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Summary

1. The ester glucuronide of *p*-aminosalicylic acid was isolated from the urine of rabbits administered with *p*-aminosalicylic acid. The methyl acetyl derivative of this compound was identified with synthetic methyl (2-acetoxybenzoyl-4-acetamido-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

2. The ester glucuronide of salicylic acid was isolated as methyl (2-acetoxybenzoyl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate from the urine of rabbits administered with salicylic acid, and the structure was confirmed with the synthetic compound.

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Hisao Tsukamoto, Keitaro Kato, Kazuo Yoshida, and Kiyoshi Tatsumi : Metabolism of Drugs. XLIII.*1 Comparison of Glucuronyl Transfer Activity between β-Glucuronidase and Uridine Diphosphate Transglucuronylase.

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Glucuronide formation is one of the most important detoxication mechanism in animal body and has been understood to be catalyzed by uridine diphosphate transglucuronylase which was first described by Dutton and Storey.[1] On the other hand, Fishman and Green have provided another conjugation mechanism, in which β-glucuronidase catalyzes the transfer of the glucuronyl group from ethereal glucuronides to acceptor alcohols.[2]

While the former mechanism is acceptable for formation of all kinds of glucuronides, the latter involves certain limitation for its general application, since β-glucuronidase have been shown to catalyze the transfer to alcohols, but not to phenolic acceptor when ethereal glucuronides were used as the donor substrate.

The present investigation has been undertaken to recheck the previous results described above and at the same time, to decide which is a preferential mechanism for glucuronide formation in animal body by comparing the activity of both systems, in which the same acceptors were used. For this purpose, the several phenolic compounds, such as 2-naphthol, *p*-cresol, *p*-nitrophenol, *m*-aminophenol, and *p*-aminosalicylic acid were employed as an acceptor and shown to be transfered glucuronyl group from uridine diphosphate glucuronic acid by the former mechanism, but not from phenolphthalein β-D-glucuronide or *p*-nitrophenyl β-D-glucuronide by β-glucuronidase system.

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It was however shown in this study, same as in Fishman's experiment, that β-glucuronidase could catalyze the transfer of glucuronyl group from *p*-nitrophenyl β-D-glucuronide to methanol.

Materials and Method

Donor Substrates and Reference Compounds——β-Glucuronides of phenolphthalein[3] and *m*-aminophenol[4] were isolated from the urine of rabbits administered with aglycons. Ester and ether type glucuronides of *p*-aminosalicylic acid[5] were purified from the urine of rabbits administered with the aglycon. β-Glucuronides of *p*-nitrophenol,[6] 2-naphthol,[7] *p*-cresol,[7] and MeOH[8] were chemically synthesized.

UDP Glucuronic Acid——The 'crude' nucleotide was used according to the method of Dutton and Storey.[1a]

Preparation of Suspension——Rats were used as the source of liver suspension. The 10% suspension was prepared according to the method of Dutton and Storey.[1a]

β-Glucuronidase——Rabbit liver β-glucuronidase was partially purified by a modification of the method of Talalay, *et al.*[9]

Paper Chromatography——Ascending development was employed with Toyo Roshi No. 50. Solvent systems employed were (A) BuOH-AcOH-H_2O (4:1:5), (B) AcOEt-AcOH-H_2O (5:2:2). Products were detected by spraying the following reagents on a chromatogram: (1) Ehrlich reagent; (2) diazobenzene sulfonic acid; (3) $NaIO_4$-benzidine;[10] (4) 10% NaOH; (5) conc. HCl-EtOH (2:1). Identification of spots was made by reference to standard compounds rather than by measurement of Rf values.

Results

Glucuronide Formation by UDP-Transglucuronylase System

The method of Dutton and Storey was used. The composition of the incubation mixtures are given in the legend to Table I. After incubation for 1.5 hours at 37°, the mixture was boiled for 3 minutes, cooled, and centrifuged to remove most of the flocculated protein. Each supernatant solution was treated as follows.

