The authors are grateful to Mr. M. Matsui, Director of this Laboratory, and Prof. K. Tsuda of the University of Tokyo for encouragement throughout this work. The measurement of infrared and ultraviolet spectra were carried out by Messrs. O. Amakasu, H. Higuchi, N. Higosaki, and Miss N. Sawamoto. Microanalyses were made by Messrs. T. Onoe and H. Nagashima, and Misses C. Furukawa and H. Ohtsuka.

Summary

It was found that methyl iodide of acetylenic Mannich base reacted with active methylene compounds to form alkylated acetylenic compounds with a loss of tertiary amine. Various acetylenic compounds may be prepared by this method. It seems likely that such alkylation reactions proceed mainly by S_N-1 mechanism in alcohol and mainly by S_N-2 mechanism in dibutyl ether.

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14. **Hisao Tsukamoto**[*1] **and Seisuke Terada**[*2]: Metabolism of Drugs. XXVI.[*3] Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (2).[*4]

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In the previous paper,3) it was reported that the ether-type glucuronide of *p*-hydroxybenzoic acid was isolated as the methylacetyl derivative on methylation and acetylation of the glucuronide fraction which was separated by lead acetate1) from the urine of rabbits receiving methyl *p*-hydroxybenzoate, and its structure had been established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (I).

In the present investigation, five metabolites; *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide(ether-type glucuronide), *p*-hydroxybenzoyl glucuronide(ester-type glucuronide), and *p*-carboxyphenyl sulfate, were identified in the urine of rabbits receiving methyl *p*-hydroxybenzoate. The three main metabolites, the ether-type glucuronide, *p*-hydroxyhippuric acid, *p*-hydroxybenzoic acid, were isolated as crystalline materials. In the present series of work, structure of the ether-type glucuronide was established as *p*-carboxyphenyl-β-D-glucopyranosiduronic acid by identification with the authentic sample prepared by saponification of (I).

Experimental

Separation of the Metabolite Fraction from the Urine of Rabbits——The animals used were male rabbits weighing 2.6～3.2 kg. They were housed in metabolism cages and fed "Okara" (soybean curd residue). A total dose of 7.2 g. of methyl *p*-hydroxybenzoate (0.8 g./kg. body wt.) was administered as a 12% solution in the form of Na salt to 3 rabbits by a stomach tube.

[*1] Katakasu, Fukuoka (塚元久雄).

[*2] Nakagawa-cho, Nagasaki (寺田精介).

[*3] Part XXV. H. Tsukamoto, M. Yoshimura: This Bulletin, **9**, 584 (1961).

[*4] Part (1). H. Tsukamoto, S. Terada: *Ibid.*, **8**, 1066 (1960).

1) I.A. Kamil, J.N. Smith, R.T. Williams: Biochem. J., **50**, 235 (1951).

The 24-hr. urine collected was filtered through cotton, the filtrate was adjusted to pH 4, and treated with saturated $(AcO)_2Pb$ solution. The filtrate separated from the precipitate was adjusted to pH 8 and saturated basic lead acetate solution was added in excess. The basic lead salt was collected, decomposed with H_2S in MeOH, and the filtrate was evaporated to dryness in a reduced pressure. A small amount of H_2O was added to the residue and the mixture was extracted with Et_2O to remove *p*-hydroxybenzoic acid (2.4 g.) excreted as a metabolite. The aqueous solution was evaporated to dryness in a reduced pressure and a crude gum (3.5 g.) was obtained.

Isolation of *p*-Hydroxyhippuric Acid and Ether-type Glucuronide of *p*-Hydroxybenzoic Acid——The crude gum was dissolved in about 30 cc. of H_2O and the solution was continuously extracted with Et_2O in a Soxhlet liquid extractor for 30 hr. Et_2O solution was dried by evaporation and the residue was treated with activated charcoal in MeOH. The filtrate was evaporated to dryness in a reduced pressure and the residual solid was recrystallized from hot H_2O to colorless needles, m.p. 236～238°. Yield, 0.35 g. *Anal.* Calcd. for $C_9H_9O_4N$: C, 55.38; H, 4.65; N, 7.18. Found : C, 55.58; H, 4.75; N, 7.16.

