ethanolic KOH and acid fraction was separated in the usual way. The fraction was distilled to give 2.5 g. of white crystals (IX), b.p_{12} 167～169° (22.5%). Crude (IX) was purified through S-benzylthiuronium salt, followed by decomposition with conc. HCl. Pure (IX), m.p. 33.0°, was obtained after recrystallization from Me_2CO. *Anal.* Calcd. for $C_{12}H_{22}O_2$: C, 72.68; H, 11.18. Found : C, 72.43; H, 11.25.

S-Benzylthiuronium salt : m.p. 155.5° (from EtOH). *Anal.* Calcd. for $C_{20}H_{32}O_2N_2S$: C, 65.90; H, 8.85. Found : C, 65.45; H, 8.90.

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Summary

Anodic synthesis of saturated alicyclic compounds was carried out. Dihydroalepric acid and homodihydroalepric acid, analogs of chaulmoogric and hydnocarpic acids, were prepared by the mixed Kolbe reaction of cyclopentylacetic acid and half-ester of dibasic acid. ω-Cyclohexylhexanoic acid was obtained by the cross-coupling of cyclohexylacetic acid with methyl hydrogenadipate. 1,2-Dicyclopentylethane and 1,2-dicyclohexylethane were identified as the symmetrical coupling product of cyclopentylacetic acid and cyclohexylacetic acid, respectively.

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

(*Pharmaceutical Institute, Medical Faculty, University of Kyushu,*[*1] *and Hygienic Research Laboratory of Nagasaki Prefecture*[*2])

The alkyl *p*-hydroxybenzoates have been widely used as a preservative for galenicals, foods, and cosmetics. In the studies on the biotransformation of *p*-hydroxybenzoic acid in the animal body, unchanged *p*-hydroxybenzoic acid and *p*-hydroxyhippuric acid[1～3] have been isolated from human and dog urine, and 3,4-dihydroxybenzoic acid[4] has been detected in the urine of rabbit as the metabolites. On the occurrence of conjugated glucuronic acid, Quick[3] also demonstrated the diglucuronide excretion in the urine of man and dog receiving *p*-hydroxybenzoic acid, but monoglucuronide of *p*-hydroxybenzoic acid has not yet been isolated as a urinary metabolite of *p*-hydroxybenzoic acid or its esters.

As *p*-hydroxybenzoic acid and its esters possess two functional groups which would conceivably be metabolized, two monoglucuronides are possible, the ether, *p*-carboxyphenyl glucuronide, and the ester, *p*-hydroxybenzoyl glucuronide. In the present paper is described the glucuronide formation in the metabolism of methyl *p*-hydroxybenzoate using

*1 Katakasu, Fukuoka (塚本久雄).
*2 Nakagawa-cho, Nagasaki (寺田精介).
*3 Part XXII. H. Tsukamoto, H. Ide, E. Takabatake : This Bulletin, 8, 236(1960).
1) E. Baumann, E. Herter : Z. physiol. Chem., **1**, 244(1877～1878).
2) C. P. Schotten : *Ibid.*, **7**, 23(1882～1883).
3) A. J. Quick : J. Biol. Chem., **97**, 403(1932).
4) H. G. Bray, W. V. Thorpe, K. White : Biochem. J., **46**, 271(1950).

rabbits. Both forms of *p*-hydroxybenzoic acid glucuronides were identified by paper chromatography in the urine and the ether-type was isolated as methylacetyl derivative whose structure has been established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

Experimental

Separation of Glucuronide from the Urine of Rabbits—Separation of glucuronide from the urine used the $(AcO)_2Pb$ method, which is a general procedure of preparing glucuronides described by Williams, *et al.*[5]

The animals used were male rabbits weighing 2.4~2.8 kg. They were housed in metabolism cages and fed 'Okara' (soybean curd residue) only. Methyl *p*-hydroxybenzoate Na salt (0.8 g./kg. body wt. as methyl *p*-hydroxybenzoate) was administered by stomach tube as a 12% (w/v) solution and a total dose of 8.2 g. of methyl *p*-hydroxybenzoate was used for 4 rabbits. The decomposition of urinary metabolites was prevented by the addition of toluene.

