[Chem. Pharm. Bull. 12 (6) 670 ~ 674]

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94. Keitaro Kato, Kazuo Yoshida, and Hisao Tsukamoto : Metabolism of Drugs. XLVI.*1 Behavior of 2-Naphthyl β-D-Glucofuranosiduronic Acid toward β-Glucuronidase.

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The action of β-glucuronidase on a furanose structure of glucuronide was described by Levvy and Marsh[1] and by Tsou and Seligman.[2] Levvy and Marsh used methyl β-D-glucofuranosiduronolactone which had been treated with alkali as a competing substrate in the hydrolysis of phenolphthalein glucuronide by mouse-liver β-glucuronidase. Since the depression of the release of phenolphthalein was not observed, they concluded that β-D-glucofuranosiduronic acids are not substrates for the enzyme. Subsequently, however, it has been observed by Osman,[3] *et al.* that this glycoside is decomposed by alkali.

Tsou and Seligman also reported later that 2-naphthyl β-D-glucofuranosiduronolactone was decomposed by alkali with liberation of 2-naphthol in an attempt to open the lactone ring with alkali. Therefore, they used 2-naphthyl β-D-glucofuranosiduronolactone as a substrate in direct hydrolysis experiments with mammalian β-glucuronidase. Since the enzyme did not liberate 2-naphthol, they reached the same conclusion as that of Levvy and Marsh. As it is assumed, however, that the enzyme would require the presence of a carboxylic acid group at C_6 for its hydrolytic activity, any lactone would not be a substrate for the enzyme.

In either experiments the substrates used were unsuitable for their purposes. Accordingly, both experiments have later been criticized by Levvy and Marsh, and the specificity of β-glucuronidase for a furanose structure of glucuronide has so far been uncertain.

The present investigation was undertaken to determine the specificity of β-glucuronidase for the furanose structure of glucuronide with the suitable test compound, 2-naphthyl β-D-glucofuranosiduronic acid, and the evidence was obtained that β-glucofuranosiduronic acid could be a substrate for β-glucuronidase.

Results and Discussion

Materials

Sodium 2-naphthyl β-D-glucofuranosiduronate was prepared by the method described in the preceding paper.[4] 2-Naphthyl β-D-glucopyranosiduronic acid was prepared by the method of Tsukamoto, *et al.*[5]

Enzyme preparation : The rabbit-liver β-glucuronidase was prepared by a modification of the method of Talalay and Fishman.[6]

Methods

After enzymatic hydrolysis of the substrates at appropriate pH and temperature, the

*1 Part XLV. K. Kato, K. Yoshida, H. Tsukamoto : This Bulletin, **12**, 664 (1964).
*2 Katakasu, Fukuoka (加藤敬太郎, 吉田和夫, 塚元久雄).

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2) K. C. Tsou, A. M. Seligman : J. Am. Chem. Soc., **74**, 5605 (1952).
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liberated 2-naphthol was determined according to the method of Goldbarg, *et al.* in which liberated aglycon was converted to a blue azo dye by reaction with tetrazotized *o*-dianisidine.[7]

In the inhibition test of the hydrolysis of *p*-nitrophenyl glucuronide, liberated *p*-nitrophenol was determined according to the method of Kato, *et al.*[8]

Hydrolysis of 2-Naphthyl β-D-Glucofuranosiduronic Acid by Rabbit-liver β-Glucuronidase

2-Naphthol liberated was determined by the method of Goldbarg, *et al.* The absorbancy at 560 mμ was measured in Hitachi photoelectric spectrophotometer. Results in Table I show that 2-naphthyl β-D-glucofuranosiduronic acid was hydrolyzed by rabbit-liver β-glucuronidase and the hydrolysis was inhibited by saccharo-1,4-lactone.

Table I. Liberation of 2-Naphthol from 2-Naphthyl β-D-Glucofuranosiduronic Acid by Rabbit-liver β-Glucuronidase

Substrate	Inhibition	2-Naphthol liberated (γ)	Inhibition (%)
$2\times10^{-2}M$	none	70	—
$2\times10^{-2}M$	$1\times10^{-4}M$ saccharate	13	81.6

Incubation mixture contained 14.4 mg. of sodium 2-napthyl β-D-glucofuranosiduronate (final concentration 0.02*M* as a free acid), 1 ml. of 0.2*M* phosphate-0.1*M* citrate buffer, pH 5.0, 0.5 ml. of H_2O and 0.5 ml. of enzyme solution. To test for inhibition of the hydrolysis, 0.5 ml. of H_2O in the incubation mixture was replaced by $4\times10^{-4}M$ saccharate solution, and the usual control was done. Each tube was incubated for 1 hr. at 38°.

