however, before this conclusion can be definitely established.

As mentioned above, gallic acid is unique as an inhibitor in that it requires preoxidation for maximum inhibition although the presence of oxygen is necessary during the reaction. The nature of the active oxidized product has not yet been elucidated. It should be pointed out, however, that gallic acid itself is also responsible for a part of the inhibition observed.

The fact that preincubation of the cells with inhibitor is necessary to attain a maximal inhibition may be explained by the time required for the inhibitor to penetrate the cells and, in the case of gallic acid inhibition, also by the time needed to produce the active oxidized form.

The authors are indebted to Dr. R. Sato of the Institute for Protein Research, University of Osaka, for valuable suggestions. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.

Summary

The enzymatic production of hydrogen sulfide by *E. coli* was inhibited by most of the tannins and related compounds tested. Inhibition by polyhydroxyl compounds lacking free carboxyl group is greater than that by polyhydroxycarboxylic acids. The inhibition is not competitive with substrate, nor is it reversed by the addition of a coenzyme.

In order to attain a maximal inhibition it is necessary to incubate the cells with an inhibitor for a certain length of time prior to addition of the substrate. Cysteine is able to partially protect the enzyme from the action of inhibitors. This protecting effect is more pronounced when the substrate and inhibitor are incubated and then added to the reaction mixture.

Inhibition by gallic acid is considerably enhanced by aerating the inhibitor solution. This suggests that the oxidized form of gallic acid is more inhibitory than gallic acid itself. There are many differences between the mechanisms of inhibition by gallic acid and *d*-catechin.

(Received October 30, 1958)

UDC 591.133.2:547.587.1:599.325.1

80. **Hisao Tsukamoto and Akira Yamamoto:** Metabolism of Drugs. XIX.[*1] Metabolic Fate of *p*-Aminosalicylic Acid in the Rabbit. (3).[*2]

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In the previous papers of this series,1,7) the occurrence of seven metabolites was reported in the urine of rabbit receiving *p*-aminosalicylic acid (PAS). They were PAS, N-acetylated PAS (Ac-PAS), *p*-aminosalicyluric acid (PASU, M_1), ester-type PAS-glucuronide (ester-PASG, M_2), *m*-aminophenyl sulfate (MAPS, M_3), ether-type PAS-glucuronide (ether-PASG, M_4), and *m*-aminophenyl glucuronide (MAPG, M_5). PAS, Ac-PAS, and PASU have been isolated by Venkataraman2) and Way,3) respectively.

[*1] Part XVIII. H. Tsukamoto, H. Yoshimura, S. Toki: This Bulletin, **6**, 88(1958).

[*2] Part (2). H. Tsukamoto, A. Yamamoto, O. Kamata: *Ibid.*, **5**, 565(1957).

[*3] Katakasu, Fukuoka (塚元久雄, 山本　陽).

1) H. Tsukamoto, A. Yamamoto: This Bulletin, **3**, 427(1955).

2) A. Venkataraman, *et al.*: J. Biol. Chem., **173**, 6(1948).

3) E. L. Way, *et al.*: J. Am. Pharm. Assoc., Sci. Ed., **44**, 65(1955).

The occurrence of reducing material in the urine of man and animals receiving PAS has been ascertained by several workers.[4~9] The isolation of ester-PASG as the acetyl-methyl derivative was proof that this reducing material might be glucuronic acid formed by the decomposition of ester-PASG with alkaline reagents.

In the metabolism of salicylic acid in man and animals,[10,11] both glucuronide, the ester and the ether, have been demonstrated as the urinary metabolites and were already isolated as the acetyl-methyl derivatives from the urine of man and rabbit receiving salicylic acid, by Williams, *et al.*[11] and by Tsukamoto, Kato, and Tatsumi,[12] respectively.

In the metabolism of PAS, two ether-type glucuronides were detected in the urine of rabbit receiving PAS, one was identified as MAPG and the other as ether-PASG.

It is shown in this report that the structure of the isolated derivative of ether-PASG was established as methyl (5-acetamido-2-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, which was also chemically synthesized, and free ether-PASG was prepared synthetically. Further observations of PASU and MAPG are discussed.