2-Naphthol, *p*-cresol, and *p*-nitrophenol: Each supernatant was concentrated to a small volume at 25~30° under reduced pressure. The concentrated solution was brought to pH 7.0 with sodium bicarbonate and extracted with ether to remove the substrate. The solution was brought to pH 3.0 with hydrochloric acid and the conjugate was extracted with ethyl acetate. The ethyl acetate solution was dried with anhyd. sodium sulfate and distilled under reduced pressure to remove most of the solvent. The residue was analyzed by paper chromatography with solvent A.

The chromatograms indicated the spots corresponding to 2-naphthyl β-D-glucuronide, *p*-cresyl β-D-glucuronide, and *p*-nitrophenyl β-D-glucuronide. These conjugates were hydrolyzed on filter paper by spraying the reagent (5) followed by heating at 75° for 7 minutes. The conjugates of 2-naphthol and *p*-cresol were then visualyzed by spraying the reagent (2). The conjugate of *p*-nitrophenyl was visualyzed with the reagent (4).

m-Aminophenol and *p*-aminosalicylic acid: Each supernatant was concentrated to a small volume at 25~30° under reduced pressure. *m*-Aminophenol and *p*-aminosalicylic acid were extracted from the solution with ether after the solution was adjusted

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to pH 7.0 and 3.5 respectively. The solution was then distilled under reduced pressure at 25~30° to remove most of the water. Each resultant syrup was then analyzed by paper chromatography with solvent A. In the case of *p*-aminosalicylic acid, the chro matogram indicated two spots corresponding to ester and ether type glucuronides of *p*-aminosalicylic acid, and in the case of *m*-aminophenol, the chromatogram indicated the spot corresponding to *m*-aminophenyl β-D-glucuronide.

TABLE I. Paper Chromatography of the Conjugates

Compound		Rf	Reagent
2-Naphthol		0.69	2
p-Cresol		0.83	2
p-Nitrophenol		0.48	4
p-Aminosalicylic acid	ester	0.28	1
	ether	0.17	1
m-Aminophenol		0.14	1

Incubation mixtures (60 ml.) contained 6 ml. of 0.5*M* potassium phosphate buffer, pH 7.8, 4 ml. of substrate solution (final concentration 1.4×10^{-4}), 2 ml. of 0.3*M* $MgCl_2$, 20 ml. of 'crude' co-factor, 10 ml. of the 10% suspension, and 18 ml. of H_2O. Controls for these experiments contained the boiled enzyme.

Glucuronyl Transfer by β-Glucuronidase System

The method of Fishman and Green was used. Donor substrates used were phenolphthalein and *p*-nitrophenol glucuronides. A large scale incubation mixtures were prepared containing 80 ml. of 0.1*M* acetate buffer, pH 4.5, 10 ml. of 0.01*M* phenolphthalein or *p*-nitrophenol glucuronide, 8.3 ml. of water, 1.7 ml. of β-glucuronidase solution (15,000 Fishman units), and crystalline acceptor phenols in the final concentration of 0.1*M* except 2-naphthol. The final concentration of 2-naphthol was 0.014*M*. After incubation for 2 hours at 38°, the mixture was boiled for 1 minute, cooled, and centrifuged to remove most of the flocculated enzyme protein. The supernatant was concentrated to a small volume under reduced pressure at 25~30°, and treated as follows.

2-Naphthol, *p*-cresol, and *p*-nitrophenol as acceptors: Each concentrated solution was brought to pH 7.0 and extracted with ether. The extract contained hydrolyzed donor aglycon and acceptor was discarded. The solution was then brought to pH 3.0 with hydrochloric acid and the transfer products were extracted with ethyl acetate. The ethyl acetate solution was dried over anhyd. sodium sulfate and distilled under reduced pressure to remove most of the solvent. The residue was analyzed by paper chromatography with solvent A.

m-Aminophenol and *p*-aminosalicylic acid as acceptors: The concentrated solution was brought to pH 7.0 and 3.5 respectively, and extracted with ether. The ether extract was discarded. These solution was brought to pH 3.0 and extracted with ethyl acetate to remove donor substrate, and then distilled under reduced pressure at 25~30° to remove most of the water. Each resultant syrup was analyzed by paper chromatography. In all experiments, the transfer products were not detected by paper chromatography. So, the phenols was also unsuccessful acceptor in our experiments.

