CP-NAG) were synthesized according to the procedures of Strachan, *et al.*,[13] and were generously supplied by them through the Merck Sharp and Dohme Research Laboratories, Rahway, N. J. The remaining glycosides were synthesized as described below.

***o*-Methoxycarbonylphenyl β-D-Glucopyranoside (*o*-MP-Gl).** Methyl salicylate was condensed with tetra-*O*-acetyl-α-D-glucopyranosidyl bromide (from Pierce Chemical Company) in the presence of silver oxide and pyridine according to the procedure of Robertson and Waters[14] to yield, after recrystallization from methanol, *o*-methoxycarbonylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (18% yield), mp 155–156°, $[\alpha]^{30}D$ −34.0° (chloroform) [lit. mp 159–160°;[14] mp 158–159°, $[\alpha]D$ −31.1° (chloroform);[15] mp 154°;[16] mp 160.5°, $[\alpha]^{21}D$ −29.3° (chloroform)[17]].

O-Deacylation of the tetraacetate was accomplished in methanolic sodium methoxide according to Helferich and Lutzmann.[17] After two recrystallizations from methanol, *o*-MP-Gl was obtained (61%), mp 105–106°, $[\alpha]^{30}D$ −64.5 (water) (lit.[17] mp 107°).

***o*-Carboxyphenyl β-D-Glucopyranoside, Sodium Salt Monohydrate (*o*-CP-Gl).** *o*-MP-Gl was added to an equimolar quantity of sodium hydroxide in a minimum volume of hot 95% ethanol in a procedure similar to that described by Fife.[18] The reaction mixture was allowed to cool to room temperature and let stand overnight. Chilling and the addition of anhydrous ether precipitated the sodium salt, which was recrystallized from absolute ethanol as the monohydrate (19%), mp 162–165° dec, $[\alpha]^{30}D$ −30.4° (water, pH 10.85).

Anal. Calcd for $C_{13}H_{17}O_9Na$: C, 45.89; H, 5.03. Found: C, 45.80; H, 5.39.

Phenyl 2-Acetamido-2-deoxy-β-D-glucopyranoside (P-NAG). A mixture of molten phenol and β-D-glucosamine pentaacetate with a trace of *p*-toluenesulfonic acid was heated at 100° under vacuum for 40 min according to the method of Weissmann.[19] The reaction mixture was taken up in chloroform, washed with 2 *M* sodium hydroxide and water, dried, evaporated to dryness, and recrystallized from 1-propanol to yield phenyl 3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (79%), mp 201–202°, transition 184°, unchanged by two recrystallizations, $[\alpha]^{30}D$ −12.5° (chloroform) [lit. mp 204.5–205°, transition at 187°, $[\alpha]^{23}D$ −12.0° (chloroform);[19] mp 201.5–205°, transition 185°, $[\alpha]^{18}D$ −10° (chloroform)[20]].

O-Deacylation in methanolic sodium methoxide[17] yielded P-NAG (75%), mp 246–247° dec, unchanged after two recrystallizations, $[\alpha]^{30}D$ −6.8° (H_2O) [lit. mp 250° dec, $[\alpha]^{23}D$ −5.5° (water);[19] mp 248–249°, $[\alpha]^{23}D$ −3° (water)[21]].

(11) B. Capon, *Tetrahedron Letters*, 911 (1963).

(12) For a discussion of this report[11] and a discussion of neighboring carboxyl group effect in glycoside, acetal, and ketal hydrolysis see T. C. Bruice and D. Piszkiewicz, *J. Am. Chem. Soc.*, **89**, 3568 (1967).

(13) R. G. Strachan, W. V. Ruyle, T. Y. Shen, and R. Hirschmann, *J. Org. Chem.*, **31**, 507 (1966).

(14) A. Robertson and R. B. Waters, *J. Chem. Soc.*, 1881 (1931).

(15) B. Capon, W. G. Overend, and M. Sobell, *ibid.*, 5172 (1961).

(16) P. Karrer and H. Weidmann, *Helv. Chim. Acta*, **3**, 252 (1920).

(17) B. Helferich and H. Lutzmann, *Ann.*, **537**, 11 (1939).

(18) T. H. Fife, *J. Am. Chem. Soc.*, **87**, 271 (1965).

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

(20) B. Helferich and A. Iloff, *Z. Physiol. Chem.*, **221**, 252 (1933).

(21) J. Findlay, G. A. Levvy, and C. A. Marsh, *Biochem. J.*, **69**, 467 (1958).