The authors are indebted to Mr. O. Kamata for technical help, to Messrs. H. Yano and H. Matsui for the determination of infrared spectra and of ultraviolet spectra (Beckman DK-2 spectrophotometer), to Miss S. Tada and Mr. M. Shirozu for the elementary analyses, and to Sankyo Co. Ltd. for their supply of PAS.

Experimental

PAS-Na was obtained from commercial sources. Ac-PAS was synthesized by the method described by Romeo.[13] MAP was prepared by decomposition of PAS. PASU was isolated from the urine of man receiving PAS by the method described by Way, *et al.*[3] MAPG was isolated from the urine of rabbit receiving *m*-aminophenol (MAP) by the method described by Williams.[14]

Preparation of the Ether-type PAS-Glucuronide—It was demonstrated by systematic studies of Tsukamoto, *et al.*[15] and Bollenback, *et al.*[16] that the method for synthesis of aryl glycosides could be applied to that of aryl glucuronides. In this experiment, the synthetic method of ether-type PAS-glucoside by Sannie and Lapin[17] was applied to the synthesis of derivative of ether-PASG.

Since PAS possesses three functional groups which would be conjugated with glucuronic acid, both amino and carboxyl groups must be blocked for the synthesis of ether-PASG, but the condensation of methyl acetamidosalicylate with methyl acetobromoglucuronate was unsuccessful, and methyl-4-nitrosalicylate (I) was used for the synthesis of methyl (5-nitro-2-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (III).

Methyl (5-amino-2-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (IV) prepared by the catalytic reduction of (III) was deacetylated and demethylated by treatment with saturated $Ba(OH)_2$ to yield free ether-PASG.

1) **Preparation of (III)**—In a solution of 1.4 g. of KOH dissolved in a small volume of water, 5 g. of methyl 4-nitrosalicylate (I) was added, 40 cc. of water and 80 cc. of acetone were stirred into the mixture, followed by 6.6 g. of methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromid)uronate (II) in 30 cc. of acetone. After shaking for a few min., the reaction mixture was allowed to stand for 24 hr. at room temperature, the crystalline product was collected, and washed successively with water and EtOH. Recrystallization from EtOH yielded 1.2 g. of colorless needles, m.p. 159~160°. There was no color reaction with aqueous $FeCl_3$ before acid hydrolysis, but a color developed in

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[5] P. Rohan, M. Polster: Biol. listy, **32**, 66(1951).
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[14] R. T. Williams: Biochem. J., **37**, 329(1943).
[15] H. Tsukamoto, *et al.*: Yakugaku Zasshi, **76**, 1282(1956).
[16] G. N. Bollenback, *et al.*: J. Am. Chem. Soc., **77**, 3310(1955).
[17] H. Sannie, H. Lapin: Bull. soc. chim. France, **1950**, 1234.

the naphthoresorcinol reaction. *Anal.* Calcd. for $C_{21}H_{23}O_{14}N$: C, 49.12; H, 4.48; N, 2.75. Found : C, 49.61; H, 4.65; N, 2.57.

2) **Preparation of (IV)**—This was prepared by catalytic reduction of (III) in AcOH over Raney Ni, which was used by Sannie.[17] Since this method gave a low yield and required a long time, 0.5 g. of (III) was hydrogenated at atmospheric pressure in 20 cc. of AcOEt with 20 mg. of PtO_2 (Adams' catalyst). The uptake of H_2 was smooth and rapid until the theoretical amount of H_2 was used and then the reaction stopped. The catalyst was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. After several recrystallizations of the white residue from EtOH, 0.3 g. of colorless needles, m.p. 142~143°, separated. This substance gave an orange color with Ehrlich's reagent and was positive to naphthoresorcinol test. *Anal.* Calcd. for $C_{21}H_{25}O_{12}N$: C, 52.17; H, 5.17; N, 2.89. Found : C, 52.17; H, 4.94; N, 2.45.