In a concentration of $1\times10^{-4}M$, saccharo-1,4-lactone caused 81.6% inhibition with $2\times10^{-2}M$ substrate.

Time Course of Hydrolysis

In an experiment to determine the course of hydrolysis with time, 0.02*M* 2-naphthyl β-D-glucofuranosiduronic acid in phosphate-citrate buffer, pH 5.0, was hydrolyzed by β-glucuronidase for the periods of time varying from 1 to 5 hour. The enzyme units used were 180 and 1000 Fishman units. The graph shown in Fig. 1 demonstrates that each of the velocities of hydrolysis is constant and linearities are maintained during the experimental period with both enzyme units.

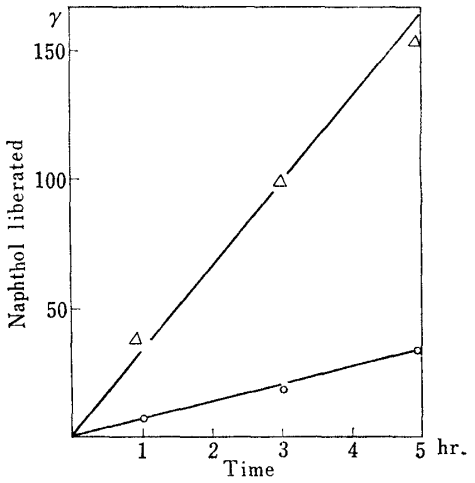

Fig. 1. Course of Enzymatic Hydrolysis of 2-Naphthyl β-D-Glucofuranosiduronic Acid in Relation to Time

The system consisted of 14.4 mg. of sodium 2-naphthyl β-D-glucofuranosiduronate, 1 ml. of 0.2*M* phosphate-0.1*M* citrate buffer, pH 5.0, and 1 ml. of enzyme solution. The incubation was carried out for 1 hr. at 38°.

—△— 1000 Fishman unit
—○— 180 Fishman unit

Inhibition of the Hydrolysis of *p*-Nitrophenyl β-D-Glucuronide

2-Naphthyl β-D-glucofuranosiduronic acid was tested as a competing substrate in the hydrolysis of *p*-nitrophenyl β-D-glucuronide by rabbit-liver β-glucuronidase. According to the

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report of Levvy and Marsh, this technique is less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis. For the enzyme assay, the method described in an earlier paper from this laboratory[9] was employed. The incubation was carried out 1 hour at 38° in acetate buffer at pH 3.8 and final concentration was 0.16*M*. The concentration of *p*-nitrophenyl β-D-glucuronide ranged from 0.01 to 0.0004*M* and that of the competing substrate ranged from 0.02 to 0.005*M*. The results were analyzed by the graphical method of Lineweaver and Burk,[10] plotting 1/S against 1/V. 2-Naphthyl β-D-glucofuranosiduronic acid depressed the release of *p*-nitrophenol and acted competitively. The value of *Ki* determined by the method of Dixon[11] was $1.70\times10^{-2}M$.

Inhibition of the hydrolysis of *p*-nitrophenyl glucuronide by 2-naphthyl β-D-glucopyranosiduronic acid was determined in the same way. The concentration of *p*-nitrophenyl glucuronide ranged from 0.01 to 0.002*M* and that of the competing substrate ranged from 0.01 to 0.001*M*. 2-Naphthyl β-D-glucopyranosiduronic acid also depressed the release of *p*-nitrophenol and acted competitively. The value of *Ki* was $1.2\times10^{-3}M$.

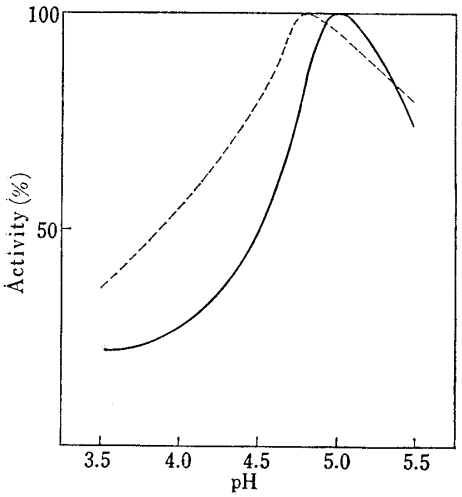

Fig. 2. Effect of pH on the Hydrolysis of 0.02*M* 2-Naphthyl β-D-Glucofuranosiduronic Acid and 0.001*M* 2-Naphthyl β-D-Glucopyranosiduronic Acid in 0.2*M* Phosphate-0.1*M* Citrate Buffer

———— 2-Naphthyl β-D-glucofuranosiduronic acid

– – – – – 2-Naphthyl β-D-glucopyranosiduronic acid

pH Optimum

The pH optimum for the hydrolysis of 2-naphthyl β-D-glucofuranosiduronic acid was determined in 0.2*M* phosphate-0.1*M* citrate buffer between pH 3.5 and 5.5. The pH optimum for 2-naphthyl β-D-glucopyranosiduronic acid was also determined under these conditions. The pH optimum for the former (final concentration 0.02*M*) is 5.0 and that for the latter (final concentration 0.001*M*) is 4.7. The pH-activity curves are given in Fig. 2.

