Summary

Thiamylal was orally administered to rabbits and its metabolites excreted in urine were examined, mainly by paper chromatography. One of the metabolites was isolated and characterized as a carboxylic acid with thiobarbituric acid ring and allyl side-chain. Comparing the paper chromatogram of urine of rabbits receiving thiamylal or secobarbital, it was apparent that thiamylal was metabolized to the compounds not only with thiobarbituric acid ring and its oxygen homologs but also without the ring structure.

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42. Keitaro Kato, Kazuo Yoshida, Hisao Tsukamoto*1; Masashi Nobunaga, Tomiichi Masuya, and Toichiro Sawada*2 : Synthesis of *p*-Nitrophenyl β-D-Glucopyranosiduronic Acid and Its Utilization as a Substrate for the Assay of β-Glucuronidase Activity.

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Phenolphthalein-mono-β-D-glucosiduronic acid has been utilized as a substrate for the assay of the hydrolytic activity of β-glucuronidase.[1] This substrate is generally prepared biosynthetically from the urine of rabbits to which phenolphthalein has been administered. *p*-Nitrophenyl β-D-glucosiduronic acid was synthesized in this laboratory for this purpose. The chemical synthesis provides a useful chromogenic substrate for β-glucuronidase which should be more practicable and more readily available than the tedious biosynthetic preparation of phenolphthalein mono-β-D-glucosiduronic acid currently in use. The latter compound has not been obtained in crystalline form but is only available as a crude cinchonidine salt. *p*-Nitrophenyl β-D-glucosiduronic acid is rapidly hydrolyzed by β-glucuronidase and the free *p*-nitrophenol may be readily determined photocolorimetrically in alkaline solution. The method of assay is similar in principle to that used for sodium phenolphthalein-mono-β-D-glucosiduronate.

The synthesis of *p*-nitrophenyl β-D-glucosiduronic acid was accomplished by alkaline hydrolysis of methyl (*p*-nitrophenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (I), which was prepared in a good yield by the condensation of methyl (1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranosid)uronate and *p*-nitrophenol using acetonitrile and freshly prepared silver oxide. By the treatment of potassium *p*-nitrophenoxide with methyl 1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranuronate in acetone-water or the fusion of *p*-nitrophenol with methyl (tetra-O-acetyl-β-D-glucopyranosid)uronate in the presence of *p*-toluenesulfonic acid, (I) was obtained only in a small amount. The method of Helferich and Berger[2] was adopted in alkaline hydrolysis of (I).

The present paper describes the use of *p*-nitrophenyl β-D-glucosiduronic acid as a chromogenic substrate for the assay of the hydrolytic activity of β-glucuronidase and preparation of the substrate.

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1) P. Talalay, W. H. Fishman, C. Huggins : J. Biol. Chem., **166**, 757(1946).
2) B. Helferich, A. Berger : Ber., **90**, 2492(1957).

Methods and Results*3

Methyl (*p*-Nitrophenyl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (I)—To a solution of 10.1 g. of methyl 1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranuronate (1 mole) and 7.1 g. of *p*-nitrophenol (2 moles) dissolved in 45 cc. of dehyd. MeCN, 6.5 g. of freshly prepared Ag_2O was added and the mixture was shaken for 30 min. After removal of the precipitate, the filtrate was evaporated under a reduced pressure. The residue was extracted with 250 cc. of hot EtOH, treated with carbon, filtered, and the filtrate was concentrated to 150 cc. After cool, (I) crystallized therefrom (7.4 g.). An additional yield of 0.6 g. was obtained from the mother liquor. The combined crops were recrystallized from EtOH to needles, m.p. 151~152°; $[\alpha]_D^{17}$ −48°(c=1.00, $CHCl_3$). Yield, 7.7 g. (66.5%). *Anal.* Calcd. for $C_{19}H_{21}O_{12}N$: C, 50.11; H, 4.62; N, 3.08. Found: C, 50.45; H, 4.72; N, 3.28.

***p*-Nitrophenyl β-D-Glucosiduronic Acid (III)**—The solution of 7.6 g. of (I) dissolved in 40 cc. of 0.1*N* MeONa was allowed to stand at room temperature for 2 days and concentrated under a reduced pressure. The resulting light yellow syrup was allowed to stand at room temperature with equimolar amount of 0.43*N* $Ba(OH)_2$. After 1 hr. EtOH was added until the precipitation of Ba glucuronate (II) no longer occurred. The Ba salt was removed by filtration; yield, 6.1 g. (95%).

