each condensation products was affected by choice of solvent, the reaction temperature, the potassium xanthate, and starting halide used.

A simple method for the preparation of bis(2,2′,3,3′,4,4′,6,6′-octa-O-acetyl-β,β′-D-glucopyranosyl)sulfide (octa-O-acetyl-isothiotrehalose) was confirmed by the reaction of potassium methyl- or benzylxanthate upon 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide. Two other isomers of octa-O-acetyl-isothiotrehalose were synthesized as crystalline forms.

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92. Kazuo Yoshida, Keitaro Kato, and Hisao Tsukamoto : Metabolism of Drugs. XLIV.*1 Glucuronyl Transfer Reaction catalyzed by β-Glucuronidase by the Use of Ester Glucuronides as Substrates.

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Glucuronide conjugation is essentially a group transfer reaction in which the donor molecule donates its glucuronyl group to a suitable acceptor molecule. Dutton and Storey have reported that microsomal UDP-transglucuronylase catalyzes the transfer of the glucuronyl group from uridine diphosphate glucuronic acid to acceptor.[1] Another enzyme which is capable of transferring the glucuronyl group is β-glucuronidase. Fishman and Green have demonstrated that, under certain conditions, β-glucuronidase can transfer the glucuronic acid residue *in vitro* from ethereal glucuronides, such as aryl and alicyclic β-glucuronides, to simple alcohols and glycols.[2]

In their experiments, however, phenolic glucuronides could not be formed by this system. Until lately, there has been no information on a pathway of glucuronyl transfer with existing glucuronides acting as donors *in vivo*. In order to establish the glucuronyl transfer reaction of β-glucuronidase *in vivo*, it is necessary to identify the native endogenous glucuronyl donors and same time to prove phenolic glucuronides formation by this system.

The present investigation has been undertaken in order to clarify whether or not ester glucuronides, such as benzoyl β-D-glucuronide (B-GA), *p*-aminobenzoyl β-D-glucuronide (PABA-GA), and 2-hydroxy-4-aminobenzoyl β-D-glucuronide (PAS-GA), whose β-glucosiduronic linkage are labil could transfer their glucuronyl group to alcohols and phenols.

Materials and Methods

Donor Substrate and Reference Compounds——B-GA,[3] PABA-GA,[4] and PAS-GA[5] were isolated

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from the urine of animals administered with the corresponding aglycones. *m*-Aminophenyl β-D-glucuronide (MAP-GA)[6] was also prepared biosynthetically. Methyl β-D-glucuronide was synthesized by the method of Helferich and Berger[7] and crystallized as a Na salt. m.p. 265° (decomp.). $[\alpha]_D^{20}$ −50° (c=0.4, H_2O). *Anal.* Calcd. for $C_7H_{11}O_7Na$: C, 36.52; H, 4.78. Found : C, 36.44; H, 5.05.

Enzyme Preparations——The rabbit liver β-glucuronidase was partially purified according to a modification of the method of Talalay, *et al.*[8] The bacterial β-glucuronidase was obtained from Sigma Chemical Co.

Paper Chromatographic Method——Ascending development was employed with filter paper, Toyo Roshi No. 50. Solvent systems employed were (A) BuOH-AcOH-H_2O (4:1:5), (B) iso-PrOH-pyridine-H_2O (1:1:1), (C) AcOEt-AcOH-H_2O (5:2:2). Products were detected by spraying the following reagents on the chromatogram : (1) $NaIO_4$-benzidine[9]; (2) Ehrlich reagent, 2% *p*-dimethylaminobenzaldehyde in MeOH-conc. HCl (3:1), (3) diazo reagent, 0.2% $NaNO_2$, 0.1*N* HCl, and 0.2% Tsuda reagent (N-(2-dimethylaminoethyl)-1-naphthylamine oxalate) were successively sprayed. Identification of spots was made by reference to standard compounds rather than by measurement of Rf values.

Paper Electrophoretic Method——Toyo Roshi No. 50 filter paper (12×24 cm.) was used with a solution of the following composition : 1% borax solution; 0.2% borax solution; 30% AcOH. The paper was freely suspended in a horizontal plane and supported by a removable plastic frame work. The whole system of electrode vessels and the paper was placed in a closed chamber. Conditions of electrophoresis are given tn the legends to Fig. 3 to 6. Upon completion, the paper was dried at room temperature and sprayed with the same reagent as described in paper chromatographic method.