This was non-reducing and developed no color with naphthoresorcinol reagent, but was positive to the Millon and Ninhydrin tests after acid hydrolysis. From the result of elemental analyses and color reactions, this compound was identified as *p*-hydroxyhippuric acid.[2]

The mother liquor, left after removal of the above *p*-hydroxyhippuric acid, was evaporated to dryness in a reduced pressure. The dried residue was dissloved in a minimum of MeOH and $(iso\text{-}Pr)_2O$ was cautiously added dropwise to the solution until a slight turbidity developed. After the filtrate was allowed to stand overnight, the crystalline mass produced was collected and recrystallized repeatedly from MeOH-$(iso\text{-}Pr)_2O$ to colorless needles, m.p. 193～195°(decomp.). Yield, 0.1 g. *Anal.* Calcd. for $C_{13}H_{14}O_9$: C, 49.69; H, 4.49. Found : C, 49.78; H, 4.31.

This compound showed no depression of m.p. when mixed with the authentic *p*-cacboxyphenyl-β-D-glucopyranosiduronic acid (II). The infrared absorption spectrum, behavior to hydrolyses, and color reactions were also the same as those of the authentic sample. The course of separation of the metabolites is shown in Chart 1.

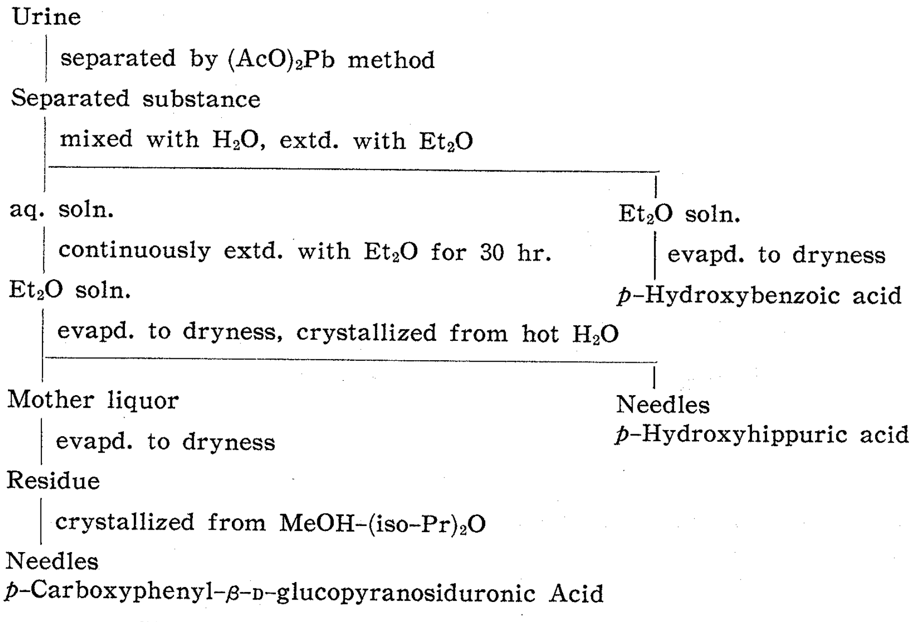

Chart 1. Separation of Metabolites from Urine

The aqueous solution, left after removal of *p*-hydroxyhippuric acid and the ether-type glucuronide by continual extraction with Et_2O, was purified by the same procedure as previously reported[3] and gave a purified gum. Attempt to isolate the ester-type glucuronide as the methylacetyl derivative from this gum was unsuccessful, this derivative being obtained only in the case of ether-type glucuronide.

Preparation of *p*-Carboxyphenyl-β-D-glucopyranosiduronic Acid (II)——To 0.5 g. of methylacetyl compound (I) 25 cc. of 2% NaOH solution was added and the mixture was heated at 70° for 2 hr. with occasional shaking. The clear hydrolyzed mixture was adjusted to pH 8 with AcOH and saturated basic lead acetate solution was added in excess. The basic lead precipitate produced was

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[2] A. J. Quick : J. Biol. Chem., **97**, 403 (1932).

