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97. Hisao Tsukamoto, Akira Yamamoto, and Osamu Kamata : Metabolism of Drugs. XIV.* Metabolic Fate of *p*-Aminosalicylic Acid in the Rabbit. (2)**

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Since *p*-aminosalicylic acid (PAS) possesses three functional groups which would be conceivably metabolized, its biotransformation is exceedingly complicated. Unchanged PAS, N-acetylated PAS (Ac-PAS),[1~3] and *p*-aminosalicyluric acid (PASU, N-(4-amino-2-hydroxybenzoyl)glycine) have been isolated from the urine of man and animals receiving PAS. The occurrence of conjugated glucuronic acid in the urine of man and animals receiving PAS has been ascertained by several workers,[5~8] but PAS-glucuronide (PASG) has not yet been isolated as a urinary metabolite of PAS.

Three monoglucuronides are possible, the ester, 4-amino-2-hydroxybenzoyl glucuronide, the ether, 5-amino-2-carboxyphenyl glucuronide, and the N-glucuronide, 4-carboxy-3-hydroxyanilino glucuronide.

In the previous communication,[9] the isolation of the derivative of ester-type PAS-glucuronide has been outlined. It is shown in this report that the structure has been established as methyl (4-acetamido-2-acetoxybenzoyl-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate. The ether-type PAS-glucuronide, *p*-aminosalicyluric acid, and *m*-aminophenyl sulfate (MAPS) has been identified by means of paper chromatography and *m*-aminophenyl glucuronide (MAPG) has been isolated from the urine of rabbits receiving PAS.

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Experimental

Sodium *p*-aminosalicylate (sodium PAS) was from commercial source. *m*-Aminophenol (MAP) was prepared by decomposition of PAS. MAPG was isolated from the urine of rabbits receiving MAP by the method described by Williams.[10] PASU was isolated from the urine of man receiving PAS by the method described by Way.[2]

Preparation of the Derivative of Ester-type PAS-Glucuronide—The method of Sannie and Lapin[11] was applied to the synthesis of derivative of ester-type PASG. Potassium *p*-acetamidosalicylate (I) (1.6 g.) was dissolved in the minimum amount of hot water, followed by 2.5 g. of methyl (2,3,4-tri-*O*-acetyl-*α*-D-glucopyranosyl bromid)uronate (II) in 8 cc. of Me_2CO. After shaking for a few mins., precipitated K *p*-acetamidosalicylate was dissolved by addition of a minimum amount of water. The reaction mixture was allowed to stand for 24 hrs. at room temperature, the crystalline product was collected, and washed succesively with water, EtOH, and Et_2O. Recrystallization from

* Part XIII. H. Yoshimura : This Bulletin, **5**, 561(1957).
** Part (1). H. Tsukamoto, A. Yamamoto : *Ibid.*, **3**, 427(1955).
*** Katakasu, Fukuoka (塚元久雄, 山本 陽, 鎌田 理).

1) A. Venkataraman, *et al.* : J. Biol. Chem., **173**, 6(1948).
2) E. L. Way, *et al.* : J. Pharmacol. Exptl. Therap., **43**, 368(1948).
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6) P. Rohan, M. Polster : Biol. Listy., **32**, 66(1951).
7) H. Tsukamoto, A. Yamamoto : This Bulletin, **3**, 427(1955).
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9) H. Tsukamoto, A. Yamamoto, O. Kamata : This Bulletin, **5**, 283(1957).
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11) H. Sannie, H. Lapin : Bull. soc. chim. France, 1234(1950).

EtOH yielded 0.7 g. of methyl (4-acetamido-2-hydroxybenzoyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate (III), m.p. 220°.