Colorimetric Method——PABA and PAS were determined with Ehrlich reagent. To 5 ml. of a test solution were added 5 ml. of 8% *p*-toluenesulfonic acid and 1 ml. of the the Ehrlich reagent. The absorbancy at 450 mμ was measured in Hitachi photoelectric spectrophotometer. BzOH was estimated by the method of Dickens, *et al.*[10] BzOH was subjected to nitration followed by reduction to aminobenzoic acid, and then diazotized. The diazotized aminobenzoic acid was coupled with the Marshall reagent, and the absorbancy at 530 mμ was measured. Free glucuronic acid and glucosiduronic acids were estimated by the method of Fishman and Green.[11]

Result

pH-Optima for Hydrolysis of Ester Glucuronides

The pH-optima for hydrolysis of ester glucuronides (B-GA, PABA-GA, and PAS-GA) by rabbit liver β-glucuronidase were determined in 0.1*M* acetate buffer between pH 3.2 and 5.0. The composition of incubation mixtures is given in the legend to Fig. 1. After enzymatic hydrolysis, 9 ml. of 0.2*M* acetate buffer, pH 3.8 and 3.5 were added to the reaction mixtures containing PABA-GA and PAS-GA as substrates respectively. Each liberated aglycon was immediately extracted twice with 10 ml. of ether. In the case of B-GA, 2 ml. of 1% hydrochloric acid and 2 ml. of water were added after incubation to the mixture. The liberated benzoic acid was extracted three times with 10 ml. of ether. Resulted ethereal extract was evaporated to dryness, and the aglycon was determined by the methods described above. All ester glucuronides examined were not extracted from aqueous solution by ether. Fig. 1 shows the pH-activity curves for β-glucuronidase from rabbit liver and *E. coli* acting on the ester glucuronides.

Action of β-glucuronidase on ester glucuronides was described by Levvy and Worgan.[12] They showed that pH-activity curve obtained for the hydrolysis of α-ethylhexanoyl glucuronide by mouse liver β-glucuronidase had two peaks at pH 4.4 and pH 5.1~5.2. In our experiment, however, the pH-optima for hydrolysis of B-GA, PABA-GA and PAS-GA by rabbit liver β-glucuronidase were 4.0, 3.8, and 3.5 respectively. The pH-optimum for the hydrolysis of PABA-GA by *E. coli* β-glucuronidase was 5.8.

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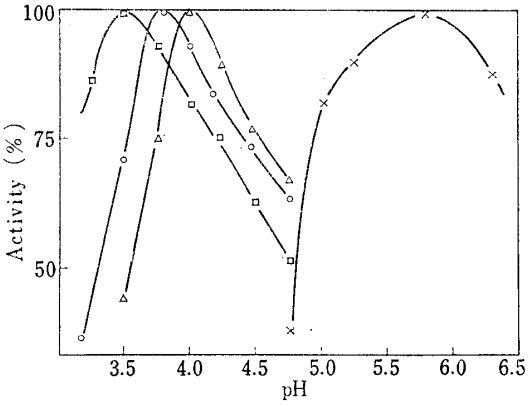

Fig. 1. Effect of pH on the Hydrolysis of 0.001*M* PABA-GA, 0.001*M* PAS-GA, and 0.01*M* B-GA by Rabbit Liver and *E. coli* β-Glucuronidase in 0.1*M* Acetate Buffer

In the case of PABA-GA and PAS-GA, the incubation mixtures contained 0.1 ml. of 0.01*M* PABA-GA or 0.01*M* PAS-GA, 0.1 ml. of enzyme solution and 0.8 ml. of 0.1*M* acetate buffer. In the case of B-GA, the incubation mixtures contained 0.6 ml. of 0.1*M* B-GA, 0.6 ml. of rabbit liver enzyme and 4.8 ml. of 0.1*M* acetate buffer. Incubation time was for 30 min. at 38°.