[3] H. Tsukamoto, S. Terada : This Bulletin, 8, 1066 (1960).

collected and washed thoroughly with H_2O. This was made into a fine suspension in MeOH and the lead salt was decomposed by treatment with H_2S. After removal of PbS by filtration, MeOH solution was evaporated to dryness in a reduced pressure and the white residue was dried in a desiccator. The dried solid was dissolved in a minimum of MeOH and $(iso\text{-}Pr)_2O$ was cautiously added dropwise to the solution until a slight turbidity developed. A gelatinous precipitate that formed was removed by filtration and the filtrate was allowed to stand overnight in a refrigerator. A crystalline material (decomp. 190∼195°) produced was collected and recrystallized twice from MeOH-$(iso\text{-}Pr)_2O$ to colorless needles, m.p. 193∼195°(decomp.); $[\alpha]_D^{14}$ −92.3°(c=0.92, $CHCl_3$). Yield, 0.2 g. *Anal.* Calcd. for $C_{13}H_{14}O_9$: C, 49,69; H, 4.49. Found : C, 49.65; H, 4.55.

This compound was non-reducing, positive to naphthoresorcinol test and Et_2O extract after its acid hydrolysis gave a red color with the Millon reagent.

Conversion of (II) to (I)——A solution of (II)(0.1 g.) in 5 cc. of MeOH was cooled in ice and an Et_2O solution of CH_2N_2, freshly prepared from 1 g. of nitrosomethylurea, was added. The mixture was allowed to stand overnight in a refrigerator, the solvent was removed by evaporation, and the residue was dissolved in 1.0 cc. of pyridine and 0.7 cc. of Ac_2O. The reaction mixture was allowed to stand overnight at room temperature and poured into 10 cc. of ice-water with stirring. The precipitate produced was collected and washed successively with 2% H_2SO_4 and H_2O. The dried white solid was recrystallized from Et_2O-petr. ether to colorless needles, m.p. 157∼158°. Yield, 0.1 g. The compound showed no depression of m.p. with the authentic (I).

The infrared absorption spectra of (I) and (II) are shown in Fig. 1 and the route of preparation correlative to those is illustrated in Chart 2.

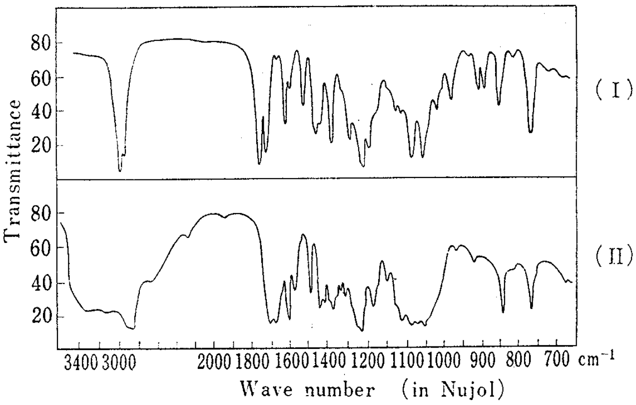

Fig. 1. Infrared Absorption Spectra

(I) Methyl (*p*-Methoxycarbonylphenyl 2,3,4-tri-O-acetyl-*β*-D-glucopyranosid)uronate

(II) *p*-Carboxyphenyl-*β*-D-glucopyranosiduronic acid

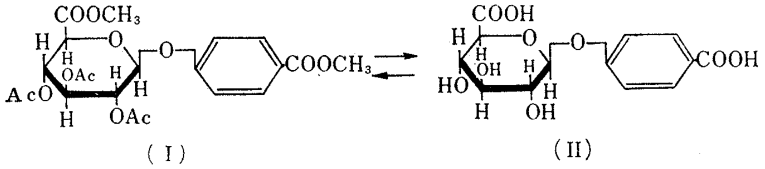

Chart 2.