The collected 24-hr. urine was filtered through cotton wool, adjusted to pH 4 with glacial AcOH, and then treated with saturated lead acetate solution until precipitation was complete. The precipitate was removed by filtration. The filtrate was adjusted to pH 8 with NH_4OH and saturated basic lead acetate solution was added in excess. The basic lead precipitate was collected and washed several times 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 at 25~30° under a reduced pressure. The residue was dissolved in a small volume of H_2O and extracted with Et_2O to remove unchanged methyl *p*-hydroxybenzoate and *p*-hydroxybenzoic acid (extract A). The aqueous solution was evaporated again to dryness at 30° under a reduced pressure and a red gum (gum B) was obtained.

This crude gum (B) was dissolved in about 30 cc. of EtOH and Et_2O was added until precipitation no longer occurred. The precipitate was filtered off and the filtrate was evaporated to dryness under a reduced pressure, giving a reddish gum (gum C).

This gum (C) was dissolved in EtOH and saturated $(AcO)_2Ba$ solution was added until precipitation was complete. The precipitate was collected, washed repeatedly with EtOH, and dried over anhyd. $CaCl_2$ *in vacuo*. The dried powder was dissolved in H_2O and filtered. The filtrate was adjusted to pH 7 and saturated basic lead acetate solution was added in excess. The basic lead precipitate was collected, washed with H_2O, 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 under a reduced pressure at 20° and the residue was dried over P_2O_5 *in vacuo*. A pale yellow glucuronide (1.3 g.) was obtained.

Isolation of the Derivative of Ether-type Glucuronide—To 1 g. of the purified glucuronide in 25 cc. of MeOH was added Et_2O solution of CH_2N_2, freshly prepared from 5 g. of nitrosomethylurea, under cooling in ice, and the mixture was allowed to stand overnight in a refrigerator. The solvent was evaporated to dryness under a reduced pressure and the pale yellow residue was dissolved in 6 cc. of pyridine and 4 cc. of Ac_2O. The mixture was allowed to stand at room temperature for 2 days and poured into 50 cc. of ice-water with stirring. After the mixture had been allowed to stand overnight in a refrigerator, the pale brown precipitate produced was collected and dissolved in Et_2O. Et_2O solution was washed successively with 2% HCl and H_2O, dried over anhyd. Na_2SO_4,

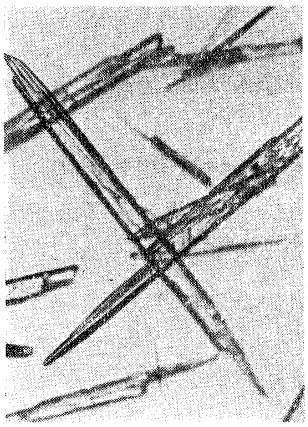

Fig. 1.
Isolated Crystals

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

Chart 1. Separation of Ether-type Glucuronide of *p*-Hydroxybenzoic Acid

Urine
↓ filtd. through cotton
Filtrate
↓ added with satd. $(AcO)_2Pb$ at pH 4 (adjusted with AcOH)
Filtrate | ppt.
↓ added with satd. basic lead acetate at pH 8 (adjusted with NH_4OH)
ppt. | Filtrate
↓ treated with H_2S in MeOH
Filtrate | PbS
↓ evapd. to dryness
Residue
↓ dissolved in H_2O, extd. with Et_2O
aq. soln. | Et_2O soln. Extract (A)
↓ evapd. to dryness
Red gum (B)
↓ dissolved in EtOH, added with Et_2O
Filtrate | ppt.
↓ evapd. to dryness
Reddish gum (C)
↓ dissolved in EtOH, added with satd. $(AcO)_2Ba$
Ba salt | Filtrate
↓ dissolved in H_2O, added with satd. basic lead acetate at pH 7
ppt. | Filtrate
↓ treated with H_2S in MeOH
Filtrate | PbS
↓ evapd. to dryness
Pale yellow glucuronide
↓ dissolved in MeOH, methylated with CH_2N_2, evapd. to dryness
Residue
↓ acetylated with pyridine and Ac_2O, poured into H_2O
ppt.
↓ dissolved in Et_2O, washed with HCl and H_2O
Et_2O soln.
↓ evapd. to dryness
White solid
↓ crystallized from Et_2O-petr. ether
Needles (Fig. 1)

and evaporated to dryness under a reduced pressure. The residue was crystallized from EtOH. The white powder (m.p. 147~152°) so obtained was recrystallized from Et_2O-petr. ether as colorless needles, m.p. 157~158°; $[\alpha]_D^{12}$ −42.0° (in $CHCl_3$). Yield, 0.35 g., having the crystal form shown in Fig. 1. *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.85; H, 5.16. Found: C, 53.85; H, 5.24. IR (in Nujol) cm^{-1}: $\nu_{C=O}$ 1760, 1718, ν_{C-O} 1230, 1083, 1044. UV: λ_{max}^{MeOH} 245 mμ.