Rabbit liver enzyme: PABA-GA ○
PAS-GA □
B-GA △
E. coli enzyme: PABA-GA ×

Identification of the Transfer Product from PABA-GA to Methanol by Paper Chromatography

The incubation mixtures contained 10 ml. of 0.01*M* PABA-GA, 10 ml. of methanol, 2 ml. of enzyme solution (14,000 Fishman units), and 78 ml. of 0.1*M* acetate buffer, pH 4.5. After incubation for 2 hours at 38°, the mixture was boiled for 1 minute, cooled, and filtered to remove most of the flocculated enzyme protein. The filtrate was neutralized and concentrated under reduced pressure. The residue showed a spot whose Rf value was similar to that of sodium methyl glucuronate when examined by paper chromatography using Solvent C. This spot could not be detected in the control experiment. For further confirmation, this product was purified by the following procedure; the residue was developed on a sheet of filter paper (40×40 cm.) using the same solvent. The area corresponding to sodium methyl glucuronate was cut out, extracted with water and freed from water. The residue was submitted again to paper chromatography. This examination gave one spot on paper chromatogram which had the same Rf value as that of sodium methyl glucuronate. The transfer product was detected with the reagent (1). This spot was not visualized by spraying with aniline phthalate reagent, but was possitive to naphthoresorcinol test after extraction with water. The transfer product thus purified underwent complete hydrolysis by β-glucuronidase; this must be a good evidence for a β-glucosiduronic linkage.

Table I. Paper Chromatography of the Transfer Product with Methanol

Compound	Solvent system C Rf	Compound	Solvent system C Rf
Glucuronolactone	0.44	Transfer product	0.30
Methyl β-D-glucuronide	0.40	Glucuronic acid	0.19
Sodium methyl β-D-glucuronate	0.30		

Enzymatic Transfer of the Glucuronyl Group from Ester Glucuronides to Simple Alcohols

PABA-GA and PAS-GA were used as donor substrates because the released aglycons could be accurately and easily measured. Enzyme reaction was performed at each pH-optimum for hydrolysis of the substrates. The liberated donor aglycons were analyzed by the same method as described in the preceding paragraph. A newly formed glucuronide was estimated by the method of Fishman and Green after decomposition of donor substrate and liberated free glucuronic acid with alkali. Percentage of glucuronyl transfer activity is indicated as the ratio of micromoles of newly formed glucuronide to micromoles of released aglycone.

The composition of the reaction mixture (2 ml. of total volume) is given in the legend to Table II. After incubation for 30 minutes at 38°, each mixture was boiled for 1 minute, cooled, made up to 5 ml. with water, and centrifuged at 2000 r.p.m. for 10 minutes. From the supernatant, a 1 ml. aliquot was removed and the released aglycon was measured by the method described above. A 1 ml. aliquot was pipetted into a test tube contained 0.1 ml. of *N* sodium hydroxide and heated in a boiling water bath for 4 minutes, and then cooled in ice bath. It was demonstrated in other experiments that, by this alkali treatment, ester glucuronides, such as PABA-GA, PAS-GA, and B-GA, and free glucuronic acid were completely destroyed, but ethereal glucuronides, such as methyl and 2-naphthyl β-D-glucuronides, were not decomposed. Therefore, only the newly formed glucuronide could remain in the test tube after this procedure. After alkali treatment, 2.9 ml. of water was added to the test tube and analysis of the glucuronide was performed as described by Fishman and Green.[11] All alcohols examined displayed an ability to serve as glucuronyl acceptors. In the case of butanol, however, transfer was less than 10 per cent from either donor substrates. In Table II (Experiment 3) is listed the extent of glucuronyl transfer with *E. coli* β-glucuronidase. *E. coli* β-glucuronidase exhibited considerably high transfer activity for all tested alcohols.

Table II. Enzymatic Transfer of the Glucuronyl Group from Ester Glucuronides to Simple Alcohols

Alcohols	Acceptor concn. (*M*)	Aglycon liberated (μ*M*/ml.)	Alcohol glucuronide formed (μ*M*/ml.)	Glucuronyl transfer (%)
Experiment 1.	Donor-PABA-GA Enzyme-rabbit liver β-glucuronidase			
MeOH	2	0.839	0.198	23.5
EtOH	1.45	0.784	0.064	8.1
PrOH	0.66	0.365	0.053	14.5
BuOH	0.54	0.656	0.038	5.8
Experiment 2.	Donor-PAS-GA Enzyme-rabbit liver β-glucuronidase			
MeOH	2	0.645	0.127	19.7
EtOH	1.45	0.409	0.064	15.6
PrOH	0.66	0.297	0.019	6.4
BuOH	0.54	0.277	0.019	6.8
Experiment 3.	Donor-PABA-GA Enzyme-*E. coli* β-glucuronidase			
MeOH	2	1.040	0.910	87.5
EtOH	1.45	1.010	0.767	75.9
PrOH	0.66	1.010	0.837	82.8
BuOH	0.54	1.076	0.881	81.8