Paper Chromatography of the Metabolites——It has previously been shown[3] that the gum, obtained from the urine of rabbits receiving methyl *p*-hydroxybenzoate, gave five spots at Rf 0.88, 0.74, 0.57, 0.48, and 0.38 on the paper chromatogram using the solvent system of BuOH-AcOH-H_2O, and that the spots of Rf 0.88, 0.57, and 0.48 are those of *p*-hydroxybenzoic acid, the ether-type glucuronide and the ester-type glucuronide, respectively.

For the chromatography, ascending development was employed with Toyo Roshi No. 50 and the solvent system used was BuOH-AcOH-H_2O(4:1:5). The spot of metabolites was detected with the following reagents : (1) 1% $NaIO_4$, then 1% $KMnO_4$, followed by benzidine reagent;[4] (2) $Hg(NO_3)_2$ reagent (20 parts of 10% $Hg(NO_3)_2$ solution mixed with 1 part of conc. HNO_3 just before spraying. The chromatogram sprayed with this reagent is placed in an oven at 90∼100° for 5∼10 min. and *p*-hydroxybenzoic acid derivative appears as lilac or pink spot on the paper); (3) diazotized sulfanilic acid[5] or *p*-nitroaniline[6]; (4) aniline hydrogenphthalate reagent[7]; and (5) Ninhydrin reagent.[8]

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[4] M.L. Wolfrom, J.B. Miller : Anal. Chem., **28**, 1037 (1956).
[5] T. Mann, E. Leone : Biochem. J., **53**, 140 (1953).
[6] H.C. Bray, W.V. Thorpe, K. White : *Ibid.*, **46**, 271 (1950).
[7] S.M. Partrige : Nature, **164**, 443 (1949).
[8] E.F. McFarren, J.A. Mills : Anal. Chem., **24**, 650 (1952).

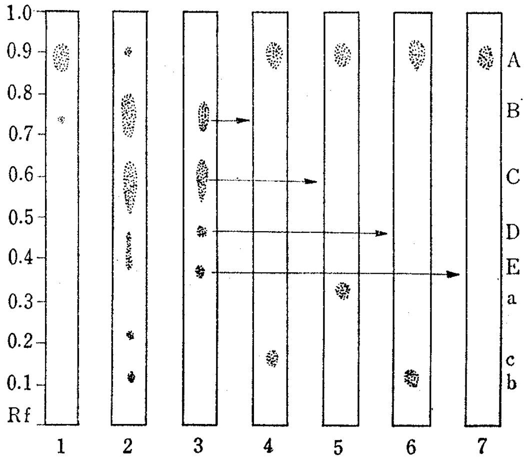
Paper chromatography of the crude gum separated from the urine revealed 2 large spots at Rf 0.74 and 0.57, and a small spot at Rf 0.42~0.46 by spraying with reagents (1), (2), and (3). However, in the case of the gum obtained after continuous extraction with Et_2O, the spots became small zones at Rf 0.74 and 0.57, and new spots appeared at Rf 0.48 and 0.38.

To detect unknown metabolites, a solution of this gum was developed on a large filter paper (40 × 40 cm.), each zone whose position was tested with color reagents, was cut out and eluted with 50% MeOH. The eluate was filtered and the filtrate was concentrated to a small volume in a reduced pressure.

The extract from Rf 0.74 section, which came in a large quantity, was resistant to alkaline hydrolysis. After hydrolysis with 5% HCl in a steam-bath for 60 min., the paper chromatogram showed 2 spots corresponding to *p*-hydroxybenzoic acid (Rf 0.88) and glycine (Rf 0.15) with reagents (2) and (5). Consequently, this substance may be considered as *p*-hydroxyhippuric acid.

In the case of a section corresponding to Rf 0.38, the original spot disappeared after acid hydrolysis under a similar condition as above and a new spot appeared at Rf 0.88. The fraction of Rf 0.38 was identified with the synthetic *p*-carboxyphenyl sulfate[9] in points of the chromatographic behavior and the acid hydrolysate being obviously positive to SO_4 test with Ba rhodizonate.

The foregoing evidence shows that a rabbit receiving methyl *p*-hydroxybenzoate excretes three main metabolites in the urine, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, and *p*-carboxyphenylglucuronide, and two minor metabolites, *p*-hydroxybenzoyl glucuronide and *p*-carboxyphenyl sulfate. Chromatographic data of urinary metabolites are summarized in Fig. 2.