0.4 g. of (III) was acetylated with 0.72 cc. of Ac_2O and 1 cc. of pyridine for 3 days at room temperature. The reaction mixture was added with $CHCl_3$ and successively washed with 5% HCl and water. $CHCl_3$ solution was dried over anhyd. $CaCl_2$ and evaporated to dryness *in vacuo*. White gummy residue was recrystallized from MeOH to methyl (4-acetamido-2-acetoxybenzoyl tri-*O*-acetyl-β-D-glucopyranosid)uronate (IV), as fine long needles, m.p. 193~194°; $[\alpha]_D^8$ −35°(c=1.01 in $CHCl_3$). Yield, 0.26 g. *Anal.* Calcd. for $C_{24}H_{27}O_{14}N$: C, 52.06; H, 4.91; N, 2.53. Found: C, 51.75; H, 4.98; N, 2.62.

The infrared spectrum of (IV) is shown in Fig. 1 and the route of its preparation in Chart 1.

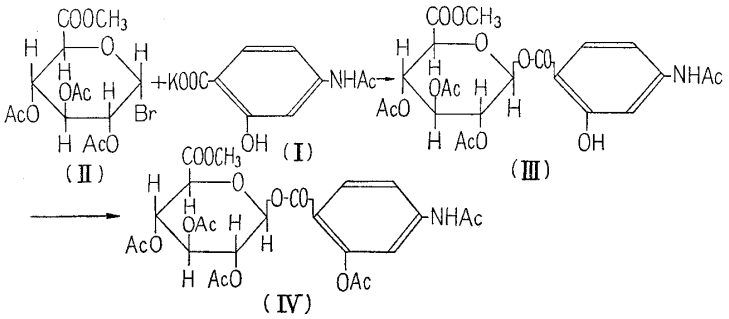

Chart 1.

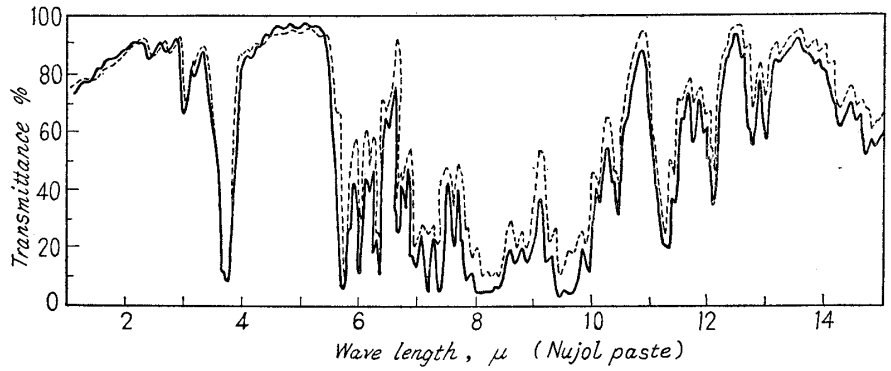

Fig. 1.

Infrared Absorption Spectra

------ Isolated methyl (4-acetamido-2-acetoxy-benzoyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate

——— Synthesized

Isolation of the Derivatives of Ester-type PAS-Glucuronide—The crude ester-type glucuronide was obtained by separation with $Pb(OAc)_2$, which is a general method of preparing glucuronides descreibed by Williams.[12]

The animals used were male rabbits weighing 2.5~3.3 kg. They were housed in metabolism cages and fed 'Okara' (soybean curd residue) only. Na-PAS (1.37 g./kg. body wt.) in water was administered by stomach tube. Urinary collections were made daily. The decomposition of metabolites was prevented by the addition of toluene and glacial AcOH.

A total dose 60 g. of Na-PAS dissolved in water was administered by stomach tube to 15 rabbits. The collected 24-hr. urine was filtered through cotton, the filtrate was brought to pH 4 with glacial AcOH, and treated with saturated aqueous *N* $Pb(OAc)_2$ until precipitation was complete. The precipitate was removed by filtration. The filtrate was brought to pH 7 with NH_4OH and aqueous basic lead acetate added in excess. The precipitate was filtered and completely washed 3 times with water. The basic lead precipitate was made into a fine suspension in water and the lead salt was decomposed by treatment with H_2S. The PbS was filtered, washed with water, and the filtrate and washing, after aeration, were evaporated to dryness *in vacuo* at 45°. The residual red gum was added with a small volume of water and then insoluble material was filtered off. PAS and Ac-PAS were isolated from this insoluble material. The filtrate was extracted with Et_2O, which contained a little PAS and MAP, and evaporated to dryness *in vacuo* at 45°. This yielded red gum, which was treated with 50 cc. of EtOH and filtered to remove an insoluble material (A). EtOH filtrate was concentrated to a small volume under reduced pressure, stirred into a large excess of Et_2O, and immediately grayish white precipitate separated. The precipitate was filtered off. The filtrate was concentrated to a small volume and the concentrate was again stirred into a large excess of Et_2O and dried over anhyd. $CaCl_2$ *in vacuo*, giving 4 g. of grayish white powder.