Each incubation mixture (2 ml. of total volume) contained 0.2 ml. of 0.01*M* donor substrate, 0.2 ml. of enzyme solution (500 Fishman unit), a volume of alcohols in the amount required to give the indicated final concentration; the remaining volume consisted of 0.1*M* acetate buffer (Experiment 1, pH 3.8; Experiment 2, pH 3.5; Experiment 3, pH 5.8). Controls for these experiments contained boiled enzyme.

Influence of pH on the Extent of Glucuronyl Transfer

PABA-GA and methanol were incubated with the rabbit liver enzyme in 0.1*M* acetate buffer between pH 3.5 and 5.75. The extent of glucuronyl transfer was measured as described above. The formation of the new glucuronide was a function of pH. The optimal range occured between 4.0 and 4.5. The maximum ratio of glucuronyl transfer to released aglycon was at pH 4.25 (Fig. 2).

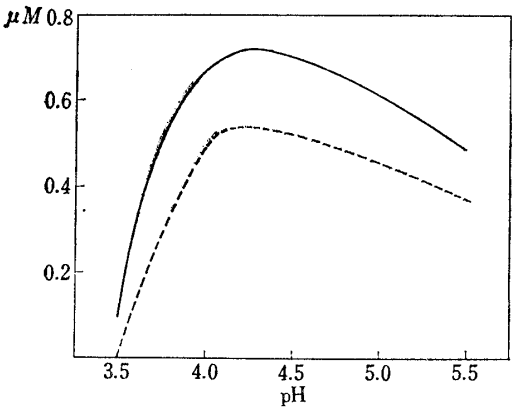

Fig. 2. Effect of pH on the Glucuronyl Transfer from PABA-GA to Methanol

Incubation mixtures contained 0.2 ml. of 0.01*M* PABA-GA, 0.17 ml. of methanol, 1.43 ml. of acetate buffer and 0.2 ml. of enzyme solution. Incubation time was for 30 min. at 38°.

——— PABA liberated
– – – – – Free glucuronic acid

Enzymatic Transfer of the Glucuronyl Group from Ester Glucuronides to *m*-Aminophenol (MAP)

In this experiment, PABA-GA and B-GA were used as donor substrates. Only a newly formed glucuronide which was not extracted from aqueous solution by ether was measured by the method of Fishman and Green after extraction of the liberated aglycon and the unreacted acceptor with ether followed by alkali treatment. The composition of the reaction mixtures (20 ml. of total volume) is given in the legends to Table Ⅲ. After incubation for 2 hours at 38°, each reaction mixture was boiled for 1 minute, cooled, and filtered. The filtrate was extracted five times with each 20 ml. of ether. The extracts containing PABA and MAP were discarded. This solution was concentrated to a small volume under reduced pressure and made up to 3 ml. with water. Since the donor substrate and free glucuronic acid were concentrated in this solution, the alkali treatment was performed in higher concentration of alkali for 15 minutes. However, *m*-aminophenyl β-D-glucuronide which was presumed as a transfer product of this reaction was not destroyed by this treatment. A 2 ml. aliquot was pipetted into a test tube which contained 2 ml. of *N* sodium hydroxide and heated in a boiling water bath for 15 minutes. A newly formed glucuronide was then measured by the same method as described above. The result obtained are shown in Table Ⅲ.

Table Ⅲ. Enzymatic Transfer of the Glucuronyl Group from Ester Glucuronides to *m*-Aminophenol (MAP)

Acceptor concn. (*M*)	Glucuronide formed (γ)	Acceptor concn. (*M*)	Glucuronide formed (γ)
Experiment 1. PABA-GA⟶MAP		Experiment 2. B-GA⟶MAP	
0.1	10	0.1	15
0.3	62	0.3	66
0.45	45	0.45	66

Each incubation mixture (20 ml. of total volume) contained 2 ml. of 0.01*M* donor substrate, 16 ml. of 0.1*M* acetate buffer (pH 4.5), crystalline MAP in the amount required to give the indicated final concentrations, and 2 ml. of rabbit liver enzyme (5000 Fishman units). Controls for these experiments contained boiled enzyme.