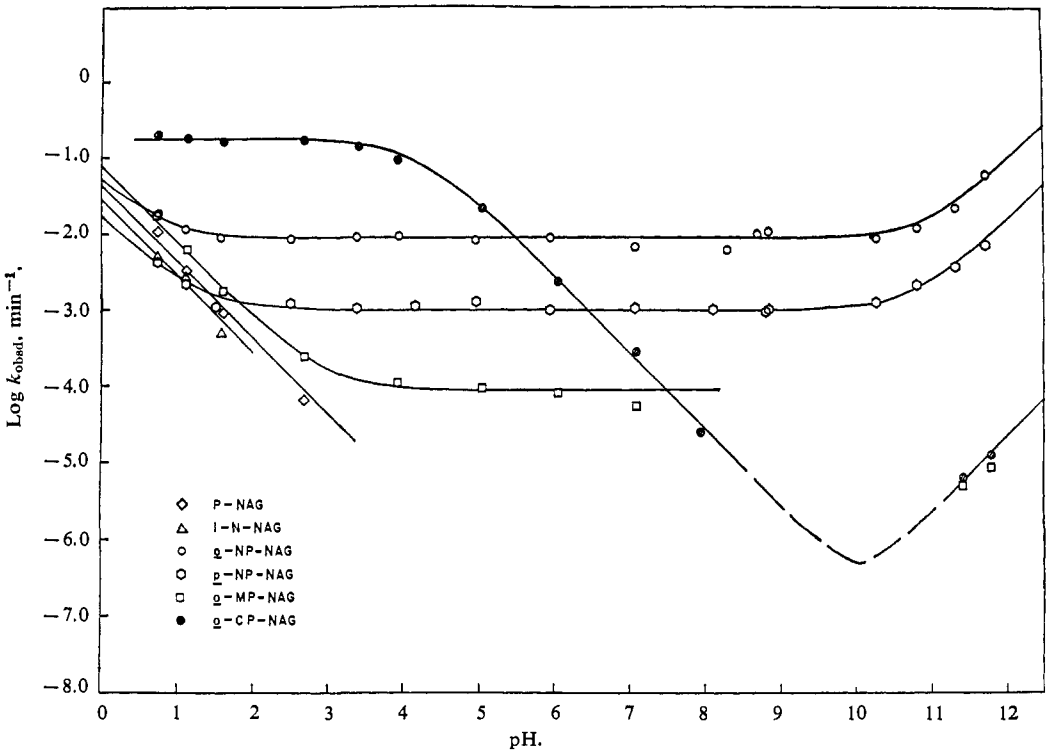


Figure 2. Spectrophotometrically determined pH–log k_{obsd} profiles for the hydrolyses of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides. Points are experimental, and the curves are calculated from the appropriate over-all rate expression 2 or 3 or 4 and the derived rate constants of Table II. Data for *o*-NP-NAG and *p*-NP-NAG is from ref 10.

Kinetics. All kinetic measurements were done at 78.2 ± 0.3° in aqueous buffers at $\mu = 0.3$ with KCl unless otherwise specified. Buffers employed were hydrochloric acid (pH 0.75–1.62), potassium formate (pH 2.70–3.42), potassium acetate (pH 3.94–6.06), potassium phosphate (pH 7.10–7.95), and potassium hydroxide (pH 11.42–11.80). With the exceptions of 1-N-Gl and 1-N-NAG where acid-catalyzed hydrolysis 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. Spectrophotometric rates were followed by observing substituted phenol formation from the corresponding glycoside at the isosbestic points of dissociated and undissociated forms. Thus, phenol formation was observed at 272 mμ, 1-naphthol at 304 mμ, methyl salicylate at 309 mμ, salicylic acid at 299 mμ below pH 7 and 297 mμ above pH 7, and *o*-nitrophenol at 372.6 mμ. The ultraviolet absorbance spectra of the product solutions of all glycosides hydrolyzed were determined at the various pH's employed (with the exceptions of *o*-CP-Gl and *o*-CP-NAG above pH 11, where reactions were followed to less than 10% completion). Hydrolysis product solutions for all glycosides which were scanned had absorbance spectra identical with the substituted phenols from which they were derived. The pseudo-first-order rate constants (k_{obsd}) were obtained by calculating the slope of plots of log $[(OD_\infty - OD_0)/(OD_\infty - OD_t)]$ *vs.* time (t) or by the method of Guggenheim.[22] All actual computations were carried out using an Olivetti-Underwood Programma 101 computer and programs were written by Dr. Donald Tanner, formerly of this laboratory.

Apparatus. The pH's of the buffer solutions employed in this study were determined at the same temperature employed for the kinetic studies by means of a Metrohm E type H high-temperature glass electrode, a Radiometer type TTT 1b autotitrator pH meter, and a previously described cell which was kept at constant temperature by refluxing 95% ethanol (78.2°) or refluxing acetone (56.2°).[23] Rates were followed using a Gilford 2000 recording spectrophotometer which was equipped with a cell holder thermostated at 78.2 ± 0.3, 56.2 ± 0.1, and 30.0 ± 0.1° by a Haake type NBe constant temperature bath.

Results

The pH–log rate profiles for the hydrolyses of substituted phenyl β-D-glucopyranosides are given in Figure 1; the pH–log rate profiles for the hydrolysis of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides are given in Figure 2. The structural formulas and abbreviations used for substituted phenyl β-D-glucopyranosides and substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides are provided in Table I.

Glycosides are generally hydrolyzed by a specific acid catalyzed mechanism.[24] Several substituted phenyl β-D-glucopyranosides were studied as control compounds (P-Gl, 1-N-Gl, *o*-MP-Gl, P-NAG, and 1-N-NAG), and log k_{obsd} was found to be a linear function of pH with slope of −1 (Figures 1 and 2), indicating that the mechanism of hydrolysis is specific acid catalysis. As determined in a previous study,[10] the hydrolysis of *o*-NP-Gl, *p*-NP-Gl, *o*-NP-NAG, and *p*-NP-NAG follow the general rate expression

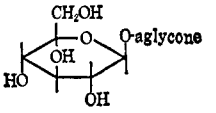
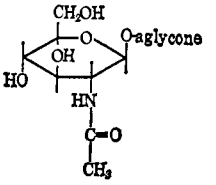
$$k_{obsd} = k_H a_H + k_{OH} K_w / a_H + k_0 \qquad (2)$$

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°[25]), and k_0 is the first-order rate constant for spontaneous hydrolysis. The hydrolysis of *o*-MP-NAG was also found to follow eq 2.