This powder was very hygroscopic and quickly converted into a red gum on exposure to air.

12) R. T. Williams: Biochem. J. (London), **50**, 235(1951).

The paper chromatograms indicated that this powder mostly consisted of a material with Rf 0.28, which was identified as PAS-glucuronide in our previous study.[7] However, the preparation of *p*-toluidine salt, benzylamine salt, and metal salts of PAS-glucuronide from this powder was attempted but no crystalline material could be obtained. Therefore attempt was made to obtain crystalline derivative of PAS-glucuronide on methylation and acetylation of this powder.

To 4 g. of the glucuronide powder in 100 cc. of MeOH was added an ether solution of CH_2N_2, obtained from 20 g. of nitrosomethylurea, and the mixture was allowed to stand overnight in a refrigerator. The solvent was removed by evaporation and the residue was dissolved in 30 g. of pyridine and 24 g. of Ac_2O. After the mixture was allowed to stand at room temperature for 3 days, it was poured into ice-water with stirring and extracted 3 times with Et_2O. The combined Et_2O extracts was washed successively with dil. AcOH and water, dried over anhyd. $CaCl_2$, and evaporated to dryness. The residue was recrystallized from EtOH containing Et_2O and once from EtOH and twice from MeOH. The yield was 0.39 g. of fine long needles, m.p. 192~194°, $[\alpha]_D^8$ −36° (c=1.03, $CHCl_3$), having the crystal form shown in Fig. 2. This compound showed no depression of m.p. with the synthesized compound (IV). *Anal.* Calcd. for $C_{24}H_{27}O_{14}N$: C, 52.06; H, 4.91; N, 2.53.

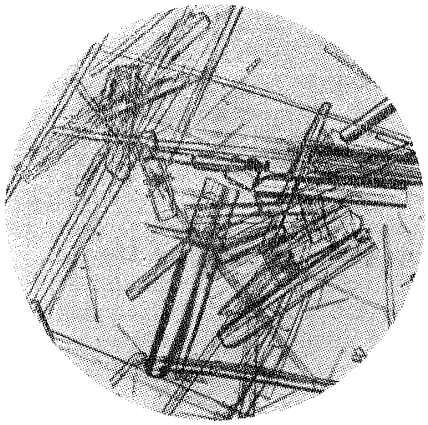

Fig. 2.

Crystals of the Isolated Derivative of Ester-type PASG

Urine
filtered
Aq. soln.
adjusted to pH 4 with AcOH, added with sat. $Pb(OAc)_2$
Aq. soln. | ppt.
adjusted to pH 7 with NH_4OH, added with sat. basic lead acetate
ppt. | Aq. soln.
suspended in H_2O, decomposed with H_2S
Aq. soln. | ppt.
evaporated to dryness
Red gum
added with H_2O
Aq. soln. | Insol. sub.
extd. with Et_2O
Aq. soln. | Et_2O soln.
evapd. to dryness
Gum
added with EtOH
EtOH soln. | Insol. sub. (A)
evaporated to 10 cc., added with 100 cc. of Et_2O
ppt. | Ether soln.
added with MeOH
MeOH soln.
methylated with CH_2N_2, evaporated to dryness
Gum
acetylated with Ac_2O and pyridine, poured into ice cold water, extracted with Et_2O
Et_2O soln. | Aq. soln.
evapd. to dryness
Gum
added. with Et_2O
White solid | Et_2O soln.
recrystallized from EtOH
Long needles (Fig. 2)

Chart 2.