5.2 g. of (II) was dissolved in a small amount of H_2O and Ba was removed as Ba oxalate by addition of the calculated amount of 2*N* oxalic acid. The solution was chilled for 1 hr., Ba oxalate was removed by filtration, and the filtrate was evaporated to dryness under a reduced pressure. The residue was dissolved in a small amount of hydr. AcOEt, Et_2O was added until the solution became turbid, and the free acid (III) crystallized as needles (3.4 g.). An additional yield of 0.5 g. was obtained from the mother liquor; total yield, 3.9 g. (86.4%).

(III) was recrystallized from hydr. AcOEt and Et_2O, and after drying over P_2O_5 for 10 hr. at 60°, melted at 137~139° with softening at 110°. In this form (III) was hygroscopic and began to cake immediately after exposure to air. Moisture apparently was absorbed to the extent of 1 mole. The m.p. of the monohydrate was about 95°. Several recrystallizations from hydr. AcOEt-Et_2O and drying over P_2O_5 failed to improve the m.p. $[\alpha]_D^{17}$ −108°(c=1.00, EtOH). *Anal.* Calcd. for $C_{12}H_{13}O_9N$: C, 45.71; H, 4.13; N, 4.44. Found: C, 45.42; H, 4.35; N, 4.22. Calcd. for $C_{12}H_{13}O_9N \cdot H_2O$: C, 43.24; H, 4.50; N, 4.20. Found: C, 43.15; H, 4.65; N, 4.12. In the experiment, *p*-nitrophenyl β-D-glucosiduronic acid monohydrate was used as the substrate.

Enzyme—The β-glucuronidase was obtained from the Worthington Biochemical Co.

Principles of Present Method—*p*-Nitrophenol and its glucuronide appear colorless in the acid range. *p*-Nitrophenol exhibits its maximum stable color intensity at pH 9.5~12.2, and it is not necessary to keep the digest at constant pH using buffer, as phenolphthalein. The absorption spectra of *p*-nitrophenol and its glucuronide in the visible range were compared at pH 11.7 with a Hitachi photoelectric spectrophotometer. The absorption curves are plotted in Fig. 1, in which the extinc-

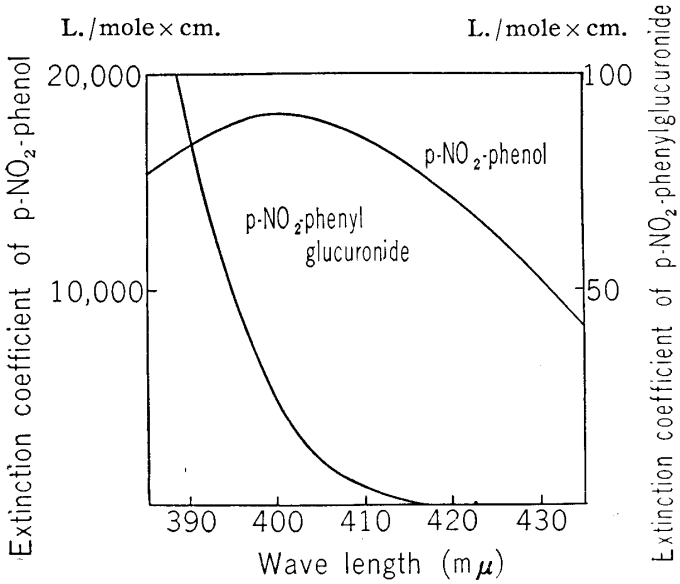

Fig. 1. Spectrophotometric Absorption Curves of *p*-Nitrophenyl Glucuronide and *p*-Nitrophenol at pH 11.7

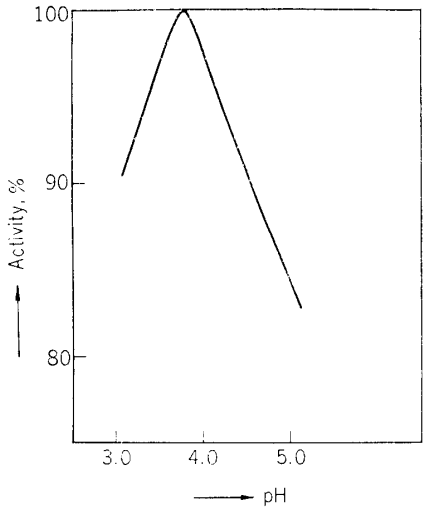

Fig. 2. Effect of pH on the Activity of β-Glucuronidase in 0.2*M* Acetate Buffer

*3 All m.p.s are uncorrected.

tion coefficient of *p*-nitrophenyl glucuronide is shown on a scale 200 times larger than the coefficient of *p*-nitrophenol. The maximum of *p*-nitrophenol is at 400 mμ (ε 18200 L./mole×cm.) and the maximum of *p*-nitrophenyl glucuronide is in the ultraviolet range.

p-Nitrophenyl glucuronide has an extinction coefficients of 23 L./mole×cm. at 400 mμ, which is the maximum of *p*-nitrophenol, so that at the absorption peak of *p*-nitrophenol, the glucuronide has only 0.12% of the absorption of the free *p*-nitrophenol for the same concentration of the two compounds (phenolphthalein, 0.18%).

