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Effect of Substituents on the Hydrolysis of Substituted Phenyl β-Acetylglucosaminides by Bovine Liver β-Acetylglucosaminidase

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Michaelis constant, Km and relative catalytic constant, k_{cat}^{rel} were determined for the β-acetylglucosaminidase-catalyzed hydrolysis of a series of substituted phenyl β-acetylglucosaminides; the substituents are 2,4-dinitro, *p*-nitro, 4-nitro-3-methyl, *m*-nitro, *p*-chloro, *m*-chloro, *p*-methyl, *m*-methyl, *p*-methoxy and *p*-hydroxyl groups. The values were evaluated in terms of the ρ-σ relationship of Hammett.

Plots of log Km with respect to Hammett substituent constant, σ and Hansch substituent constant, π showed the enzyme-substrate affinity to be dependent on the electronic nature of the substituents ($\rho=-0.42$, correlation coefficient $r=0.943$) but not on the hydrophobic nature.

The log k_{cat}^{rel} value was only slightly dependent on σ value ($\rho=+0.16$, $r=0.626$). An experiment on nucleophilic competition with methanol was carried out in an attempt to explain the small ρ value for k_{cat}^{rel}. Methanol competes with water for the glycosyl enzyme to some extent but does not increase k_{cat}^{rel} for the glycosides examined, indicating that the deglycosylation is not rate-limiting and hence the ρ value for k_{cat}^{rel} pertains to the reaction of glycoside bond cleavage.

The electronic effect of substituents on the enzymatic reaction of a series of *p*- and *m*-substituted phenyl compounds have been studied in terms of a ρ–σ relationship of Hammett to elucidate the nature of enzymatic reaction. Emulsin β-glucosidase,[2] yeast α-glucosidase,[3] Taka-amylase,[4] *E. coli* β-galactosidase,[5] β-glucuronidase[6] and lysozyme[7] are glycoside hydrolases so far studied in this line.

Leaback[8] briefly described the substituent effect on the hydrolysis catalyzed by pig epididymal β-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30), although the number of the substituents examined was limited. The present paper deals with a similar study on the hydrolysis of a more extensive series of substituted phenyl β-acetylglucosaminides by bovine liver β-acetylglucosaminidase, and also with a preliminary experiment on nucleophilic competition with methanol carried out in order to interpret the ρ–σ relationship obtained.

Materials and Methods

Preparation of Substrates——Phenyl, *p*-nitrophenyl[9], *p*- and *m*-chlorophenyl, *p*- and *m*-tolyl, and *p*-methoxyphenyl[10] β-acetylglucosaminide were prepared by deacetylation of the corresponding acetates with sodium methoxide. The 2,4-dinitrophenyl glycoside was prepared by deacetylation of the acetate with

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hydrogen chloride–methanol–chloroform mixture.[11] The *m*-nitrophenyl, 4-nitro-3-methylphenyl and *p*-hydroxyphenyl glycoside were synthesized by employing the procedure of Leaback:[9] the properties of these glycosides were as follows:[12] *m*-Nitrophenyl glycoside, mp 186° (from ethanol–water), $[\alpha]_D^{20}$ −7.5° (c=0.2, water). *Anal.* Calcd. for $C_{14}H_{18}O_8N_2 \cdot 3/4H_2O$: C, 47.3; H, 5.5; N, 7.9. Found: C, 46.9; H, 5.0; N, 7.3. The tetraacetate crystallized from methanol, mp 222°, $[\alpha]_D^{20}$ −17.5° (c=1.0, chloroform). *Anal.* Calcd. for $C_{20}H_{24}O_{11}N_2$: C, 51.3; H, 5.2; N, 6.0. Found: C, 50.8; H, 5.1; N, 6.5. 4-Nitro-3-methylphenyl glycoside, mp 203° (from water), $[\alpha]_D^{20}$ −7.5° (c=0.4, water). *Anal.* Calcd. for $C_{15}H_{20}O_8N_2 \cdot 1/3H_2O$: C, 49.7; H, 5.7; N, 7.7. Found: C, 49.4; H, 5.2; N, 7.4. The tetraacetate crystallized from ethanol, mp 191° $[\alpha]_D^{20}$ −21° (c=1.0, chloroform). *Anal.* Calcd. for $C_{21}H_{26}O_{11}N_2$: C, 52.3; H, 5.4; N, 5.8. Found: C, 52.2; H, 5.4; N, 5.8. *p*-Hydroxyphenyl glycoside, mp 222° (from ethanol), $[\alpha]_D^{20}$ 0° (c=0.5, water). *Anal.* Calcd. for $C_{14}H_{19}O_7N$: C, 53.7; H, 6.1; N, 4.5. Found: C, 53.4; H, 6.0; N, 4.4. The tetraacetate was synthesized in a nitrogen atmosphere and crystallized from methanol–ether, mp 196°, $[\alpha]_D^{20}$ −11° (c=0.5, methanol). *Anal.* Calcd. for $C_{20}H_{25}O_{10}N$: C, 54.7; H, 5.7; N, 3.2. Found: C, 54.9; H, 5.7; N, 3.2. The free glycoside was obtained by deacetylation in a mixture of aqueous sodium hydroxide solution and acetone (1: 1) and by subsequent treatment with Amberlite IR 120 (H^+ form).