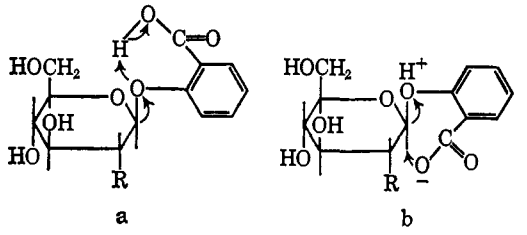
The object of this investigation is to determine: (a) if intramolecular carboxyl group catalysis [whether by gen-

(22) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).
(23) T. C. Bruice and F. H. Marquardt, *J. Am. Chem. Soc.*, **84**, 365 (1962).
(24) C. A. Bunton, T. A. Lewis, D. R. Llewellyn, and C. A. Vernon, *J. Chem. Soc.*, 4419 (1955).
(25) 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. Structures of Glycosides

Compound	Aglycone
Substituted Phenyl β-D-Glucopyranosides	
	
P-Gl	Phenyl
1-N-Gl	1-Naphthyl
o-NP-Gl	*o*-Nitrophenyl
p-NP-Gl	*p*-Nitrophenyl
o-MP-Gl	*o*-Methoxycarbonylphenyl
o-CP-Gl	*o*-Carboxyphenyl
Substituted Phenyl 2-Acetamido-2-deoxy-β-D-glucopyranosides	
	
P-NAG	Phenyl
1-N-NAG	1-Naphthyl
o-NP-NAG	*o*-Nitrophenyl
p-NP-NAG	*p*-Nitrophenyl
o-MP-NAG	*o*-Methoxycarbonylphenyl
o-CP-NAG	*o*-Carboxyphenyl

eral acid catalysis or nucleophilic displacement by carboxylate anion on the protonated glycoside linkage (3)] takes place; and (b) if the 2-acetamido group can make



(3)

a b

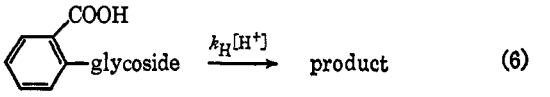
R = OH, $NHCOCH_3$

contribution in concert with the carboxyl group to the rate of hydrolysis of *o*-CP-NAG. The pH–log rate profiles for the hydrolyses of *o*-CP-Gl and *o*-CP-NAG may be derived from the kinetically equivalent eq 4 and 5,

$$k_{obsd} = k_H a_H\left[\frac{a_H}{K_{app} + a_H}\right] + k_c\left[\frac{a_H}{K_{app} + a_H}\right] + k_{OH}\frac{K_w}{a_H} \quad (4)$$

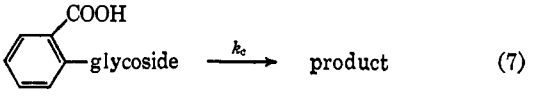
$$k_{obsd} = k_H a_H\left[\frac{a_H}{K_{app} + a_H}\right] + k_b a_H\left[\frac{K_{app}}{K_{app} + a_H}\right] + k_{OH}\frac{K_w}{a_H} \quad (5)$$

where $k_c = k_b K_{app}$. In (4) and (5) the constant K_{app} is the kinetically apparent acid dissociation constant of the carboxyl groups obtained from the best fit of the equations to the pH–log k_{obsd} profile; a_H is the observed hydrogen ion activity as measured by the glass electrode. The rate constants of (4) and (5) may be interpreted in the following manner: (a) k_H is the second-order rate constant for specific acid catalyzed hydrolysis of the undissociated carboxyl form of the glycoside (*o*-CP-Gl or *o*-CP-NAG)



(6)

(b) k_c is the first-order rate constant for the spontaneous hydrolysis of glycoside with an undissociated carboxyl group



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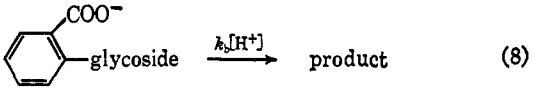
and (c) k_b is the second-order rate constant for specific acid catalyzed hydrolysis of glycoside with a dissociated carboxyl group.



(8)

An alternate interpretation of k_b (and, hence eq 8) would be intramolecular nucleophilic displacement by carboxylate anion on the protonated glycoside linakage (3a).

In the pH–log k_{obsd} profiles for the hydrolyses of substituted phenyl β-D-glucopyranosides (Figure 1) and substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (Figure 2), the points are experimental and the curves constructed from the most satisfactory solution of the appropriate over-all rate expression, eq 2 or eq 4 (or 5). For derived rate constants see Table II.