Optimum pH—The optimum pH of the hydrolysis of this substrate was determined in 0.2*M* acetate buffer between pH 3.1 and 5.1. To develop the color and arrest the reaction, 2 cc. of 0.1*N* NaOH was added to the digest at each pH, and the final pH of each digest was brought to the range of pH 9.5~12.2. It is needless to keep the digest at constant pH using buffer, because *p*-nitrophenol exhibits the same color intensity in the range of pH 9.5~12.2.

The optimum pH for the hydrolysis of 0.01*M* *p*-nitrophenol glucuronide determined under these conditions is 3.8. This optimum pH is lower than those of other glucuronides reported by other workers. The pH-activity curve is given in Fig. 2.

Effect of Enzyme Concentration—The hydrolysis of *p*-nitrophenol glucuronide by serial dilutions of enzyme is shown in Fig. 3. The amount of hydrolysis was directly proportional to the enzyme concentration, other factors being constant.

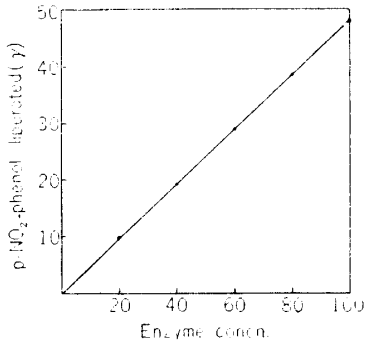

Fig. 3. Effect of Enzyme Concentration on the Rate of Hydrolysis of *p*-Nitrophenyl Glucuronide

The system consisted of 0.8 cc. of 0.2*M* acetate buffer, (pH 3.8), 0.1 cc. of 0.1*M* *p*-phenyl glucuronide, and 0.1 cc. of various dilutions of β-glucuronidase. Incubated for 1 hr. at 38°; 2 cc. of 0.1*N* NaOH and 3 cc. of H_2O were added to develop the color and arrest the reaction. Read with Hitachi photoelectric spectrophotometer against a control; 400 mμ.

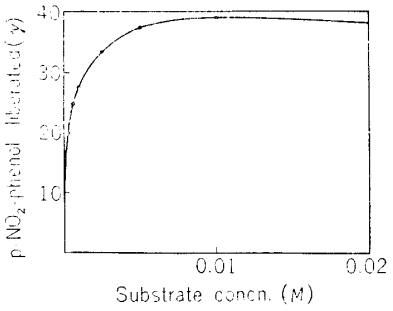

Fig. 4. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of *p*-Nitrophenyl Glucuronide

The reaction rate is expressed in γ of *p*-Nitrophenol liberated during 1 hr. at 38°. The system consisted of 0.8 cc. of 0.2*M* acetate buffer (pH 3.8), 0.1 cc. of *p*-Nitrophenyl glucuronide, and 0.1 cc. of β-glucuronidase. Reaction was stopped and color developed by addition of 2 cc. of 0.1*N* NaOH and 3 cc. of H_2O. Read with Hitachi photoelectric spectrophotometer.

Influence of Substrate Concentration on Activity of β-Glucuronidase—Fig. 4 shows the effect on the activity of the enzyme at pH 3.8 varying the substrate concentration. The velocity of the reaction was determined from the amount of *p*-nitrophenol liberated in 1 hr.

The maximum velocity was found to be reached at a substrate concentration of about 0.01*M* and a slight inhibitory effect was found at 0.02*M*. In the present work a substrate concentration of 0.01*M* was chosen which lies on the flat part of the curve and at which the velocity is maximum.

The results were analysed by the graphical method of Lineweaver and Burk,[3] plotting s against s/v (Fig. 5). The Michaelis-Menten constant was $2\times10^{-4}M$. This value of the Michaelis-Menten constant is larger than that of phenolphthalein monoglucuronide (5.3×10^{-5}).

The rate of hydrolysis of *p*-nitrophenyl glucuronide and phenolphthalein monoglucuronide was compared, the same enzyme dilution and optimum conditions being employed for each substrate. In this experiment *p*-nitrophenyl glucuronide was hydrolyzed more rapidly than phenolphthalein monoglucuronide.