3) **Acetylation of (IV)**—0.5 g. of (IV) was acetylated with 1 cc. of Ac_2O and 1.4 cc. of pyridine for 3 days at room temperature. The reaction mixture was stirred into 20 cc. of ice-cold water and allowed to stand for a few hr. in a refrigerator. The crystalline material was collected and recrystallized from hydr. MeOH to 0.46 g. methyl (5-acetamido-2-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (V), as silky needles melting at 110~115° with resolidification on further heating and again melting at 169~170°. $[\alpha]_D^{30}$ −80.4°(c=0.435 in $CHCl_3$). It gave no color with Ehrlich's reagent before acid hydrolysis, but the naphthoresorcinol test was positive. The ultraviolet and infrared absorption spectra are shown in Fig. 2 and Table II. *Anal.* Calcd. for $C_{23}H_{27}O_{13}N \cdot H_2O$: C, 50.82; H, 5.34; N, 2.57. Found : C, 50.96; H, 5.51; N, 2.33.

4) **Preparation of the Free Ether-PASG (VI)**—A suspension of 0.1 g. of (IV) in 5 cc. of saturated $Ba(OH)_2$ solution was allowed to stand for 24 hr. at room temperature. The excess $Ba(OH)_2$ was removed by treatment with CO_2, the filtrate was brought to pH 8 with NH_4OH, and saturated basic lead acetate solution was added in excess. The basic lead precipitate was collected by filtration, washed repeatedly with water, made into a fine suspension in 50 cc. of MeOH, and Pb was removed by saturation with H_2S. After removal of PbS by filtration, the MeOH solution was evaporated to dryness under reduced pressure at 15° and dried over P_2O_5 *in vacuo* to yield 0.05 g. of a white-yellowish amorphous substance, which was slightly hygroscopic in air and resistant to alkaline hydrolysis. After acid hydrolysis, it was decomposed to PAS, MAP, MAPG, and glucurone. Furthermore, it was hydrolyzed enzymatically with β-glucuronidase preparation to yield PAS and glucuronic acid. The evidence mentioned above shows that this substance consists of ether-PASG having the configuration of β-D-glucopyranuronoside. However, attempts to crystallize this compound was yet unsuccessful. These results are described in detail in later sections. The route of its preparation is shown in Chart 1.

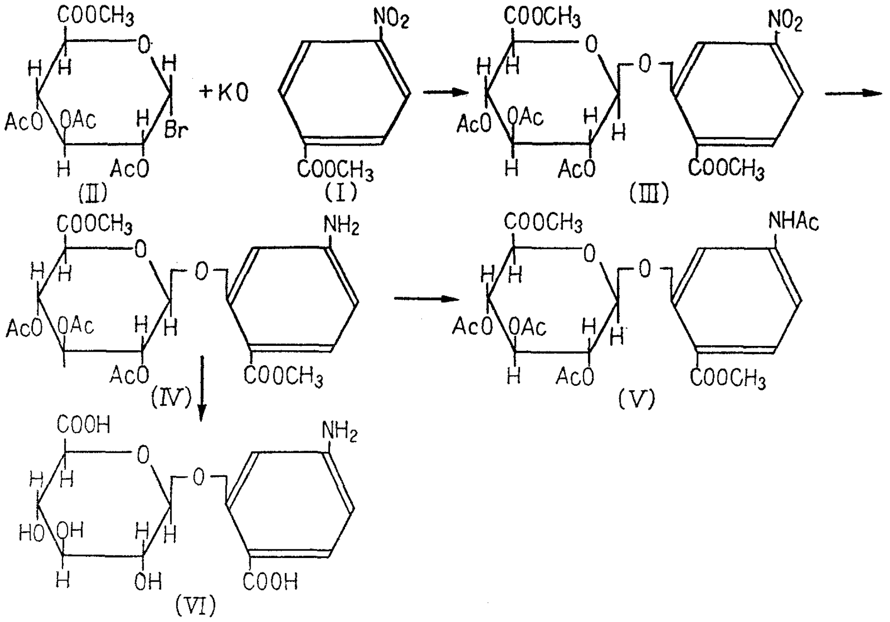


Chart 1. Preparation of Ether-type PAS Glucuronide

Separation of Ether-type PASG Fraction from the Urine of Rabbits—The animals used were male rabbits weighing 2.7~3.2 kg. They were housed in metabolism cages and fed "Okara" (soybean curd residue). PAS-Na (1.37 g./kg. body wt.) in water was administered by stomach tube. Urinary collection was made daily. The decomposition of metabolites was prevented by the addition of toluene.