Purification of the Product formed by Glucuronyl Transfer from B-GA to MAP

The incubation mixture contained 29.8 mg. of B-GA (final concentration 0.001*M*), 3.27 g. of MPA (final concentration 0.3*M*), 98 ml. of 0.1*M* acetate buffer (pH 4.5), and 2 ml. of the rabbit liver enzyme (25,000 Fishman units). The control for this experiment contained boiled enzyme. After incubation for 2 hours at 38°, the reaction mixture was boiled for 1 minute, cooled, and centrifuged at 3000 r.p.m. for 15 minutes to remove most of the flocculated enzyme protein. The supernatant solution was extracted five times with each 50 ml. of ether.

The extracts containing released aglycon and MAP were discarded. This solution was concentrated to about 30 ml. under reduced pressure and passed through the column

packed with 100 ml. of Amberlite IR-120. The column was washed with 300 ml. of water and eluted with 4% ammonia. About 150 ml. of the effluent was collected and concentrated to about 30 ml. at 25~30° under reduced pressure. This solution was extracted with 30 ml. of ether and evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml. of water and rechromatographed on a column, in which 70 ml. of Amberlite IRC-50 was packed. The column was eluted with water. 150 ml. of the effluent was collected and concentrated to a small volume under reduced pressure. The concentrated solution was analyzed by paper electrophoresis. This examination showed the presence of the transfer product which had the same migration rate as that of authentic MAP-GA. In Fig. 3, Experiment 1 and 2 show the electrophoretic pattern of this product. Paper chromatography of the product, however, revealed the different Rf value from that of authentic MAP-GA. The Rf values of the product and authentic MAP-GA are shown in Table Ⅳ. For further confirmation, the product was methylated with diazomethane and the methylated product was analyzed by paper electrophoresis and paper chromatography. The methylated transfer product had the same migration rate as that of authentic methyl *m*-aminophenyl β-D-glucuronate in

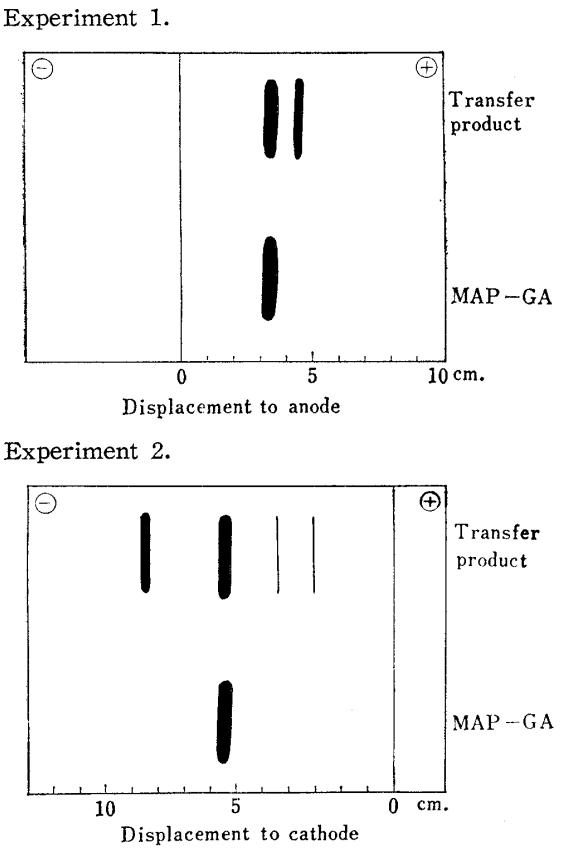

Fig. 3. Paper Electrophoresis of the Product formed by Glucuronyl Transfer from B-GA to MAP

Experiment 1: Electrophoresis was carried out for 1 hr. with a potential of 400 v. between the electrodes and 1% borax solution.