When mixed with synthesized methy (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, the m.p. was not depressed. This compound was non-reducing and was positive to both naphthoresorcinol reaction and Millon reaction after acid-hydrolysis. The infrared spectrum of this compound also indicated that it was identical with authentic sample. The course of separation of the glucuronide is shown in Chart 1. After hydrolysis of crude red gum with alkaline solution, the derivative of ether-type *p*-hydroxybenzoic acid glucuronide was also obtained by a method similar to the above.

Paper Chromatography of the Metabolites—Ascending development was employed with Toyo Roshi No. 50. Solvent system used was BuOH-AcOH-H_2O (4:1:5). Urinary metabolites were detected on paper chromatograms by spraying with the following reagents: (1) 1% $NaIO_4$, 1% $KMnO_4$, followed by benzidine reagent;[6] (2) Lintner's reagent[7] (after spraying with 10% $Hg(NO_3)_2$ solution, the paper is heated for a few min. at 60°, then sprayed with a mixture of equal volumes of 5% $NaNO_2$ and 5% H_2SO_4, and again heated at 60°); (3) diazotized sulfanilic acid[8] and *p*-nitroaniline;[4] and (4) aniline hydrogenphthalate.[9]

The red gum (B) obtained by $(AcO)_2Pb$ separation indicated 5 blue spots (Rf 0.88, 0.74, 0.57, 0.18~0.24, 0.10~0.14) and 3 pink spots (Rf 0.88, 0.74, 0.57) on paper strips on spraying the reagents (1) and (2), respectively. The spot of Rf 0.10~0.14 was also obtained from the gum of normal urine. Paper chromatography of the purified gum (C) showed the presence of a new substance whose Rf value was 0.42~0.46, and the spots of 0.18~0.24 and 0.10~0.14 all disappeared. A new spot of Rf 0.42~0.46 was also detected with the reagents (1) and (2), and the spots of 0.88, 0.74, 0.57 and 0.42~0.46 colored with the reagent (3).

To detect the glucuronides, MeOH solution of the gum (B) was developed on a large filter paper (40×40 cm.) using the same solvent. The sections of Rf 0.88, 0.74, 0.57 and 0.42~0.46 spots were cut out, each section was eluted with hot H_2O, and the eluate was filtered. The filtrate was concentrated to a small volume under a reduced pressure.

When the extract of Rf 0.88 was developed using BuOH saturated with 5*N* NH_4OH, it was identical with *p*-hydroxybenzoic acid indicating a spot at Rf 0.08~0.10, and the extract (A) also gave the same result. In this case, unchanged methyl *p*-hydroxybenzoate (corresponding to Rf 0.56~0.58) was not detected.

The naphthoresorcinol test was positive in both extracts of Rf 0.57 and 0.42~0.46.

The section of Rf 0.57 was resistant to alkaline-hydrolysis. After hydrolysis with 5% HCl in a steam-bath for 20 min., paper chromatography using BuOH-AcOH solvent system showed 2 spots corresponding to *p*-hydroxbenzoic acid and glucuronolactone (Rf 0.30~0.32) with reagents (2) and (4).

After developing repeatedly on a filter paper, the section of Rf 0.42~0.46 separated into Rf 0.50 and 0.40. When the extract of Rf 0.50 was hydrolyzed with 0.2% NaOH at 60° for 1 hr., 2 spots corresponding to *p*-hydroxybenzoic acid and glucuronic acid (Rf 0.12~0.14) were revealed by paper chromatography using the same solvent system. On the other hand, after hydrolysis with 5% HCl at 80° for 1 hr., the section of Rf 0.50 was partially decomposed into *p*-hydroxybenzoic acid and glucurone, and the spot of unchanged Rf 0.50 still remained. The amount of the extract of Rf 0.40 was so small compared to Rf 0.57 and 0.50, that its structure has not been clarified.

The section of Rf 0.74 may be considered a conjugated glycine, because it was positive to Ninhydrin test after acid-hydrolysis.

Synthesis of Methyl (*p*-Methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III)—The method of Lunsford and Murphy[10] was applied to the preparation of derivative of ether-type *p*-hydroxybenzoic acid glucuronide, in which quinoline was used instead of isoquinoline.