Ether-PASG was excreted in only small amounts in comparison with other metabolites and was furthermore acid labile. Therefore, its isolation procedures were very difficult and required great care.

A total dose of 120 g. of PAS-Na dissolved in water was administered to 30 rabbits. The 24-hr. urine collected was filtered through cotton, the filtrate was adjusted to pH 11 with 10% NaOH, and heated on a water bath for 15 min. at 100° to destroy the ester-PASG, which interferes with the isolation of ether-PASG. When cool, the urine was brought to pH 4 with glacial AcOH and treated with saturated $Pb(OAc)_2$ solution until precipitation was complete. The precipitate was removed by filtration.

The filtrate was brought to pH 8 with NH_4OH and saturated basic $Pb(OAc)_2$ solution was added in excess. The precipitate was collected, washed successively with water and MeOH, made into a fine suspension in 50% MeOH, and the Pb salt was decomposed by treatment with purified H_2S. PbS was removed by filtration and washed with MeOH. The filtrate and washing, after aeration, were neutralized with NH_4OH and evaporated to dryness *in vacuo* at 30°. The residual gummy substance was dried over P_2O_5 for 24 hr. *in vacuo*. The dried residue was added with 300 cc. of EtOH and heated on a water bath with vigorous agitation. A white solid was separated from EtOH solution (A) by filtration and washed with warm EtOH. The white solid (8 g.) was added to 20 cc. of water and the insoluble material was filtered off. To the filtrate, basic lead acetate solution was added in excess. The basic lead precipitate was collected and washed successively with water and MeOH. The dried precipitate was made into a fine suspension in 200 cc. of MeOH and again treated with H_2S. After PbS was removed by filtration, the filtrate was evaporated to dryness *in vacuo* at 15°. The partially crystalline residue was dried over P_2O_5 *in vacuo* overnight, 50 cc. of warm MeOH was added, and the MeOH solution filtered to remove insoluble materials (B). The filtrate was allowed to stand overnight in a refrigerator. After the removal of the colorless crystals (C) that separated, the mother liquor was evaporated to dryness *in vacuo* at 15°. The residual gummy substance solidified on addition of EtOH; yield, 0.12 g. The yellowish white powder obtained was slightly hygroscopic in air. The results of paper chromatography and electrophoresis indicated that this powder mostly consisted of a material (M_4) which showed the same behavior as (VI), and furthermore was enzymatically hydrolyzed by β-glucuronidase to liberate PAS.

Isolation of the Derivative of Ether-type PASG from the Urine of Rabbits—To 0.1 g. of the glucuronide powder in 50 cc. of MeOH, an ethereal solution of CH_2N_2 (obtained from 10 g. of nitrosomethylurea) was added and the mixture was allowed to stand overnight in a refrigerator. The solvent was removed by evaporation *in vacuo* after the insoluble material was filtered off and the gummy residue was dissolved in 1 cc. of Ac_2O and 1.4 cc. of pyridine. The mixture was allowed to stand at room temperature for 5 days, poured into 30 cc. of ice-cold water with stirring, and allowed to stand at 0° for 3 hr. The yellowish orange colored material which separated, was collected by filtration, washed with water, and dried over P_2O_5 *in vacuo* overnight. This material weighing 0.04 g. was dissolved in 5 cc. of MeOH, after which water was carefully added dropwise until the orange-colored substance precipitated. After a few hr., the clear yellow supernatant was decanted and treated with charcoal. To the decolorized supernatant water was added until a slight cloudiness appeared. It was allowed to stand overnight in a refrigerator. White crystalline material that separated was collected and recrystallized from hydr. MeOH. The yield was only 0.006 g. of pure silky needles, which melted at 115° with resolidification (crystallization in microscope) on further heating and again melted at 167~169°. This compound showed no depression of m.p. with the synthetic compound (V). *Anal.* Calcd. for $C_{23}H_{27}O_{13}N \cdot H_2O$: C, 50.82; H, 5.34. Found : C, 50.71; H, 5.64. This compound showed the same behavior in chemical hydrolysis and ultraviolet absorption spectra as the synthetic compound (V). These results are described in later sections.