Time Course of Hydrolysis—In an experiment to determine the course of hydrolysis with time, 0.01*M* *p*-nitrophenyl glucuronide in 0.2*M* acetate buffer (pH 3.8) was hydrolyzed by glucuronidase for periods of time varying from 1 to 20 hr. The graph shown in Fig. 6 demonstrates that during this period, the velocity of hydrolysis is constant and linearity is maintained. There was no ap-

3) H. Lineweaver, D. Burk : J. Am. Chem. Soc., **56**, 658(1934).

preciable inhibition by the product of the reaction under these conditions.

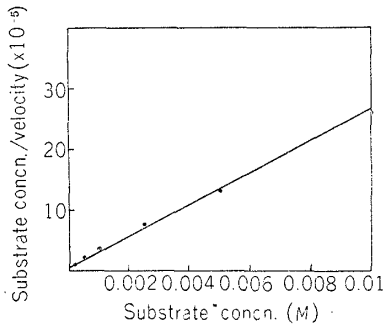

Fig. 5. Effect of Substrate Concentration on the Rate of Enzymatic Hydrolysis of *p*-Nitrophenyl Glucuronide

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 $2\times10^{-4}M$.

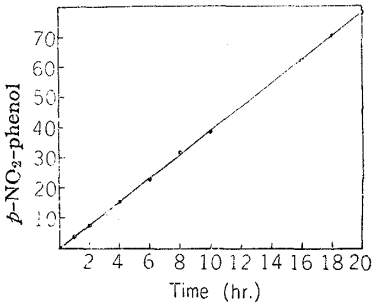

Fig. 6. Course of Enzymatic Hydrolysis of *p*-Nitrophenyl Glucuronide in Relation to Time

The system consisted of 0.8 cc. of 0.2*M* acetate buffer (pH 3.8) and 0.1 cc. of glucuronidase. Incubated at 38°; color developed by addition of 2 cc. of 0.1*N* NaOH and 3 cc. of H_2O. Read with Hitachi photoelectric spectrophotometer against a control; 400 mμ.

Method of Performing Assay -Each determination was run in duplicate with a single control. 0.8 cc. of 0.2*M* acetate buffer was pipetted into test tubes and 0.1 cc. of 0.1*M* *p*-nitrophenyl glucuronide was added to the two experimental tubes but not the control. The tubes were placed in a water bath at 38° and allowed to come to temperature. 0.1 cc. of enzyme solution was added to each tube and the content was mixed by whirling the tube. The tubes were stoppered and incubated for an exact period of time, usually 1 hr. At the end of this time, 2 cc. of 0.1*N* NaOH and 3 cc. of H_2O were added to each tube, including the control, and then 0.1 cc. of the substrate was added to the control tube. Precipitation of tissue proteins in the tube, if any, was centrifuged off. The content of the tube was decanted into spectrophotometer tubes and read in a photoelectric spectrophotometer at 400 mμ, the control tube in each case being set at 100. The color remained stable under these conditions over a period of several hours. The amount of spontaneous hydrolysis of the substrate in the control tube was so small as to be undetectable in the spectrophotometer.

The *p*-nitrophenol calibration curve was prepared in the same buffer mixture which the experimental tubes finally contained. Spectrophotometer tubes were prepared to contain 0.8 cc. of 0.2*M* acetate buffer, 2 cc. of 0.1*N* NaOH, 3 cc. of H_2O, and 0.2 cc. of *p*-nitrophenol solution of varying dilutions. Linearity was maintained in the range of 5 to 80 γ of *p*-nitrophenol.

The authors are indebted to Miss S. Tada and Mr. M. Shirauzu for the elemental analysis.

Summary

1. *p*-Nitrophenyl β-D-glucosiduronic acid is proposed as a substrate for the assay of the hydrolytic activity of β-glucuronidase. The liberated *p*-nitrophenol is measured photocolorimetrically in alkaline solution.

2. The chemical synthesis of *p*-nitropenyl β-D-glucopyranosiduronic acid was accomplished by alkaline hydrolysis of methyl (*p*-nitrophenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate which was prepared by the condensation of methyl 1-bromo-2,3,4-tri-O-acetyl-α-D-glucopyranuronate and *p*-nitrophenol using acetonitrile and freshly prepared silver oxide.

3. The kinetics of the hydrolysis of *p*-nitrophenyl β-D-glucopyranosiduronic acid by β-glucuronidase were investigated. The optimum pH is 3.8 in 0.2*M* acetate buffer at 38°. The reaction velocity is constant with time and varies linearly with enzyme concentrations in the presence of excess substrate. The Michaelis-Menten constant is $2\times10^{-4}M$.

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