Enzyme——The partially purified enzyme was prepared by the method of Langley.[13] The fraction retained in the DEAE-cellulose column, "DEA II" as designated by Langley, was stored at pH 4.2 (0.01M citrate) in a frozen state. The enzyme (specific activity 9—10 μmoles/min/mg at 37°) prepared as above was stable in this form at least for six months.

Kinetic Measurements——A mixture (0.5 ml) containing a glycoside, enzyme, 0.01% bovine serum albumin and 0.05M citric acid–sodium citrate buffer was incubated in a tube at 37° for 5—30 min. The hydrolysis was followed by determining the phenol liberated except in the case of the *p*-hydroxyphenyl glycoside where acetylglucosamine liberation was followed by the method of Morgan–Elson.[14] Nitrophenols were determined by the method of Levvy and Conchie.[15] For the other phenols, the method of Folin and Ciocalteu[16] modified by Arita, *et al.*[17] was further modified as follows to give better results for 0.05—1 mM phenols especially for *p*-hydroxyanisole: to 0.5 ml of an incubation mixture was added 1 ml of 2-fold diluted Folin's reagent and 5 ml of 5% sodium carbonate solution. After keeping at 37° for 20 min, the color density was measured at 650 nm. Initial velocity was calculated from a linear time course including at least four points which were found mostly within 5% hydrolysis.

The 2,4-dinitrophenyl glycoside was labile and hydrolyzed non-enzymatically in the buffer solution of pH 4.2 at 37° with a pseudo-first-order rate constant of 2.30×10^{-2} min^{-1}. The measurement was carried out as follows with a recording spectrophotometer equipped with a thermostated cell compartment: three ml of buffer solution of pH 4.2 containing the enzyme and 0.01% bovine serum albumin was equilibrated at 37° and the reaction was initiated by adding an appropriate amount of the glycoside (solid). The reaction was followed at 360 nm, and the correction was made for the nonenzymatic hydrolysis.

Results

All substrates used exhibited a Michaelian behaviour, some of which are depicted in Fig. 1. Michaelis parameters were calculated from Lineweaver-Burk plots and the values obtained at the optimum pH 4.2 for Km and relative catalytic constant k_{cat}^{rel} are listed in Table I.

The ρ–σ relationships for Km and k_{cat}^{rel} are shown in Fig. 2 and 3, respectively, where the point for 2,4-dinitrophenyl is excluded as it has *ortho*-substituent and the σ value is not available. As seen in Fig. 2, the plot of log Km at the optimum pH 4.2 *versus* Hammett substituent constant, σ shows a linearity with a ρ value of -0.42 ± 0.06 (S.D.) (size n=10 and correlation coefficient r=0.934). On the other hand, when the values of log Km at pH 4.2 are plotted against Hansch substituent constant, π,[18] a measure of hydrophobic nature of substituents, the points are scattered widely (n=9, r=−0.449 in phenols, and n=9, r=−0.198 in phenoxyacetic acids) (figure not presented). The plots for the other pH values than 4.2

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Table I. Michaelis–Menten Parameters for the Hydrolysis of β-Acetylglucosaminides by Bovine Liver β-Acetylglucosaminidase at pH 4.2