To evaluate the significance of changes in electronic effects with dissociation of carboxyl groups on the hydrolyses of *o*-CP-Gl and *o*-CP-NAG, Hammett plots were constructed using the equation log k_H or $k_b = \rho\sigma + C$.[26] If k_H and k_b pertain to the same mechanism of hydrolysis, specific acid catalysis, an essentially linear plot would be expected. If, however, intramolecular carboxyl group catalysis were contributing significantly to the rates of hydrolysis of *o*-CP-Gl and *o*-CP-NAG, the points corresponding to k_b for ionized carboxyl substituted glycosides would be expected to show a positive deviation from the Hammett plot. For the specific acid catalyzed hydrolyses of substituted phenyl β-D-glucopyranosides (Figure 3) all k_H values congregate about a line ($\rho = -0.06$) with k_b for *o*-CP-Gl (dissociated) being the only point to show a significant positive deviation. The point corresponding to k_b for *o*-CP-Gl (dissociated) falls 3.77 log units above a least-squares line calculated from the values of k_H, and corresponds to a rate acceleration at pH's above the pK_{app} of *o*-CP-Gl of 5.9×10^3-fold. Thus, neighboring carboxyl group catalysis in the hydrolysis of *o*-CP-Gl is confirmed[11] to be one of the kinetically equivalent mechanisms of intramolecular general acid catalysis or intramolecular nucleophilic displacement by carboxylate anion on the protonated glycoside linkage (3).

The Hammett plot for k_H and k_b values of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (Figure 4) again shows all k_H values congregated about a line ($\rho = -0.11$) with k_b for *o*-CP-NAG (dissociated) falling 4.91 log units above the least-squares line for the

(26) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p 184.

Table II. Kinetic Constants for the Hydrolyses of Substituted Phenyl β-D-Glucopyranosides and Substituted Phenyl 2-Acetamido-2-deoxy-β-D-glucopyranosides (78.2°; H_2O; 0.3 μ with KCl)

Compound	σ[b]	k_H, M^{-1} min^{-1} × 10^2	k_{OH}, M^{-1} min^{-1}	k_0, min^{-1}	$k_c = k_b K_{app}$, min^{-1} × 10	$k_b = k_c/K_{app}$, M^{-1} min^{-1} × 10^{-2}	K_{app}, M
P-Gl	0.00	1.66	...	...	...	. .	...
1-N-Gl	0.04	1.41	...	...	...	...	...
o-NP-Gl[a]	0.78	2.00	9.33	4×10^{-5} [d]	...	...	...
p-NP-Gl[a]	0.78	0.832	1.99	3×10^{-6} [d]	...	...	. .
o-MP-Gl	0.45	5.01	...	...	...	...	...
o-CP-Gl							
(undissociated)	0.45	6.3	...	...	0.251	...	2.24×10^{-4}
(dissociated)	0.00	...	1.59×10^{-4}	...	...	1.12	(pK_{app} = 3.65)
P-NAG	0.00	4.47	...	...	...	...	...
1-N-NAG	0.04	2.95	...	...	...	...	...
o-NP-NAG[a]	0.78	4.47	3.80×10^{-1}	8.72×10^{-3}	...	...	...
p-NP-NAG[a]	0.78	1.78	6.60×10^{-2}	1.03×10^{-3}	...	...	...
o-MP-NAG	0.45	7.94	10^{-4} [c,d]	8.91×10^{-5}	...	...	...
o-CP-NAG							
(undissociated)	0.45	...	...	...	1.78	...	6.31×10^{-5}
(dissociated)	0.00	...	10^{-4} [d]	...	...	34.7	(pK_{app} = 4.2)

[a] Data for these compounds was originally presented in ref 10. [b] σ values from D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23**, 420 (1958). Values for *ortho* substituents were considered to be the same as for *para* substituents. The σ value for CO_2CH_3 was considered as identical with that given for $CO_2C_2H_5$. [c] The value of k_{OH} for o-MP-NAG is approximately equal to k_{OH} for o-CP-NAG when followed at the same wavelength (297 mμ). This is probably due to rapid hydrolysis of the methyl ester in base, followed by slow specific base catalyzed hydrolysis of glycoside linkage. [d] Not known with great accuracy.

values of k_H. This corresponds to a rate acceleration at pH's above the pK_{app} of o-CP-NAG of 8.1 × 10^4-fold. Thus, intramolecular carboxyl group catalysis in the hydrolysis of o-CP-NAG is established also. Since σ_I values for the 2-hydroxyl (+0.25) and 2-acetamido substituents (+0.28) are similar,[27] o-CP-Gl and o-CP-NAG have similar electronic properties, and a direct comparison of the accelerations in their rates of hydrolysis may be made. Thus, o-CP-NAG has an acceleration in $k_b = k_c/K_{app}$ which is 14 times greater than that for o-CP-Gl. In addition k_c for o-CP-NAG is 7.1 times greater than k_c for o-CP-Gl (see Table II). A reasonable conclusion which may be drawn from the relatively rapid rate of hydrolysis of o-CP-NAG in comparison to o-CP-Gl in the acid region is that o-CP-Gl hydrolyzes with neighboring carboxyl group catalysis and o-CP-NAG hydrolyzes with concerted intramolecular acetamido and carboxyl group catalysis.

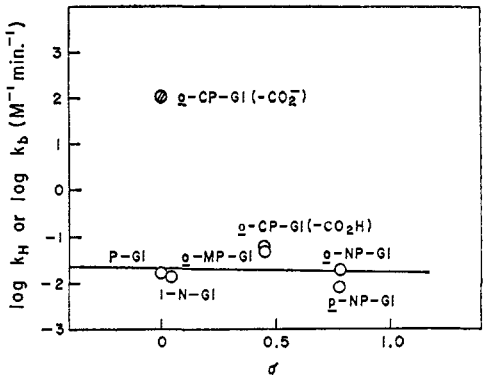


Figure 3. Hammett plot for the specific acid catalyzed hydrolyses (k_H or k_b) of substituted phenyl β-D-glucopyranosides. Values of all constants employed are provided in Table II.