Experiment 2: Electrophoresis was carried out for 40 min. with a potential of 700 v. between the electrodes and 30% acetic acid.

The compounds were detected with the reagents (2) and (3).

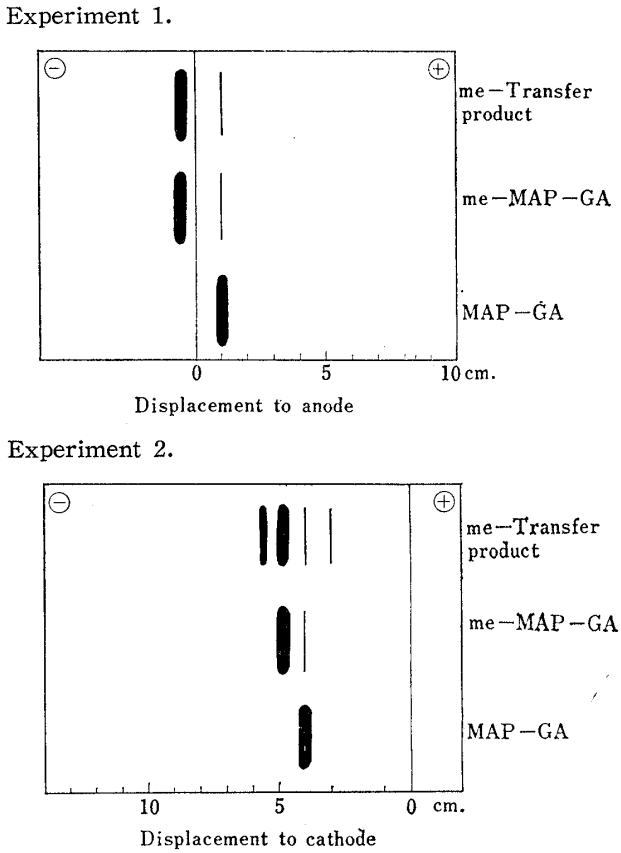

Fig. 4. Paper Electrophoresis of the methylated Transfer Product

Experiment 1: Electrophoresis was carried out for 1 hr. with a potential of 400 v. between the electrodes and 0.2% borax solution.

Experiment 2: Electrophoresis was carried out for 40 min. with a potential of 700 v. between the electrodes and 30% acetic acid.

me-MAP-GA was used without further purification after the methylation of MAP-GA with diazomethane.

The compounds were detected with the reagents (2) and (3).

paper electrophoresis, but the Rf value of the former was different from that of the later in paper chromatography. The Rf value of these compounds are shown in Table Ⅳ. In Fig. 4, the electrophoretic pattern of the methylated transfer product was shown. In all above paper chromatographic and paper electrophoretic examinations, the product was visualized on papers by spraying with Ehrlich and diazo reagents, and water extracts of this area gave a positive naphthoresorcinol test. The same transfer product was not obtained from the control and from the experiments using *p*-nitrophenyl glucuronide and phenolphthalein glucuronide as donor substrates.

Table Ⅳ. Paper Chromatography of the Transfer Product

Compound	Rf values with solvents	
	A	B
Transfer product	0.09	0.49
MAP-GA	0.12	0.56
MAP	0.61	0.86
Methylated transfer product	0.50	0.82
me-MAP-GA	0.53	0.84

The compound were detected with the reagnts (2) and (3).

The purified transfer product was incubated with rabbit liver β-glucuronidase in 0.1*M* acetate buffer, pH 4.5, for 2 hours at 38°, but this was not hydrolyzed by the enzyme. After heating at 100° in 10% hydrochloric acid for 1 hour, it was partially hydrolyzed and a small amount of MAP was detected in the hydrolyzate by paper chromatography and paper electrophoresis. On the other hand, authentic MAP-GA underwent complete hydrolysis by β-glucuronidase and acid treatment under the same conditions. In order to investigate whether the transfer product was a conjugated compound of MAP and glucuronic acid or not, the MAP-glucuronic acid ratio of this compound was analyzed. The molar ratio of MAP to glucuronic acid was close to 1. On the basis of above findings, it was likely that the transfer product was a conjugated compound of MAP and glucuronic acid, but not the β-glucosiduronic acid of MAP. However, there is not yet enough evidence for a α-glucosiduronic linkage.