Influence of Substrate Concentration on Activity of β-Glucuronidase

Fig. 3 shows the effect of the enzyme at pH 5.0 varying 2-naphthyl β-D-glucofuranosiduronic acid concentration. The velocity of the reaction was determined from the amount of 2-naphthol liberated in 1 hour.

The results were analyzed by the graphical method of Lineweaver and Burk,[10] plotting S against S/V (Fig. 4). The Michaelis-Menten constant was $1.75\times10^{-2}M$. 2-Naphthyl β-D-glucopyranosiduronic acid was also tested in the same method as above (Figs. 5, 6). The Michaelis-Menten constant was $1.3\times10^{-3}M$. From comparison of the *Km* values, it was considered that the D-glucofuranosiduronic acid has a lower affinity for enzyme than the D-glucopyranosiduronic acid. The rate of hydrolysis of the D-glucofuranosiduronic acid was more slowly than that of the D-glucopyranosiduronic acid.

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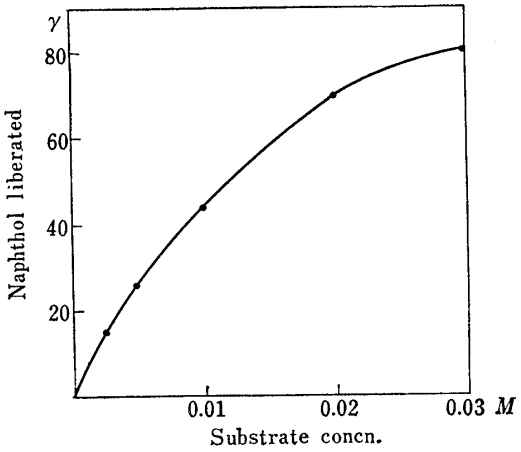

Fig. 3. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of 2-Naphthyl β-D-Glucofuranosiduronic Acid

The reaction rate is expressed in γ of 2-naphthol liberated during 1 hr. at 38°. The system consisted of 1 ml. of 0.2*M* phosphate-0.1*M* citrate buffer, pH 5.0, 0.5 ml. of the glucofuranosiduronic acid, and 0.5 ml. of the enzyme (500 Fishman unit).

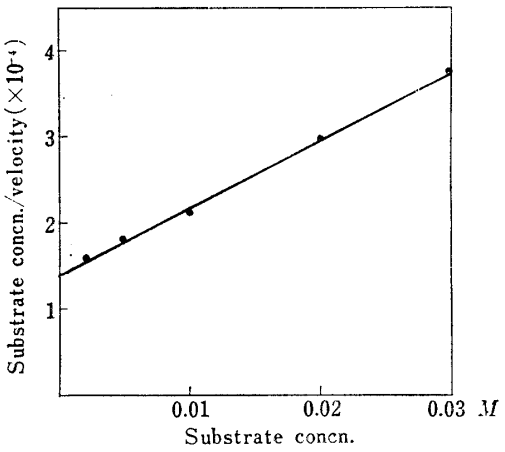

Fig. 4. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of 2-Naphthyl β-D-Glucofuranosiduronic Acid

Data of Fig. 3 plotted according to Lineweaver and Burk. From the slope and intercept of the graph, the Michaelis-Menten constant was calculated to be $1.75\times10^{-2}M$.

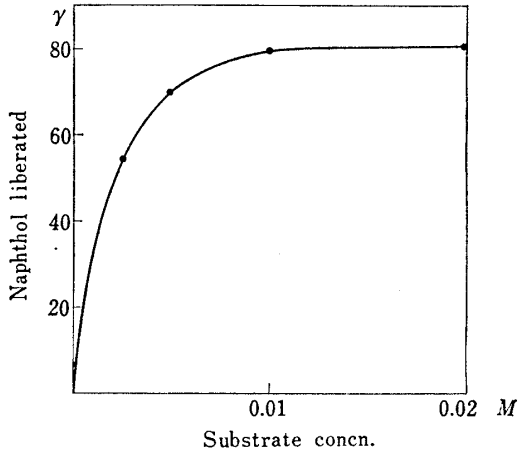

Fig. 5. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of 2-Naphthyl β-D-Glucopyranosiduronic Acid

The system consisted of 1 ml. of 0.2*M* phosphate-0.1*M* citrate buffer, pH 5.0, 0.5 ml. of the glucopyranosiduronic acid, and 0.5 ml. of the enzyme (20 Fishman units).

