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The Effect of Various Substituents on the Hydrolysis of Mono-substituted Phenyl-β-D-glucuronic Acids by β-Glucuronidase

β-Glucuronidase is an enzyme catalyzing the hydrolysis of a wide variety of alkyl- and aryl-β-D-glucuronic acids. In spite of the accumulation of a great deal of information about this enzyme, difficulties encountered in purifying it have limited the information available about its physicochemical properties.[1] Many aryl-β-D-glucuronides have been investigated and demonstrated to be hydrolyzed with different *K*m and reaction velocities.[2] However, no systematic investigation into the factors which determine substrate specificity has been reported. A promising approach to this problem was to focus attention on a series of mono-substituted phenyl-β-D-glucuronic acids, the substituents of which would have properties varying from electron-attracting to electron-releasing. In view of the fact that earlier investigations on the effect of various substituents have produced some evidence regarding the mechanism of the enzymatic hydrolysis of other glycosides[3], it is important to determine whether there is a correlation between the structure of glucuronides and their rate of hydrolysis. In this communication we wish to report evidence that the hydrolysis of mono-substituted phyenyl-β-D-glucuronides by calf liver β-glucuronidase is affected in a predictable way according to the nature of the substituents in the benzene ring.

Phenyl-β-D-glucuronic acid and five mono-substituted derivatives thereof, prepared by the previous methods,[4] were incubated with a commercial β-glucuronidase preparation (Tokyo

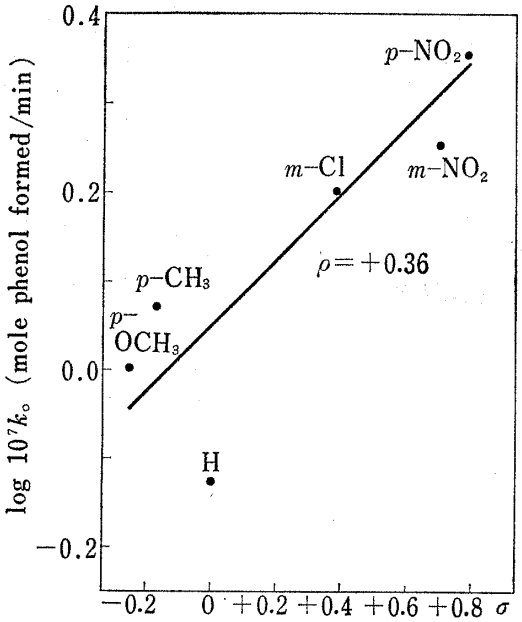

Fig. 1. Correlation of Structure of Mono-substituted Phenyl-β-D-Glucuronides with k_0 in the Hydrolysis by β-Glucuronidase

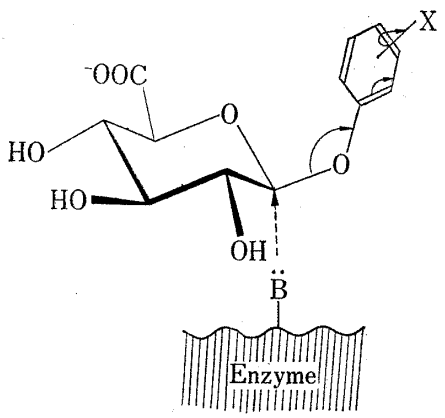

Fig. 2. A Possible Mode of Hydrolysis of Substituted Phenyl-β-D-glucuronides by β-Glucuronidase

B: an anionic active site of the enzyme
X: an electronattracting group.