Discussion

Fishman and Green reported that β-glucuronidase catalyzed the transfer of the glucuronyl group *in vitro* from donor-glucuronides to simple alcohols and glycols.[2] In their experiments, glucuronides of stilbesterol, 2,4-dichloro-1-naphthol *p*-chlorophenol, phenolphthalein, menthol, 8-hydroxyquinoline were used as donor substrates. These glucuronides transferred from 50 to 80 per cent of the released glucuronyl group to propylene glycol. Subsequently, however, Levvy and Marsh have criticized this report for several reasons,[13] one of which is that attempts to employ phenols or terpene alcohols as acceptors were unsuccessful.

In the well known types of glucuronides, which appear in an animal body and are susceptible to the hydrolytic action of β-glucuronidase, ester glucuronides, have most labil β-glucosiduronic linkage. It is conceivable that this type of glucuronide may play a broad role in metabolism due to its lability. Therefore, it would seem more suitable to use ester glucuronides as donor substrates in the glucuronyl transfer reaction of β-glucuronidase. In the present investigation it was provided that ester glucuronides

13) G. A. Levvy, C. A. Marsh : Advances in Carbohydrate Chem., **14**, 381 (1959).

functioned as donors and simple alcohols tested served as glucuronic acid acceptors *in vitro*. Of the data listed in Table II, it is of interest that *E. coli* β-glucuronidase exhibited the high extent of glucuronyl transfer and the donor transferred from 75 to 90 per cent of the released glucuronic acid to all alcohols tested.

Since the glucuronyl transfer reaction catalyzed by β-glucuronidase requires high acceptor concentrations, it is necessary to employ suitable phenols which have greater solubility in water. However, it is difficult to attain the desired concentration of phenols and that higher concentration of phenols causes their inhibiting action on the enzyme. In this connection, *m*-aminophenol (MAP) fitted as the best acceptor amongst the phenols examined. Furthermore, in the case of this acceptor, a newly formed glucuronide can be easily and sensitively detected with the Ehrlich and diazo reagents. The amount of the transfer product from ester glucuronides to MAP was small because of lower solubility of MAP than simple alcohols. In this instance, therefore, the conditions were less favorable for the transfer raction. The transfer product which was purified by ion exchange resin contained equimolar amounts of MAP and glucuronic acid. The paper electrophoretic behaviors of this transfer product and the methylated transfer product were similar to those of authentic MAP-GA and methylated MAP-GA. However, in paper chromatography, the former compounds showed different Rf values from those of the latter compounds. This product was not hydrolyzable by β-glucuronidase and more stable in acidic milieu than authentic MAP-GA. These facts suggested the presence of β-glucosiduronic linkage in the product but the exact proof remained obscure.

The experiment using PAS-GA as a doner substrate also performed. In this instance, the incubation mixture lacked acceptor compounds, for PAS-GA had a phenolic hydroxyl group in its molecule. So, inter- or intramolecular transfer reactions were expected in this experiment. However, this attempt have been unsuccessful so far. This is due in part to the difficulty of attaining the desired concentration of the acceptor phenolic hydroxyl group because of limited solubility of PAS-GA.

Summary

1. The glucuronyl transfer reaction from ester glucuronides to aliphatic alcohols were examined with partially purified rabbit liver β-glucuronidase and *E. coli* β-glucuronidase. The enzymes transferred the glucuronic acid moiety of ester glucuronides to aliphatic alcohols. The product with methanol was identified to be methyl glucuronide by paper chromatography. The product was completely hydrolyzable by β-glucuronidase, proving it to be a β-glucosiduronic acid of methanol.

2. The glucuronyl transfer from ester glucuronides to *m*-aminophenol was examined. The product formed by glucuronyl transfer from benzoyl β-D-glucuronide to *m*-aminophenol was purified with ion exchange resin. It contained equimolar amounts of *m*-aminophenol and glucuronic acid. The paper electrophoretic and paper chromatographic behavior of this purified product was presented. In paper chromatography, the Rf value of this product was different from that of authentic *m*-aminophenyl β-D-glucuronide. This product was not hydrolyzable by β-glucuronidase and more stable in acidic milieu than authentic *m*-aminophenyl β-D-glucuronide. These facts suggested the presence of α-glucosiduronic linkage in its molecule.

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