β-Acetylglucosaminide	σ[a]	Km (mM) ± S.D.[b]	k_{cat}^{rel}[c] ± S.D.[b]	Substrate conc. examined (mM)
2,4-Dinitrophenyl	—	2.14±0.113	103±4.3	0.4—1.0
p-Nitrophenyl	0.778	0.90±0.014	100±1.1	0.63—2.5
m-Nitrophenyl	0.710	1.04±0.030	83±1.8	0.63—2.5
4-Nitro-3-methylphenyl	(0.709)[d]	1.16±0.020	86±1.2	0.63—2.5
m-Chlorophenyl	0.373	1.41±0.047	65±1.7	0.6—2.4
p-Chlorophenyl	0.227	2.36±0.198	92±7.2	0.6—2.4
Phenyl	0	2.38±0.088	79±2.5	1—4
m-Tolyl	−0.069	2.04±0.048	75±1.4	1—4
p-Tolyl	−0.170	3.29±0.269	86±6.3	1—4
p-Methoxyphenyl	−0.268	2.33±0.201	66±4.6	1—4
p-Hydroxyphenyl	−0.37	3.04±0.251	42±2.9	1.3—4

a) Hammett substituent constant, σ[19]
b) Standard deviation calculated from a Lineweaver-Burk plot.
c) relative to the value for the *p*-nitrophenyl glycoside
d) The sum of the σ's for the two substituents, regardless of the substituents being *ortho* to each other (refer to ref. 20).

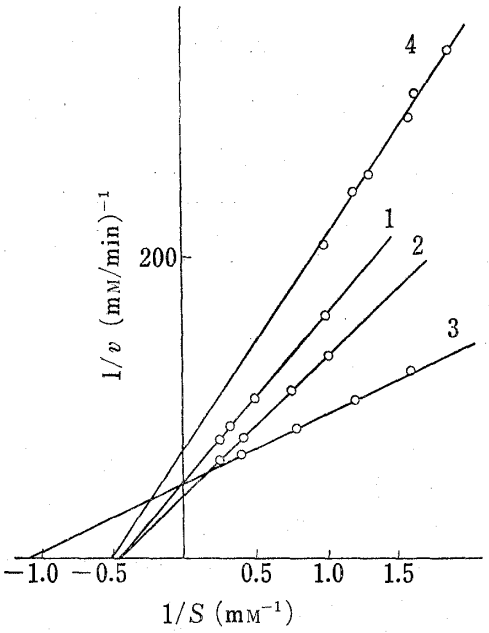


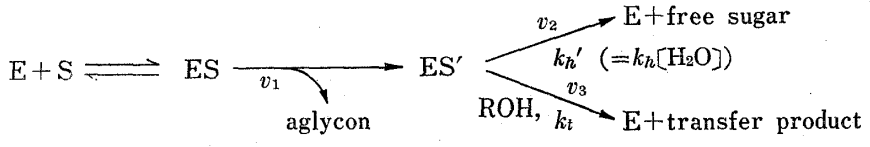
Fig. 1. Lineweaver–Burk Plots for the β-Acetylglucosaminidase-catalyzed Hydrolysis, at pH 4.2, 37°

Substrates are *p*-methoxyphenyl (1), phenyl (2), *p*-nitrophenyl (3) and 2,4-dinitrophenyl β-acetylglucosaminide (4).

show similar trends. These findings indicate that the Km values are dependent on the electronic nature of substituents but not on the hydrophobic nature.

Fig. 3 shows that the log k_{cat}^{rel} values at the optimum pH 4.2 are only slightly dependent on σ values ($\rho=+0.16\pm0.069$, $n=10$, $r=0.626$): the point for the most susceptible nitrophenyl glycoside is found only *ca.* 0.4 log unit above the point for the least susceptible *p*-hydroxyphenyl glycoside. The ρ value of +0.16 is much smaller than those reported for β-glucosidase ($\rho=+1.23$ and +1.02 for *m*- and *p*-substitution, respectively),[2] Taka-amylase (+1.66),[4] lysozyme (+1.23),[7a] and −2.96 with lower σ values and +0.55 with higher σ values[7b]) but comparable to that for pig epididymal β-acetylglucosaminidase (+0.3).[8]

An experiment on the effect of methanol on the hydrolysis of the phenyl and *p*-nitrophenyl glycosides was carried out and analyzed, assuming that the steps of hydrolysis can be expressed by the following scheme as shown by Mega, *et al.*[20] for the hydrolysis by *Aspergillus oryzae* β-acetylglucosaminidase:



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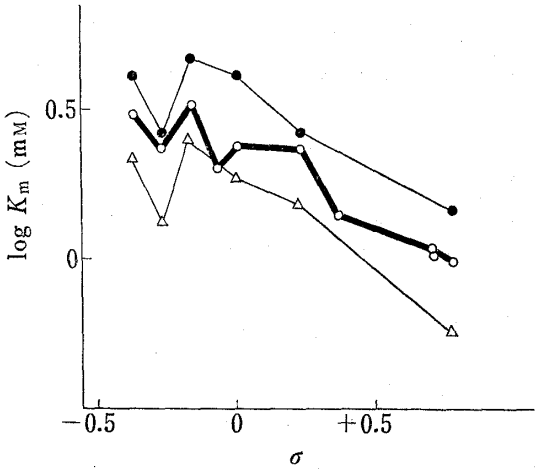

Fig. 2. log Km—σ Plots for the β-Acetylglucosaminidase-catalyzed Hydrolysis of Substituted Phenyl β-Acetylglucosaminides at 37°

—○—: pH 4.2 (optimum); —●—: pH 3.6; —△—: pH 5.0

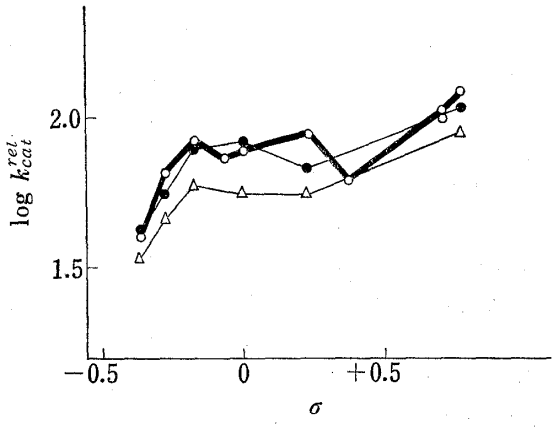

Fig. 3. log k_{cat}^{rel}—σ Plots for the β-Acetylglucosaminidase-catalyzed Hydrolysis of Substituted Phenyl β-Acetylglucosaminides at 37°

—○—: pH 4.2 (optimum); —●—: pH 3.6; —△—: pH 5.0

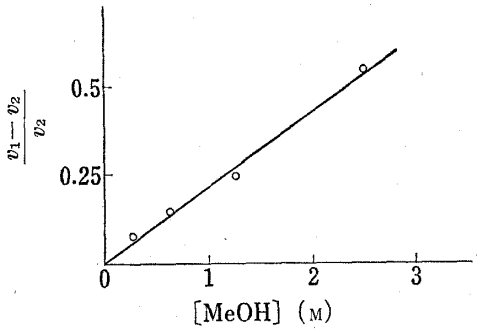

Fig. 4. Effect of Methanol on the Initial Velocities for the Appearance of Phenol (v_1) and Acetylglucosamine (v_2) in the Enzymatic Hydrolysis of Phenyl β-Acetylglucosaminide

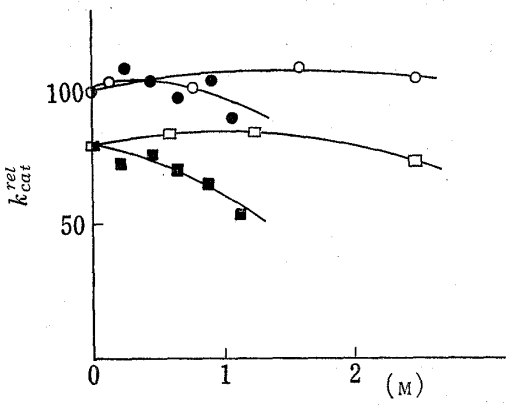

Fig. 5. Effects of Methanol and Dioxane on k_{cat}^{rel} for the Enzymatic Hydrolyses of Phenyl and p-Nitrophenyl β-Acetylglucosaminide

phenyl β-acetylglucosaminide: methanol (□), dioxane (■)
p-nitrophenyl β-acetylglucosaminide: methanol (○), dioxane (●)