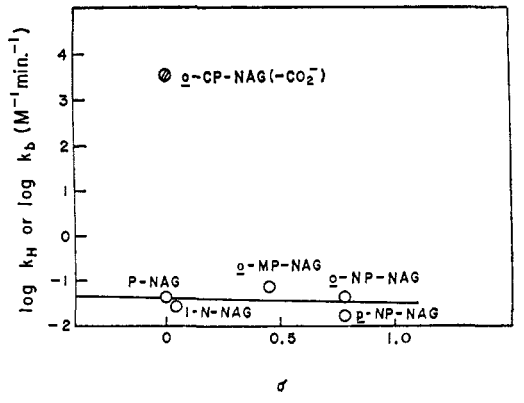


Figure 4. Hammett plot for the specific acid catalyzed hydrolyses (k_H or k_b) of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides. Values of all constants employed are provided in Table II.

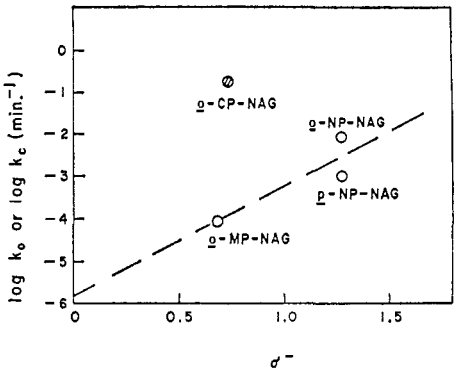


Figure 5. Hammett plot (σ^-) for the spontaneous hydrolyses (k_0 or k_c) of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides. Values of k_0 and k_c employed are provided in Table II.

To evaluate the significance of electronic effects on the rate of liberation of aglycone in the spontaneous hydrolyses of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (k_0 in eq 2), a partial Hammett plot was again constructed (Figure 5). Since the substit-

(27) M. Charton, *J. Org. Chem.*, **29**, 1222 (1964).

uents used are capable of resonance-electron withdrawal which would stabilize the leaving group (substituted phenolate ion) σ^- values were employed.[28] As seen from Figure 5 the rate of spontaneous hydrolysis of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides (k_0) is very dependent upon the electron-withdrawing nature of the leaving group. The plot has a ρ^- value of +2.6. A positive value of approximately this magnitude would be expected in a reaction mechanism involving intramolecular nucleophilic attack by a neighboring acetamido group as proposed previously.[10] Similar values of ρ are observed for the inter- and intramolecular nucleophilic attack of amines on *meta*- and *para*-substituted phenyl acetates.[29]

When plotted on the same graph (Figure 5) k_c (from eq 3 and 7), corresponding to the plateau rate of hydrolysis of undissociated *o*-CP-NAG, falls approximately four orders of magnitude above the line. Thus, *o*-CP-NAG has a spontaneous rate of hydrolysis (k_c) greater than would be expected on the basis of 2-acetamido participation alone. Once again, intramolecular carboxyl group catalysis must be invoked to account for this increased rate of hydrolysis.

The spontaneous rates of hydrolysis of *o*-CP-Gl (k_c), *o*-CP-NAG (k_c), and *o*-NP-NAG (k_0) were determined at several temperatures for calculation of the activation parameters of these reactions. These rates and the calculated values of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ are given Table III. It is interesting to note that as the temperature is decreased, the ratio of k_c for *o*-CP-NAG to k_c for *o*-CP-Gl increases. Thus, *o*-CP-NAG hydrolyses 7.1-fold faster than *o*-CP-Gl at 78.2° and 16.5-fold faster at 30°.

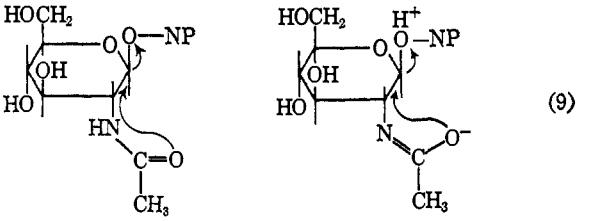
Table III. Activation Parameters for Spontaneous Hydrolyses of Glycosides (Solvent, H_2O; μ = 0.3)

Compd	pH	T, °C	k_c or k_0, min^{-1}	$\Delta H^{\ddagger}$,[a] kcal/mol	$T\Delta S^{\ddagger}$,[a] kcal/mol
o-CP-Gl	2.62	78.2	2.51×10^{-2}		
		56.2	1.14×10^{-3}		
		30.0	3.19×10^{-5}	28.4	1.9
o-CP-NAG	2.62	78.2	1.78×10^{-1}		
		56.2	2.12×10^{-2}		
		30.0	5.26×10^{-4}	24.6	0.0
o-NP-NAG	7.05	78.2	8.72×10^{-3}		
		56.2	6.26×10^{-4}		
		30.0	1.42×10^{-5}	27.0	0.2

[a] Calculated from $\Delta H^{\ddagger} = E_a - RT$, $-T\Delta S^{\ddagger} = \Delta F^{\ddagger} - \Delta H^{\ddagger}$, and $\Delta F^{\ddagger} = RT2.303 \log (KT/hk_r)$ (A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms," John Wiley and Sons, Inc., New York, N. Y., 1953, pp 95–97); standard state used was T = 25°, with time in seconds.