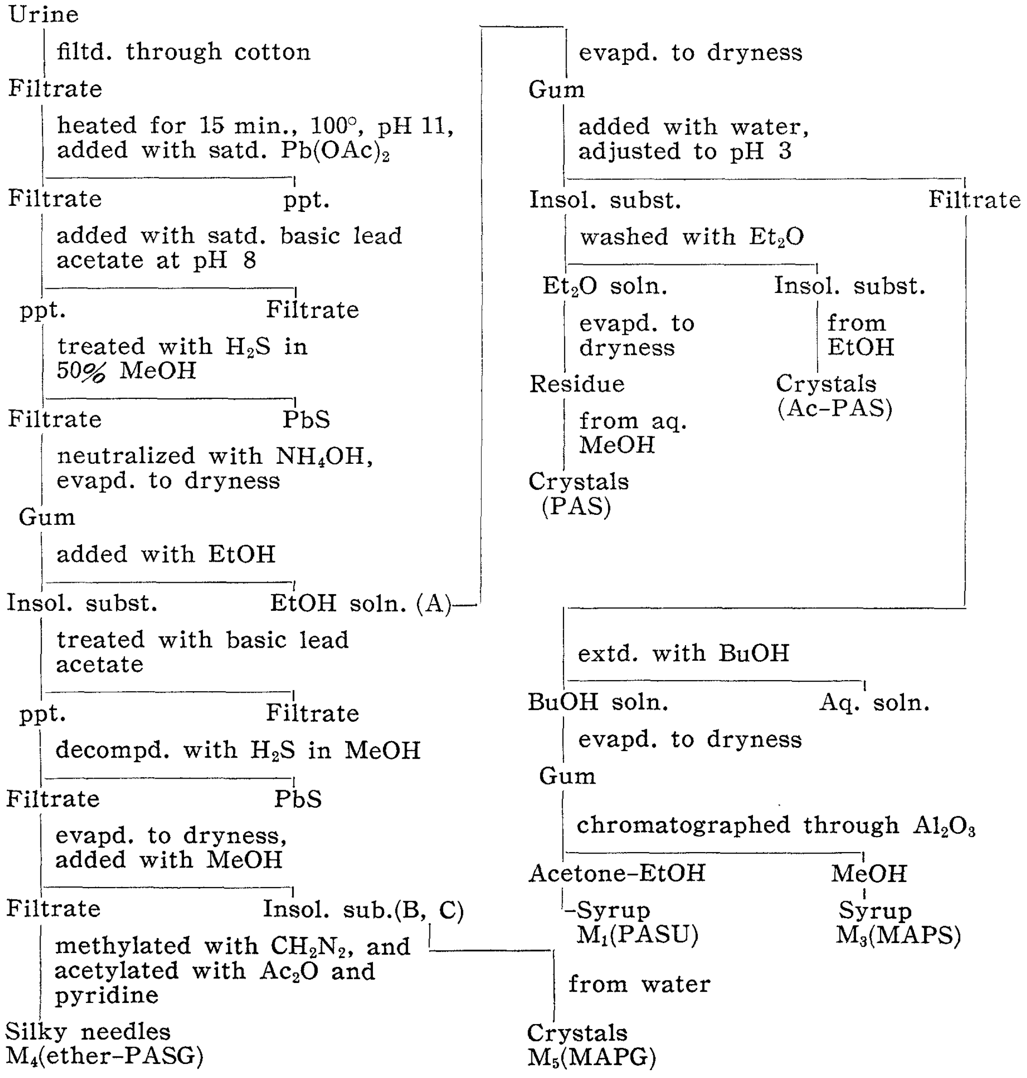
Identification of Other Metabolites—EtOH solution (A), which is mentioned above, was evaporated to dryness *in vacuo*. The residue was dissolved in 100 cc. of water and adjusted to pH 3 with 10% HCl, by which white crystals (E) separated immediately. After collecting the crystals by filtration, the filtrate (D) was extracted 3 times with 5 volumes of ether, which was washed with water and dried over Na_2SO_4. The combined ether extract was evaporated to dryness. Crystals (E) and above residue were washed with Et_2O, the sparingly soluble solid remained on the filter. Et_2O solution was evaporated and the residue crystallized from hydr. MeOH to yield 17.4 g. of PAS, m.p. 145°(decomp.). The ether-insoluble solid was recrystallized from EtOH to yield 15 g. of Ac-PAS, m.p. 220°(decomp.). The two isolated compounds showed no depression of m.p. with the respective authentic samples.