With the phenyl glycoside, initial velocities, v_1 for the appearance of aglycon and v_2 for that of free sugar, were determined and the value for $(v_1-v_2)/v_2$ were plotted against methanol concentrations (Fig. 4). The slope gives the ratio k_t/k_h' under stationary state conditions. The value for k_t/k_h' thus obtained is 0.21M^{-1} so that an increment in k_{cat}^{rel} is expected to be approximately 40% in the presence of 2M methanol assuming that the deglycosylation of glycosyl enzyme ES′ is rate-limiting. As seen in Fig. 5, however, no significant increment in k_{cat}^{rel} was observed in the hydrolyses of both the phenyl and the p-nitrophenyl glycoside, while dioxane markedly decreased k_{cat}^{rel} in both glycosides as expected by much larger effect of dioxane to decrease the dielectric constant of the medium at the same molar concentration.

Discussion

The Km value decreases with the increase in σ value in accordance with similar observations on α-[3] and β-glucosidase,[2] and Taka-amylase.[4] The dependence of Km on the electronic nature of substituents can be explained by the assumption of Nath, *et al.*[2] that, in the enzyma-

tic hydrolysis of substituted phenyl β-glucosides, hydrogen bonds are formed between hydrogen atoms of the hydroxyl groups in the glycon moiety and hydrogen-accepting groups of the enzyme, and the electron-attracting groups on the benzene ring fastens the hydrogen bonds to increase the enzyme-substrate affinity.

Leaback[8] described that the Hammett plot of the V_{max} exhibits a positive slope ($\rho=+0.3$) in the pig epididymal β-acetylglucosaminidase-catalyzed hydrolysis of substituted phenyl β-acetylglucosaminides, suggesting the participation in the catalyzed reaction of basic or nucleophilic groups on the enzyme. The ρ values for the acidic and the alkaline hydrolysis have previously been shown to be -0.52 and $+1.69$, respectively in the substituted phenyl β-acetylglucosaminides.[10] In the present study an intermediate ρ value between the above two values was anticipated, as several enzymatic hydrolyses are known to involve concerted catalysis, showing a ρ value between those for the acid- and the base-catalyzed hydrolysis.

Of the substrates studied, the 2,4-dinitrophenyl glycoside having the most acidic aglycon, 2,4-dinitrophenyl (pK_a 4.1), hydrolyzes non-enzymatically at room temperature and is, therefore, expected to hydrolyze enzymatically much more rapidly than the other glycosides. However, the k_{cat}^{rel} value for this glycoside is approximately equal to that for the *p*-nitrophenyl glycoside (the pK_a value for the parent phenol is 7.2). It has been reported[21] that E.coli β-galactosidase hydrolyzes the galactosides having usual or moderate leaving groups, such as phenoxyl and *p*-nitrophenoxyl, with the rate-limiting step of galactosylation (or cleavage of the glycoside bond), whereas the enzyme hydrolyzes the glycosides having extremely good leaving groups, such as 2,4- and 3,5-dinitrophenoxyl, with the rate-limiting step of degalactosylation, and that in the latter case addition of methanol markedly enhances the k_{cat} value by increasing the rate of degalactosylation owing to the nucleophilic competition with the solvent, a much more effective nucleophile than water.

As far as the results shown in Fig. 3 indicate, k_{cat}^{rel} is only slightly affected by electronic nature of the aglycones, and hence the deglycosylation of the glycosyl enzyme, a step common to various substrates, is tentatively presumed to be rate-limiting, taking the above discussion[21] into consideration. This presumption is, however, demonstrated to be unlikely because of the following facts. The effect of methanol on the β-acetylglucosaminidase-catalyzed hydrolysis was evaluated by comparing with the effect of dioxane which does not act as a nucleophile but influences the value of k_{cat}^{rel} by decreasing the dielectric constant of the medium by about 5.2 times as much as methanol at equimolar concentration.[22] Fig. 5 does not show significant enhancement of k_{cat}^{rel} by adding methanol when the points for the two solvents are compared at similar dielectric constants. This does not indicate the occurrence of rate-limiting deglycosylation, and therefore, the small ρ value for k_{cat}^{rel} is presumed to pertain to the reaction of glycoside bond cleavage. Methanol rather acted as a poor glycosyl acceptor and experiments with more effective nucleophiles are desired.

Acknowledgement The authors are deeply indebted to Dr. T. Morikawa for his performance in elemental analysis.

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