Discussion

The β anomers *o*-NP-NAG and *p*-NP-NAG, where acetamido and nitrophenoxy groups are in a *trans*-1,2 disposition, hydrolyze with a spontaneous rate (k_0) whereas their α anomers, where acetamido and nitrophenoxy groups are in a *cis*-1,2 disposition, exhibit only specific acid (k_H) and specific base (k_{OH}) catalyzed hydrolysis.[10] This study has shown that the β anomer of *o*-MP-NAG also shows a plateau rate of hydrolysis (k_0), though of lesser magnitude. In our previous study[10] the spontaneous rate constants (k_0) for the β anomers were attributed to stereospecific anchimeric participation by the 2-acetamido group. For 2-acetamido group participation the spontaneous rate of hydrolysis (k_0) was found to be insensitive to the concentrations of buffers employed and did not exhibit a significant deuterium solvent kinetic isotope effect. Two kinetically equivalent mechanisms were offered (9): (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 was considered the most reasonable on the basis of the calculated magnitude of the rate constant for b.



(9)

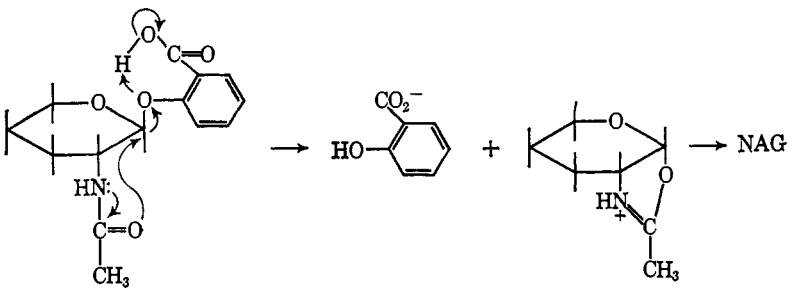
This study has show that *o*-CP-Gl and *o*-CP-NAG have pH–log k_{obsd} profiles that show a dependence on groups with pK_{app}'s of 3.65 and 4.2, respectively. In addition, the rates of hydrolysis of *o*-CP-Gl and *o*-CP-NAG below their pK_{app}'s were 5.9×10^3 and 8.1×10^4 times faster than would be anticipated for specific acid catalyzed hydrolyses of compounds of their electronic properties. The only reasonable explanation for these rate accelerations is intramolecular carboxyl group catalysis. Two kinetically equivalent mechanisms can be proposed: (a) intramolecular general acid catalysis (eq 7), and (b) intramolecular nucleophilic displacement by the carboxylate anion on the protonated glycoside linkage (as expressed by eq 8). These possibilities may be shown pictorially as eq 3a and 3b, respectively. Since kinetic equations do not stipulate the position of the proton in the transition state (*i.e.*, [glycoside-CO_2H] is kinetically equivalent to [glycoside-CO_2^-][H^+]), a choice between eq 3a and 3b may not be made on a kinetic basis.

The relatively greater rate of hydrolysis of *o*-CP-NAG over *o*-CP-Gl in the acid region suggests intramolecular acetamido as well as carboxyl group catalysis in its hydrolysis. We have shown previously[10] that the 2-acetamido group acts in the un-ionized form as a nucleophile in displacing the aglycone. Thus, the carboxyl group of *o*-CP-NAG must act as other than a nucleophile. The most reasonable mechanism for the hydrolysis of *o*-CP-NAG in the acid region involves concerted intramolecular acetamido nucleophilic and carboxyl group general acid participation, *viz.* eq 10. This mechanism would lead to a protonated oxazoline intermediate, which would rapidly hydrolyze.[30] Also, by analogy, the rapid hydrolysis of *o*-CP-Gl in the acid

(28) J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 88.

(29) T. C. Bruice and S. J. Benkovic, *J. Am. Chem. Soc.*, **86**, 418 (1964).

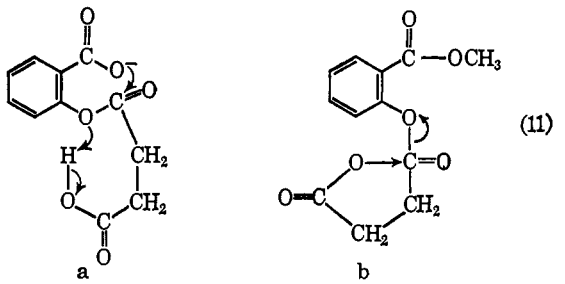
(30) It should be noted that in the hydrolysis of *o*-NP-NAG[10] no formation of the oxazoline intermediate could be detected polarimetrically. In addition, the structurally analogous compound 2-methyloxazoline has been shown to hydrolyze rapidly in the acid region at 25° (R. B. Martin and A. Parcell, *J. Am. Chem. Soc.*, **83**, 4835 (1961)). Thus in the hydrolysis of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides at 78.2°, no oxazoline intermediate would be expected to be found if formed.