The filtrate (D) left after extraction with ether was again extracted five times with 5 volumes of BuOH, the combined BuOH was washed with a small amount of water, dried over Na_2SO_4, and evaporated to dryness *in vacuo*. The residue was chromatographed through an alumina column and eluted successively with acetone-EtOH (1 : 1) and MeOH. One fraction of acetone-EtOH eluate was evaporated *in vacuo* to a viscous syrup. The results of paper chromatography and electrophoresis indicated that this syrupy substance consisted of (M_1), which was identified as PASU in the previous work.[7]

MeOH eluate consisted of (M_3) only, which was identified as MAPS in the previous study.[7] It was attempted to crystallize 0.4 g. of the syrup obtained after evaporation of MeOH eluate, but no crystalline material could be obtained. The syrup was hydrolyzed with *N* HCl for 30 min. at 100° to isolate MAP, m.p. 122°. This showed no depression of m.p. with the authentic sample.

The insoluble material (B) and colorless crystals (C) were recrystallized from water to yield pure crystals (1.9 g.) of (M_5), which was identified as MAPG in the previous work.[7] The structure of (M_5) was confirmed by comparison of infrared absorption spectrum with that of MAPG isolated from the urine of rabbit receiving MAP.

The systematic separation of metabolites is shown in Chart 2.



Chrt 2. Separation of Metabolites

Paper Chromatography and Paper Electrophoresis of Metabolites—Ascending development was employed with Toyo Roshi No. 50. Solvent systems used were (A) BuOH-AcOH-H_2O (4:1:5), (B) BuOH-0.5*N* NH_4OH-EtOH (6:3:2), and (C) pyridine-AcOEt-H_2O (1:2:2). For the paper electrophoresis, the horizontal open-strip type apparatus (plastic frame support) was used with constant voltage. Electrolyte systems used were (D) 5*N* AcOH, (E) 0.1*M* tartaric acid, and (F) McIlvaine buffer (0.2*M* Na_2HPO_4+0.1*M* citric acid, pH 2~8). Electrolyte (F) was diluted to six volumes. The current of 700 V for the electrolyte (D) and (E) was applied in the experiment for a filter paper strip (24×12 cm.) and charged for 1 hr. For the electrolyte (F), the current of 500 V was charged for a filter paper strip (30×4.5 cm.) for 1 hr.

Metabolites were visualized on filter paper by spraying the following reagents: (1) Ehrlich's reagent, 2% *p*-dimethylaminobenzaldehyde in MeOH-conc. HCl (3:1), (2) diazo reagent, 0.2% $NaNO_2$, then 0.1*N* HCl, followed by 0.2% Tsuda reagent[18,19] (N-(2-diethylaminoethyl)-1-naphthylamine oxalate); (3) 1% $FeCl_3$; (4) diazotized sulfanilic acid;[20] (5) aniline hydrogen phthalate;[21] (6) ninhydrin reagent; and (7) fluorescence under ultraviolet light. The results are listed in Table I.