1) W.H. Fishman and P. Bernfeld, "Methods in Enzymology," Vol. 1, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1955, p. 262.
2) K. Kato, K. Yoshida, H. Tsukamoto, M. Nobunaga, T. Masuya and T. Sawada, *Chem. Pharm. Bull.* (Tokyo), **8**, 239 (1960); K. Kato, K. Yoshida and H. Tsukamoto, *ibid*, **12**, 670 (1964); G.A. Levvy and C.A. Marsh, *Advan. Carbohydrate Chem.*, **14**, 423 (1959).
3) R.L. Nath and H.N. Rydon, *Biochem. J.*, **57**, 1 (1954); S. Matsubara, *J. Biochem.*, **49**, 232 (1957).
4) H. Tsukamoto, M. Hamana, K. Kato and T. Kuroda, *Yakugaku Zasshi*, **76**, 1282 (1956); B. Helferich and A. Berger, *Chem. Ber.*, **90**, 2492 (1957).

Zoki Chemicals) in 0.1 M acetate buffer at pH 5.0 and 38°. The incubation mixture included 10^{-2} to 10^{-4} M concentrations of the sodium salt of each glucuronide, 200 to 500 Fishman units of the enzyme, and sufficient buffer solution to make a final volume of 2 ml. The reaction was stopped at appropriate intervals by adding 1 ml of 0.5 N HCl to the mixture, and the phenols formed were extracted with isopropyl ether, following saturation of the mixture with sodium chloride. The phenols were then transfered into 0.1 N NaOH, in which they were determined spectrophotometrically at the wave lengths of 235 (p–OCH_3), 237 (p–CH_3), 235 (H: phenol), 240 (m–Cl), 226 (m–NO_2), and 400 mμ (p–NO_2). Blank experiments which were carried out by using a mixture consisting of the heat-deactivated enzyme and the glucuronate did not show the formation of any detectable amount of phenol. The overall velocity constant (k_0) for the hydrolysis of each glucuronide, was calculated in the usual way from the result of the time-course study of the reaction, and the Michaelis constant (Km) of the substrate was obtained by the Lineweaver-Burk method. In order to obtain systematic information on the enzymatic hydrolysis of the glucuronides, the common logarithms of k_0 and Km were plotted against the Hammett substituent constants, σ,[5] which are known to represent the electronic effects of substituents in the benzene ring.

As shown in Fig. 1, the slope of the line formed by plotting log k_0 against σ was positive. This indicates that the hydrolysis is facilitated by the presence of electron-attracting groups and that its rate is determined by the relative strength of their electron-attracting properties, while the unsubstituted substrate does not fit this pat tern. Furthermore, this fact suggests that the enzymatic hydrolysis may be initiated by the attack of some nucleophiles such as a hydroxyl anion or a basic nitrogen of the enzyme protein, but not by protonation of the ethereal oxygen atom between the phenyl and glucuronyl moieties.

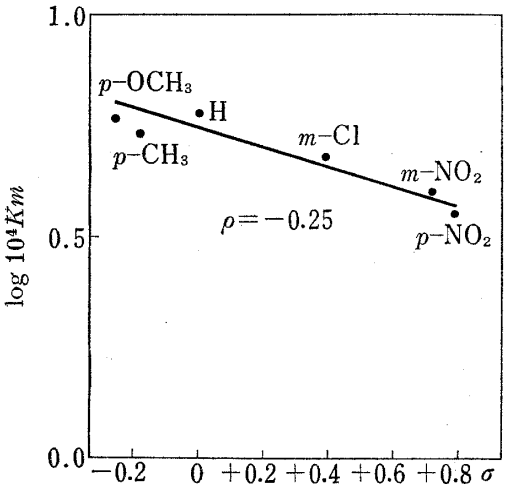

Fig. 3. Correlation of Structure of Mono-substituted Phenyl-β-D-glucuronides with Km in the Hydrolysis by β-Glucuronidase

When log Km is plotted against σ, the resulting line has a negative slope (Fig. 3), indicating that the formation of the enzyme-substrate complex is facilitated by the pre-sence of electron-attracting substituents in accordance with their relative electronic affinities. This seems to imply that in the substrate molecules the site of interaction with the enzyme may be located near the glucuronyl C_1, although on the basis of the present evidence alone it is difficult to reach a conclusion as to the real site of the interaction.

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