Found : C, 51.87; H, 5.08; N, 2.56.

Infrared spectral analysis (Fig. 1) also indicated that the substance was identical with the synthesized methyl (4-acetamido-2-acetoxybenzoyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)uronate. The separation of glucuronide is shown in Chart 2.

Isolation of MAP-Glucuronide—The insoluble material (A) mentioned above indicated Rf 0.14 on the paper chromatogram by Ehrlich's and diazo reagents. This was identical with MAPG isolated from the urine of rabbits receiving MAP.[10] After three crystallizations from water, the insoluble material (A) yielded 1.1 g. of white leaflets, which did not melt below 360°, but turned red at 220° and black at 300°. $[\alpha]_D^{13}$ −92°(c=1.01, *N*/12 HCl). *Anal.* Calcd. for $C_{12}H_{15}O_7N$: C, 50.52; H, 5.26; N, 4.91. Found : C, 49.96; H, 5.42; N, 5.08.

It was non-reducing, but yielded *m*-aminophenol after acid hydrolysis and gave a rapid positive naphthoresorcinol reaction.

Paper Chromatography of the Metabolites—Ascending development was employed with Toyo Roshi No. 50 chromatography paper. Solvent used was BuOH-AcOH-H_2O (4:1:5, by vol.). Compounds were detected on paper chromatograms by spraying with Ehrlich's reagent, iron reagent ($Fe(NO_3)_2$), diazo reagent (1-(2-diethylaminoethylamino)naphthalene=Tsuda reagent), aniline phthalate, or ninhydrin reagent.

The red gum obtained by $Pb(OAc)_2$ procedure indicated 6 spots (Rf 0.81～0.82, 0.54～0.59 (X_1), 0.28～0.34 (X_2), 0.24～0.27 (X_3), 0.17～0.22 (X_4), 0.14～0.16 (X_5)) on paper chromatograms on spraying the Ehrlich's or diazo reagents. Rf 0.81～0.82 and X_1 gave a positive iron reaction. It had already been identified that Rf 0.81 is a mixture of PAS and Ac-PAS,[7] X_2 is an ester-type PASG,[7] and X_5 is MAPG. The spot of X_3 was identical with MAPS in the urine of rabbits receiving MAP.

MeOH solution of red gum was developed on a large filter paper (40×40 cm.), and the sections of X_1 and X_4 spots, which were previously tested by color reagents, were cut off, each section was extracted twice with 5-cc. portions of hot water, and the extract filtered. The filtrate was concentrated to a small volume *in vacuo* at 45°. The extract of X_1 was identical with *p*-aminosalicyluric acid isolated from the urine of man receiving PAS. On acid hydrolysis, it indicated 2 spots of Rf 0.67 of MAP by Ehrlich's reagent and Rf 0.16 of glycine by ninhydrin reagent.

The extract of X_4 gave no aniline phthalate reaction before acid hydrolysis. It was not hydrolyzed with alkaline solution, but it indicated 3 spots of MAP, glucurone, and glucuronic acid on acid hydrolysis, and it gave a positive naphthoresorcinol reaction. This substance may be the ether-type PASG.

Since the content of X_1, X_3, and X_4 was very small in comparison with other metabolites, attempts to isolate these compounds are yet unsuccessful. The chromatographic data are summarized in Fig. 3.

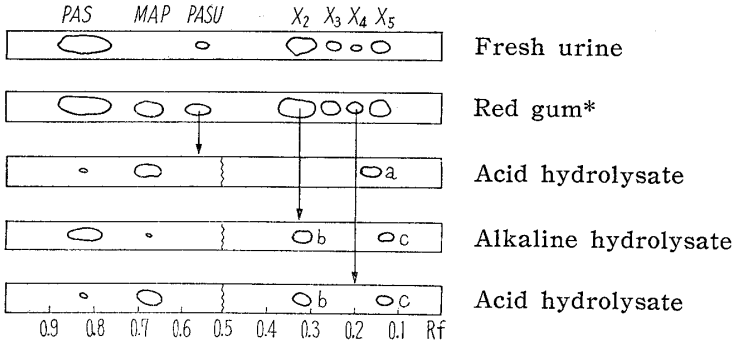

Fig. 3.