Table I. Paper Chromatography and Paper Electrophoresis of Metabolites

	Paper Chromatography			Paper Electrophoresis		Color Reaction				
Compd.	Solvent System A	B	C	Electrolyte D	E	Ehrlich reagent	$FeCl_3$ reagent	Diazo reagent	Diazotized sulfanilic acid	UV-light
		Rf		MD						
PAS	0.80	0.36	0.54	− 62	− 52	Orange-yellow	Red-brown	Red-violet	Brown	
Ac-PAS	0.81	0.48	0.38	− 40	− 18	—	Blue-brown	—	Yellow-brown	Blue-white
M_1 and PASU	0.54	0.2	0.32	− 69	− 65	Orange-yellow	Red-brown	Red-violet	〃	
M_2	0.32	—	0.21	− 47	− 39	〃	〃	〃	〃	
M_3 and MAPS	0.25	0.41	0.45	− 42	− 31	Yellow	—	Blue-violet	—	
M_4 and Ether-PASG	0.22	0.03	0.00	− 51	− 45	Orange-yellow	—	Red-violet	Yellow-brown	
M_5 and MAPG	0.16	0.25	0.11	− 75	− 71	Yellow	—	Blue-violet	Orange	
MAP	0.65	0.74	0.94	−123	−120	〃	—	〃	Brown	

In the electrolyte (F), the start line was drawn in the center of a filter paper. With the electrolyte higher than pH 8, all metabolites migrated to the anode. MAP indicated no migration at pH 7 and migrated to the cathode at pH 6, 5, and 4. MAP and MAPG migrated to the cathode and the other metabolites to the anode at pH 3. All metabolites migrated to the cathode at pH 2.

Aqueous 0.5% solution of (VI) was adjusted to pH 1 with 10% HCl and immersed in a water bath at 100°. The degradation products were investigated by means of paper chromatography and paper electrophoresis at intervals of 3 min. These results indicated that (VI) was decomposed to PAS, MAP, and MAPG as intermediates of hydrolysis. MAPG was acid-resistant and behaved the same as an authentic sample in all solvent systems and required 2 hr. for perfect hydrolysis. This seems to be an evidence that the hydrolysis of (VI) proceeds in both routes, splitting of the ether bond and decarboxylation, at the same time. Purified (M_4) fraction behaved the same as (VI) on hydrolysis. In *N* AcOH solution, (V) was hydrolyzed only in small amounts at 100°. The results of degradation of (V) on acid hydrolysis by electrolyte (D) are summarized in Fig. 1.

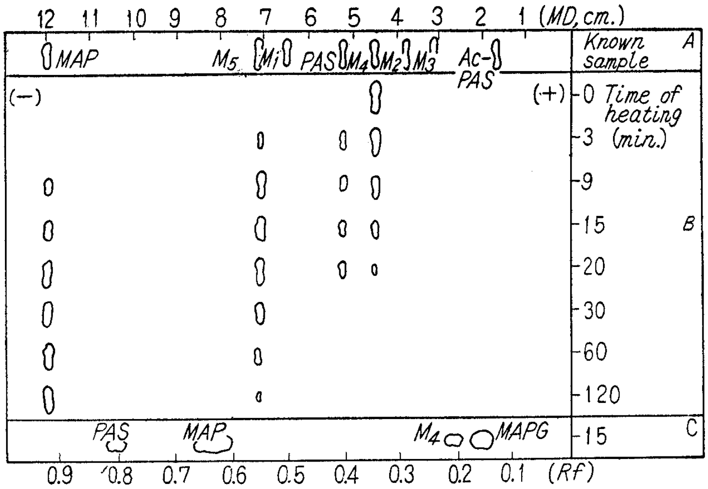


Fig. 1.
Degradation Products of M_4 and Synthetic Ether-PASG in the Acid Medium

A, B: Paper electrophoresis, 0.1*M* tartaric acid, 700 V/10 mA/hr.
C : Paper chromatography with solvent (A)

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18) K. Tsuda, *et al.* : Yakugaku Zasshi, **62**, 462(1942).
19) K. Tsuda, *et al.* : *Ibid.*, **67**, 239(1947).
20) R. A. Evans, W. H. Parr, W. C. Evans : Nature, **164**, 674(1949).
21) S. M. Partrige : *Ibid.*, **164**, 443(1949).