Solvent System : BuOH-AcOH-H_2O(4:1:5)
1) Et_2O extract
2) Gum before continuous extraction with Et_2O
3) Gum after continuous extraction with Et_2O
4) Acid hydrolysate of Rf 0.74 section
5) Acid hydrolysate of Rf 0.57 section
6) Alkaline hydrolysate of Rf 0.48 section
7) Acid hydrolysate of Rf 0.38 section
A, *p*-hydroxybenzoic acid
B, *p*-hydroxyhippuric acid
C, *p*-carboxyphenyl glucuronide
D, *p*-hydroxybenzoyl glucuronide
E, *p*-carboxyphenyl sulfate
a, glucurone b, glucuronic acid c, glycine

Fig. 2. Paper Chromatograms of the Gum obtained from the Urine of Rabbits.

Discussion

Since methyl *p*-hydroxybenzoate possesses two functional groups which could be metabolized, eight different metabolites are theoretically possible. The hydroxyl group may remain free or be combined with glucuronic acid or sulfuric acid, while the methoxycarbonyl group may be unconverted or become free carboxyl group on saponification, and in some case this free carboxyl group might combine with glycine or glucuronic acid. However, this compound was not excreted in the urine of rabbits receiving methyl *p*-hydroxybenzoate, as already reported[3] and this fact shows that the ester-decomposition would probably be very easy *in vivo*. It would be reasonable to suspect that methyl *p*-hydroxybenzoate will be converted to five metabolites in the body of rabbit.

On the metabolic fate of *p*-hydroxybenzoic acid using a dog, Quick[2] stated that a large quantity of *p*-hydroxyhippuric acid was isolated from the urine.

In the present experiment on feeding rabbits with methyl *p*-hydroxybenzoate, *p*-hydroxyhippuric acid and the ether-type glucuronide were successfully isolated on continual extraction with Et_2O from a gum separated by the lead acetate method from the urine. This ether-type glucuronide was structurally identified with the authentic *p*-car-

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[9] K. Yamaguchi : Nippon Kagaku Zasshi, **80**, 171 (1959).

boxyphenyl-β-D-glucopyranosiduronic acid. This compound was converted by methylation and acetylation to the methylacetyl derivative; methyl (*p*-methoxycarbonylphenyl 2,3,4-O-acetyl-β-D-glucopyranosid)uronate.

By the isolation of the ether-type glucuronide, there is no doubt that saponification of the methoxycarbonyl group in methyl *p*-hydroxybenzoate and the glucuronic acid conjugation with hydroxyl group take place.

In the study on the metabolism of methyl salicylate,[10] the ether-type glucuronide of salicylic acid has been isolated as methylacetyl derivative from the urine of rabbits, but the ester-type has not been obtained.

Although a portion of the ether-type glucuronide was isolated as crystalline material in the present work on the metabolism of methyl *p*-hydroxybenzoate, it was not possible to isolate the derivative of ester-type glucuronide from the gum obtained after separating the ether-type, contrary to the expectation.

The above facts suggest that the main metabolites of methyl *p*-hydroxybenzoate in rabbit are *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid and the ether-type glucuronide.

The present investigation showed that the ester-type glucuronide and *p*-carboxyphenyl sulfate occur in the urine only to a small amount. Further observation on metabolic fate of *p*-hydroxybenzoic acid will be discussed in a later paper.

The authors are indebted to Mrs. H. Mazume of Pharmaceutical Faculty, University of Nagasaki, for the determination of infrared spectra and for elemental analyses.

Summary

The ether-type glucuronide of *p*-hydroxybenzoic acid was isolated from the urine of rabbits receiving methyl *p*-hydroxybenzoate and its structure was established as *p*-carboxyphenyl-β-D-glucopyranosiduronic acid. Three main metabolites, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, and the ether-type glucuronide, were identified in the urine, and the ester-type glucuronide and *p*-carboxyphenyl sulfate were proved as minor metabolites by means of paper chromatography.

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[10] D. Robinson, R. T. Williams: Biochem. J., **62**, 23 (1956).