(10)

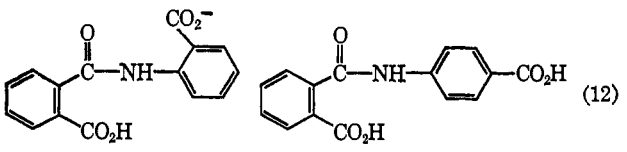
region could be explained on the basis of neighboring general acid catalysis.

This study has shown that nieghboring carboxyl group participation in the hydrolysis of *o*-CP-Gl gives an apparent rate acceleration in $k_b = k_c/K_{app}$ of approximately 6×10^3 above that which would be expected for specific acid catalyzed hydrolysis (Figure 3). In addition, intramolecular nucleophilic attack by the 2-acetamido group in the spontaneous hydrolyses of *o*-NP-NAG and *p*-NP-NAG may give rate accelerations of 218- and 344-fold over the similar spontaneous rates k_0) for hydrolyses of *o*-NP-Gl and *p*-NP-Gl, respectively.[10] Yet, when the possibility of intramolecular 2-acetamido participation is added to intramolecular carboxyl participation in the hydrolyses of *o*-CP-NAG, a rate acceleration in k_c of only 7.1-fold above that for *o*-CP-Gl is observed (at 78.2°). Clearly, the rate accelerations attributed to intramolecular carboxyl and intramolecular 2-acetamido group participation in the hydrolysis of *o*-CP-NAG are less than additive. Similar rate accelerations by the participation of two neighboring carboxyl groups over one carboxyl group have been reported previously. In the hydrolysis of monoionized succinyl salicylate (11a) the participation of two carboxyl groups causes a rate acceleration of 66-fold above the rate of hydrolysis of dissociated succinyl methyl salicylate (11b) where only one carboxylate group participates.[31] Also, in the hydrolysis of monoionized



(11)

o-carboxyphthalanilic acid (12a) participation of two carboxyl groups causes it to be hydrolyzed approximately 40-fold faster than its *para* isomer 12b where only one carboxyl group may give intramolecular catalysis.[32]



(12)

The question may be raised as to why the rate accelerations attributed to intramolecular carboxyl and intramolecular acetamido group participation in the hydrolysis of *o*-CP-NAG are less than additive. A solution to this problem may be found by examining the activation parameters for the spontaneous hydrolyses of several glycosides (Table III). Inspection of Table III reveals that though the value of $\Delta H^{\pm}$ for k_c for *o*-CP-NAG is less by *ca.* 4 kcal/mole than for *o*-CP-Gl, the value of $-T\Delta S^{\pm}$ is greater by *ca.* 2 kcal/mol. The most reasonable explanation for these findings is obtained by considering $\Delta H^{\pm}$ a measure of potential energy and $T\Delta S^{\pm}$ a measure of kinetic energy changes on reaching the transition state. This is, of course, a common but not totally warranted assumption since $\Delta H^{\pm}$ possesses both potential and kinetic energy terms, whereas $\Delta S^{\pm}$ is composed of only kinetic energy terms.[33] With this assumption, it follows that the bringing of an additional catalytic group into the transition state should lower the potential energy barrier ($\Delta H^{\pm}$), but that the necessity to orient this catalytic group should increase the kinetic energy barrier ($-T\Delta S^{\pm}$). This is what is observed.[29] The cost in kinetic energy actually amounts to one-half the decrease in $\Delta F^{\pm}$ obtained by the change in mechanism brought about by participation of the second functional group. In effect, the decrease in $T\Delta S^{\pm}$ necessary to orient the second functional group is only one-half of that predicted if the second functional group were part of another molecule and participated through a bimolecular reaction (*i.e.*, translational entropy for a single species).[34]

If carboxyl and acetamido groups could be oriented in the model compound, *o*-CP-NAG, as possible at the active site of lysozyme, $-T\Delta S^{\pm}$ would be lowered to approximately that of *o*-CP-Gl. A minimum theoretical value of $\Delta F^{\pm} = 22.7$ kcal/mol for k_c for *o*-CP-NAG could then be calculated using the observed values of $\Delta H^{\pm}$ for *o*-CP-NAG and $T\Delta S^{\pm}$ for *o*-CP-Gl (25°). Further, a maximum possible value for k_c could be calculated for *o*-CP-NAG when both carboxyl and acetamido groups were properly oriented. Thus, at 25°, a maximum value of $k_c = 8.5 \times 10^{-3}$ min^{-1} would be calculated, which is 26-fold greater than the actual value of k_c at 25°, $k_c = 3.31 \times 10^{-4}$ min^{-1} (extrapolated from an Arrhenius plot, not shown).

The dependence of $T\Delta S^{\pm}$ on the electronic nature of the substituent on the aglycone (Table III) is not unanticipated. Previous studies on neighboring carboxyl[35] and dimethylamino group[36] participation in both bimolecular and intramolecular displacement reactions on sub-

(31) H. Morawetz and I. Oreskes, *J. Am. Chem. Soc.*, **80**, 2591 (1958).
(32) H. Morawetz and J. Shafer, *ibid.*, 84, 3783 (1962).
(33) R. W. Taft in "Steric Effects in Organic Chemistry," M. S. Newman, Ed., John Wiley and Sons, Inc., New York, N. Y., 1956, pp 556–675.
(34) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1966, p 123.
(35) E. Gaetjens and H. Morawetz, *J. Am. Chem. Soc.*, **82**, 5328 (1960).
(36) T. C. Bruice and S. J. Benkovic, *ibid.*, **85**, 1 (1963).

stituted phenyl esters have shown that $T\Delta S^{\mp}$ is dependent on the electronic nature of the leaving phenolic moiety.