In the present investigation glucuronyl transfer from *p*-nitrophenyl glucuronide to methanol was also examined. The transfer product was identified by paper chromatography and percentage of glucuronyl transfer activity was measured. Percentage of glucuronyl transfer activity was defined as the ratio of micromoles of glucuronide formed to micromoles of *p*-nitrophenol liberated.

A large scale incubation mixture was prepared containing 78.3 ml. of 0.1*M* acetate buffer, pH 4.5, 10 ml. of 0.01*M* *p*-nitrophenyl glucuronide, 10 ml. of methanol (final concentration 2.48*M*), and 1.7 ml. of the enzyme solution (15,000 Fishman units). The

controls for this experiment contained boiled enzyme. After incubation for 2 hours at 38° the mixture was treated as described above. In this case, in order to purify the transfer product, the concentrated solution was chromatographed on a sheet of filter paper (40×40 cm.) using solvent B. The area corresponding to sodium methyl glucuronate was cut out, eluted with water, and the eluate was evaporated to dryness under reduced pressure. The residue was again developed on a paper strip using the same solvent. The spot which had the same Rf value as that of sodium methyl glucuronate (Rf 0.30) was obtained. This spot was detected with the reagent (1) and was positive to naphthoresorcinol test after extraction with water.

Percentage of glucuronyl transfer activity was measured by a modification of the method of Fishman and Green.[2] The incubation mixture (5 ml. of total volume) contained 0.5 ml. of 0.01*M* *p*-nitrophenyl glucuronide, 0.5 ml. of enzyme solution (500 Fishman units), 0.5 ml. of methanol, and 3.5 ml. of *M*/15 phosphate buffer, pH 6.0. The control for this experiment contained boiled enzyme. After incubation for 30 minutes at 38°, the mixture was boiled for 1 minute, cooled, and centrifuged. From the supernatant, a 0.2 ml. aliquot was removed and the free aglycon was measured by the method of Kato, *et al.*[6] A 3 ml. aliquot was acidified with 0.3 ml. of 6*N* sulfuric acid and made up to 15 ml. with water. This was extracted four times with ethyl acetate followed by the addition of 0.15 ml. of conc. sodium hydroxide (100 g. plus 100 ml. H_2O) and then restored to 15 ml. with water. Analysis of total glucuronic acid and glucuronide formed were performed on 5 ml. of aliquots. The result of this experiment was shown in Table II.

Table II. Glucuronyl Transfer by β-Glucuronidase from *p*-Nitrophenol Glucuronide to Methanol

p-Nitrophenol glucuronide concentration (*M*)	Methanol concentration (*M*)	*p*-Nitrophenol liberated (μ*M*/ml.)	Free glucuronic acid (μ*M*/ml.)	Methyl glucuronide		Glucuronyl transfer (%)
				Unextracted (μ*M*/ml.)	Computed (μ*M*/ml.)	
0.001	2.48	0.276	0.209	0.064	0.067	24.2

Summary

1. UDP-transglucuronylase catalyzed the transfer of the glucuronyl group from UDP glucuronic acid to phenolic compounds, such as 2-naphthol, *p*-cresol, *p*-nitrophenol, *m*-aminophenol, and *p*-aminosalicylic acid, but the glucuronyl transfer by β-glucuronidase from phenolphthalein β-D-glucuronide or *p*-nitrophenyl β-D-glucuronide to these phenolic compounds was not observed.

2. The transfer product by β-glucuronidase from *p*-nitrophenyl β-D-glucuronide to methanol was identified by paper chromatography, and percentage of glucuronyl transfer activity was also measured. This glucuronide transferred 24.2 per cent of the liberated glucuronic group to methanol.

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