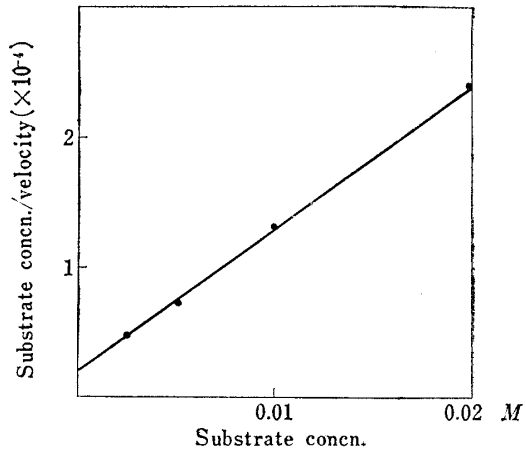

Fig. 6. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of 2-Naphthyl β-D-Glucopyranosiduronic Acid

Data of Fig. 4 plotted according to Lineweaver and Burk. From the slope and intercept of the graph, the Michaelis-Menten constant was calculated to be $1.3\times10^{-3}M$.

Summary

1. 2-Naphthyl β-D-glucofuranosiduronic acid was hydrolyzed by a rabbit-liver β-glucuronidase preparation, and this hydrolysis was inhibited by saccharo-1,4-lactone. Moreover, this glucuronide inhibited competitively the hydrolysis of *p*-nitrophenyl β-D-glucopyranosiduronic acid by the enzyme.

2. The kinetics of the hydrolysis of 2-naphthyl β-D-glucofuranosiduronic acid by β-glucuronidase were investigated. The pH optimum of the β-D-glucofuranosiduronic acid is 5.0 in phosphate-citrate buffer at 38°. The reaction velocity is constant with time. The Michaelis-Menten constant is $1.75\times10^{-2}M$. The kinetics of the hydrolysis of 2-naphthyl β-D-glucopyranosiduronic acid by the enzyme were also investigated. The pH optimum is 4.7. The Michaelis-Menten constant is $1.3\times10^{-3}M$.

3. In view of the above facts, it was concluded that β-D-glucofuranosiduronic acids could be a substrate for β-glucuronidase.

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95. Minoru Sekiya, Keiichi Ito, and Minako Saito : Reaction of Amide Homologs. XII.*1 Reaction of N-Arylmethylene-1-acylamino-1-arylmethylamine with Active Methylene Compound.

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1-Acylaminoarylmethylations of ethyl malonate were found to proceed with some N-arylmethylene-1-acylamino-1-arylmethylamines reported by Sekiya, *et al.*[1,2] by the action of alkaline catalyst. In the present paper we wish to report this reaction and related studies. Previously, there were some reports[3,4] in which 1-acylaminoarylmethylation of active methylene compound is effected in some cases only by treatment with N,N'-arylmethylenebisamide or a mixture of an aromatic aldehyde and acetamide in acetic anhydride.

The following N-arylmethylene-1-acylamino-1-arylmethylamines were used for the reaction : N-Benzylidene-α-acetamidobenzylamine, N-(4-methoxybenzylidene)-α-acetamido-4-methoxybenzylamine and formamido homolog, N-(3,4-methylenedioxybenzylidene)-α-acetamido-3,4-methylenedioxybenzylamine and formamido homolog. Among these compounds the latter two 3,4-methylenedioxybenzylidene derivatives have not been described previously. Both the compounds were prepared by the reaction of 3,4:3',4':3'',4''-tris(methylenedioxy)hydrobenzamide with acetamide or formamide according to the previously reported method.[2]

The N-arylmethylene-1-acylamino-1-arylmethylamines reacted with active methylene compound such as ethyl malonate, ethyl cyanoacetate, phenylacetonitrile, and malononitrile. The general procedure for carring out the reaction is to reflux a toluene solution of an active methylene compound and N-arylmethylene-1-acylamino-1-arylmethylamine with suspended sodium hydroxide powder. All reactions examined of N-arylmethylene-1-acylamino-1-arylmethylamines with ethyl malonate proceeded smoothly yielding diethyl (α-acylaminobenzyl)malonate effecting the replacement of the

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*2 Oshika, Shizuoka (関屋 実, 伊藤敬一, 斎藤美奈子).
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2) M. Sekiya, A. Hara : *Ibid.*, **11**, 901 (1963).
3) G. Stefanović, *et al.* : J. Org. Chem., **17**, 816, 1110, 1114, 1305 (1952).
4) G. Stefanović, S. Mihajlović, M. Stefanović : *Ibid.*, **18**, 1467 (1953).