A mixture of 1.2 g. of methyl (tri-O-acetyl-α-D-glucopyranosyl bromid)uronate (I), 0.91 g. of methyl *p*-hydroxybenzoate (II), and 1.5 cc. of quinoline was triturated with a pestle in a mortar, cooling the mortar in an ice-bath. To this mixture, 1.5 g. of freshly prepared Ag_2O was added in small portions with continuous mixing. After complete addition, mixing was continued for 15 min., the viscous mixture was allowed to stand in a desiccator for 2 hr., and then was extracted several times with 20-cc. portions of Et_2O. Et_2O solution was washed successively with 2% H_2SO_4 and H_2O, dried over

6) M. L. Wolfrom, J. B. Miller: Anal. Chem., **28**, 1037(1956).
7) C. J. Lintner: Z. angew. Chem., **13**, 707(1900).
8) T. Mann, E. Leone: Biochem. J., **53**, 140(1953).
9) S. M. Partrige: Nature, **164**, 443(1949).
10) C. D. Lunsford, R. S. Murphey: J. Org. Chem., **21**, 580(1956).

anhyd. Na_2SO_4, and evaporated under a reduced pressure. The residue was dissolved in EtOH and treated with activated charcoal. The precipitate produced was collected and recrystallized from Et_2O-petr. ether as colorless large needles, m.p. 157~158°; $[\alpha]_D^{12}$ −42.5° (in $CHCl_3$). Yield, 0.4 g. *Anal.* Calcd. for $C_{21}H_{24}O_{12}$: C, 53.85; H, 5.16. Found: C, 53.81; H, 5.16. IR (in Nujol) cm^{-1}: $\nu_{C=O}$ 1758, 1715, ν_{C-O} 1227, 1082, 1044.

This compound was non-reducing and positive to naphthoresorcinol reaction and Millon reaction after acid-hydrolysis. The course of its synthesis is shown in Chart 2.

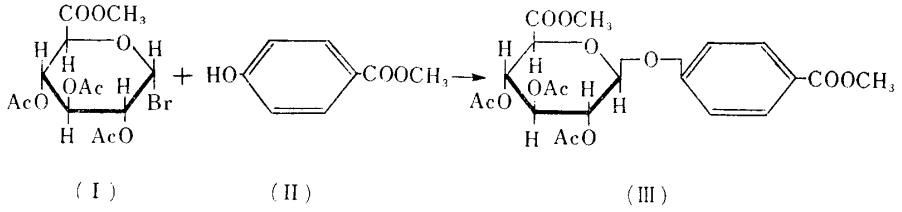

Chart 2. Preparation of Ether-type *p*-Hydroxybenzoic Acid Glucuronide

Discussion

In the present study, excretion of unchanged methyl *p*-hydroxybenzoate was not identified qualitatively by paper chromatography. Previous to this investigation, quantitative study on the metabolism of methyl *p*-hydroxybenzoate in rabbit showed that, after a dose of 0.4 g./kg. body wt. of methyl *p*-hydroxybenzoate, the excretion was concluded in almost 6 hr., about ⅓ portion being excreted as free *p*-hydroxybenzoic acid and the rest was discharged as conjugated form, without the unchanged methyl *p*-hydroxybenzoate. Schotten[2] also found that after rabbit received *p*-hydroxybenzoic acid, during 24 hr., 35% of it was excreted as unchanged compound. This fact shows that the ester decomposition is very easy in the animal body.

Robinson and Williams[11] isolated a derivative of the ether-type glucuronide of salicylic acid from the urine of rabbits receiving methyl salicylate. In the present experiment using methyl *p*-hydroxybenzoate, although the result could not be quantitated, the ether-type glucuronide of *p*-hydroxybenzoic acid appeared to be present in greater amount than the ester-type on paper chromatogram. Therefore, it could be generally supposed that the ether-type occurs in larger amount in the urine of rabbit receiving alkyl esters of hydroxybenzoic acid.

From the glucuronide fraction obtained by lead acetate method, the ether-type glucuronide was successfully isolated as methylacetyl derivative and its structure was established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate with the synthetic sample, but the isolation of the ester-type has so far been unsuccessful and now further investigation is under way.

The authors are indebted to Mr. H. Matsui for determination of infrared spectra and to Miss S. Tada for the elemental analyses.

Summary

The ether-type glucuronide of *p*-hydroxybenzoic acid was isolated as methylacetyl derivative from the urine of rabbits receiving methyl *p*-hydroxybenzoate and its structure was established as methyl (*p*-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)-uronate with the synthetic sample. Ester-type glucuronide was detected in the urine by paper chromatography.

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