* Red gum obtained by the lead acetate procedure.
X_2, ester-type PASG; X_3, MAPS; X_4, ether-type PASG; X_5, MAPG.
a, glycine; b, glucurone; c, glucuronic acid.

Discussion

In our previous quantitative study of the metabolism of PAS in rabbit, no considerable decarboxylation of PAS was observed and the paper chromatogram of fresh urine of rabbit receiving PAS showed that the position corresponding to MAP was negative to color reagents. On the paper chromatogram of fresh urine of rabbit receiving MAP at a dose level of 0.29 g./kg. body wt., no unchanged MAP was identified, but MAPS and MAPG were identified. Williams[10] reported that MAP was excreted mainly in a conjugated form and therefore the failure to find a considerable

amount of MAP excreted may be explained by the fact that MAP formed by the decomposition of PAS *in vivo* may immediately undergo detoxication.

The aqueous solution of PAS is easily decarboxylated by heat, acid, or light. Way and Smith[4] have found that PAS is readily decarboxylated when incubated with human blood at 38°, at a considerably greater rate (about one-half in 24 hrs.) than could be accounted for *in vivo*. Therefore, it may be considered that PAS is decarboxylated when in contact with the acidity in the stomach and the activity of intestinal fluid.[1]

To the best of our knowledge, there has been no report on the conclusive determination of decarboxylation of PAS *in vivo*. In our present study, by the isolation of MAPG and the identification of MAPS on the paper chromatogram, there is no doubt that the decarboxylation of PAS occurred *in vivo*, through enzymatic or other processes.

In our experiments, MAP formed from the decarboxylation of 1~1.5% of PAS administered was conjugated with glucuronic acid. The samples of Na-PAS used contained MAP below 0.03%. Therefore, the isolated MAPG was not due to MAP contaminated in the original sample.

In the studies on the metabolism of salicylic acid,[13] it has been reported that two conjugates of salicylic acid with glucuronic acid, ether- and ester-types, have been identified in human urine, the ether-type glucuronide occurring in greater amounts than the ester-type.[14] Although the results could not be quantitated, ether-type PASG appeared to be present in greater amount than the ester-type on the paper chromatogram of the urine of man receiving PAS. In rabbits, on the contrary, the ester-type was much greater than the ether-type and the main metabolites in rabbit were Ac-PAS and ester-type PASG.

Ester-type PASG was very labile in alkaline solution and was almost completely decomposed by heating for 15 minutes at 60° or allowing to stand for 24 hours at room temperature after the urine was adjusted to pH 11 with ammonia, but stable in the treatment with acetic acid at pH 3 and 60 minutes. In the present experiments, the decomposition of PASG was prevented by the previous addition of a little amount of acetic acid into the collection bottles. By the administration of 60 g. of Na-PAS, 0.39 g. of a derivative of the ester-type PASG was obtained which corresponded to 0.25% of the dose of PAS. The derivative isolated from the urine was identified with the synthetic sample by the elemental analysis, optical rotation, infrared absorption spectrum, chemical properties, and by admixture with the synthetic sample. The structure was established as methyl (4-acetamido-2-acetoxybenzoyl 2,3,4,-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate. Since this was very labile in alkaline solution, the preparation of free glucuronide by its demethylation and deacetylation was impossible.

Venkataraman, *et al.*[1] found no evidence for glycine conjugated with PAS in the rabbit. The present investigation suggests that PASU usually occurred in the urine of rabbit receiving PAS but much less than in the human urine.

Summary

The ester-type PAS-glucuronide was isolated from the urine of rabbit receiving PAS and the structure was established as methyl (4-acetamido-2-acetoxybenzoyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosid)uronate, which was synthesized. *m*-Aminophenyl glucuronide was isolated from the urine of rabbit. *p*-Aminosalicyluric acid, *m*-aminophenyl sulfate, and the ether-type PAS-glucuronide were detected in the urine by means of paper chromatography. (Received July 11, 1957)

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