Recently, Lowe, *et al.*,[7b] have reported a detailed kinetic analysis of the lysozyme-catalyzed hydrolyses of several β-aryl di-*N*-acetylchitobiosides. The Michaelis constants, K_m, for these compounds were found to be almost independent of the aglycone, whereas the catalytic constants (k_{cat}) showed a marked dependence, giving a Hammett reaction constant ρ^- equal to +1.2. This study has shown that the spontaneous hydrolyses of substituted phenyl 2-acetamido-2-deoxy-β-D-glucopyranosides, which involves nucleophilic participation by the acetamido group, gives a Hammett plot with ρ^- of approximately +2.6. Both enzymatic and nonenzymatic hydrolyses show a dependence on leaving group ability of the aglycone, though of different magnitudes. Thus, it seems likely that the enzymatic reaction, like the nonenzymatic reaction, may involve catalytic nucleophilic participation by a neighboring group, either a carboxylate at the active site or the acetamido group itself.

The relative efficiencies of the model enzyme–substrate complexes *o*-CP-Gl and *o*-CP-NAG may be determined by comparing their spontaneous rates of hydrolysis (k_c) to the determined[7b] catalytic rates (k_{cat}) for the hydrolyses of β-aryl di-*N*-acetylchitobioses in the lysozyme–substrate complex. Such a comparison would be based on the reasonable assumption that the difference in substituents at the 4 position of the model compounds (–OH) and enzyme substrates (NAG–) would have negligible steric and inductive effects on the rates of nonenzymatic and enzymatic hydrolysis. A glycoside with an aglycone of the same electronic properties as the models (*i.e.*, having the same σ^- value as the *o*-carboxyphenyl group) would hydrolyze with a value of $k_{cat} = 1.2 \times 10^{-4}$ min^{-1} at 35° (interpolated from the Hammett plot presented by Lowe, *et al.*[7b]). The model compound *o*-CP-Gl, which hydrolyzes with intramolecular carboxyl group catalysis, has a value of $k_c = 6.0 \times 10^{-5}$ min^{-1} at 35° (interpolated from an Arrhenius plot of log k_c *vs.* $1/T$°K, not shown). Thus, k_c for the model *o*-CP-Gl and k_{cat} for a substrate of similar electronic nature are of similar magnitude. It should be noted, however, that *o*-CP-Gl with a pK_{app} of 3.65 has a substantially more acidic carboxylic acid group than does lysozyme, which has at the active site glutamic acid 35 with a pK_a of approximately 6.5.[37] If the carboxyl group of *o*-CP-Gl had a less acidic carboxyl with a pK_a similar to that of the enzyme, the value of k_c for *o*-CP-Gl would be proportionally lowered to a value dependent on the Brønsted β for this process. It would appear unlikely, therefore, that carboxyl group general acid catalysis *alone* could account for the catalytic efficiency of lysozyme. In the hydrolysis of *o*-CP-NAG, where both intramolecular general acid and acetamido group catalyses are present, a value of $k_c = 1.15 \times 10^{-3}$ min^{-1} at 35° is obtained (interpolated from an Arrhenius plot of log k_c *vs.* $1/T$°K, not shown). This value of k_c is an order of magnitude above k_{cat} for the enzyme-catalyzed hydrolysis of a substrate of similar electronic properties. Again, the model enzyme has a more strongly acidic carboxyl group ($pK_{app} = 4.2$) than does the enzyme. If the spontaneous hydrolysis process described by k_c had *any* reasonable β value (0.0 to 0.8), compensation for the difference in pK_a's of *o*-CP-NAG and lysozyme would give a value of k_c for the model within an order of magnitude of k_{cat} for the enzymatic reaction. As a first approximation, then, it appears that a hydrolytic mechanism involving concerted carboxyl and acetamido participation, as in the hydrolysis of *o*-CP-NAG, could possibly explain the catalytic efficiency of lysozyme.

It should be noted that the β-aryl di-*N*-acetylchitobiosides[7b] used in the above comparison are much less readily hydrolyzed than *N*-acetylglucosamine oligomers and cell wall oligosaccharides. The hexamer of *N*-acetylglucosamine shows a turnover number (k_{cat}) of at least 15 min^{-1}.[6a] Cell wall oligosaccharides have been estimated to hydrolyze with a turnover number (k_{cat}) of similar magnitude.[6a] This fact may be due to the difference in chemical natures of the substrates, more nonproductive binding of the smaller β-aryl di-*N*-acetylchitobiosides, or poor orientation of the β-aryl di-*N*-acetylchitobiosides in the productive enzyme–substrate complex. Therefore, the values k_{cat} for the hydrolyses of β-aryl di-*N*-acetylchitobioses by lysozyme may give an erroneously low measure of the enzyme's catalytic ability, and the comparison of the model's k_c and enzyme's k_{cat} values should be judged accordingly.

Acknowledgments. This work was supported by a grant from the National Institutes of Health. We wish to thank Drs. L. H. Sarett, R. G. Strachan, W. V. Ruyle, T. Y. Shen, and R. Hirschmann of the Merck Sharp and Dohme Research Laboratories, Rahway, N. J., for a generous gift of *o*-MP-NAG and *o*-CP-NAG.

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