Ultraviolet and Infrared Absorption Spectra of Metabolites—The ultraviolet absorption spectra were determined by the Beckman DK-2 apparatus. The same samples as used in paper chromatography were used for M_1, M_2, M_3, and M_4. These samples were developed with solvent (A) on a filter paper (40×40 cm.), suitable sections were cut out, and extracted with 5 cc. of 0.5*M* phosphate buffer (pH 7). The following eluates were considered for ultraviolet spectra. PAS, Ac-PAS, and MAPG in 0.5*M* phosphate buffer of pH 7 and the derivatives of ester and ether-PASG in MeOH. Since the absorption spectra of all metabolites indicated the closest resemblance with known samples, these spectra were omitted from Fig. 2 to avoid complexity. These results are summarized in Table II and Fig. 2.

Table II. Ultraviolet Absorption Spectra of Metabolites

Compd.	Solvent	λ_{max} (mμ)	log ε	λ_{max} (mμ)	log ε
PAS	Acetate buffer pH 7	264	4.44	299	4.28
Isolated	〃	265	4.44	300	4.30
Ac-PAS	〃	261	4.25	301	3.80
Isolated	〃	261	4.25	302	3.80
PASU	〃	274		302	
M_1	〃	272		302	
M_2	〃	240		305	
MAPS	〃	232		280	
M_3	〃	232		280	
Ether-PASG	〃	260			
M_4	〃	260			
MAPG	〃	233	3.92	282	3.36
Isolated	〃	233	3.92	282	3.35
MAP	〃	230	3.87	282	3.34
Deriv. of ester-PASG	MeOH	277	4.22		
Isolated	〃	277	4.23		
Deriv. of ether-PASG	〃	265	3.97		
Isolated	〃	265	3.98		

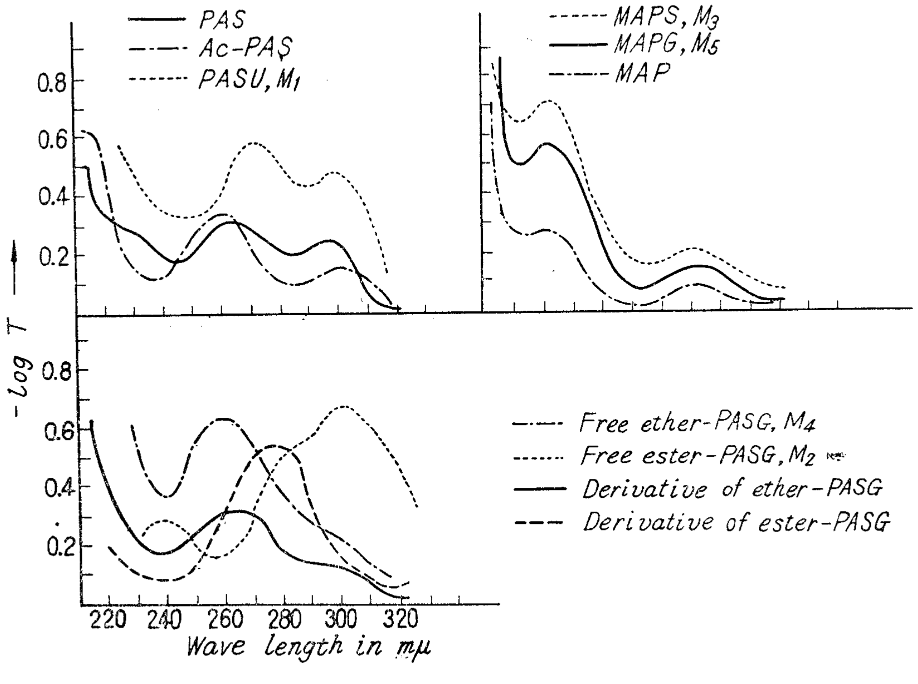

Fig. 2. Ultraviolet Absorption Spectra of Metabolites

The infrared absorption spectra of authentic PAS, Ac-PAS, MAPG, and the derivative of ether-PASG are shown in Fig. 3. PAS, Ac-PAS, and MAPG isolated from the urine of rabbit indicated complete agreement with authentic samples but the isolated derivative of ether-PASG was too small in quantity for the estimation of infrared absorption spectrum.

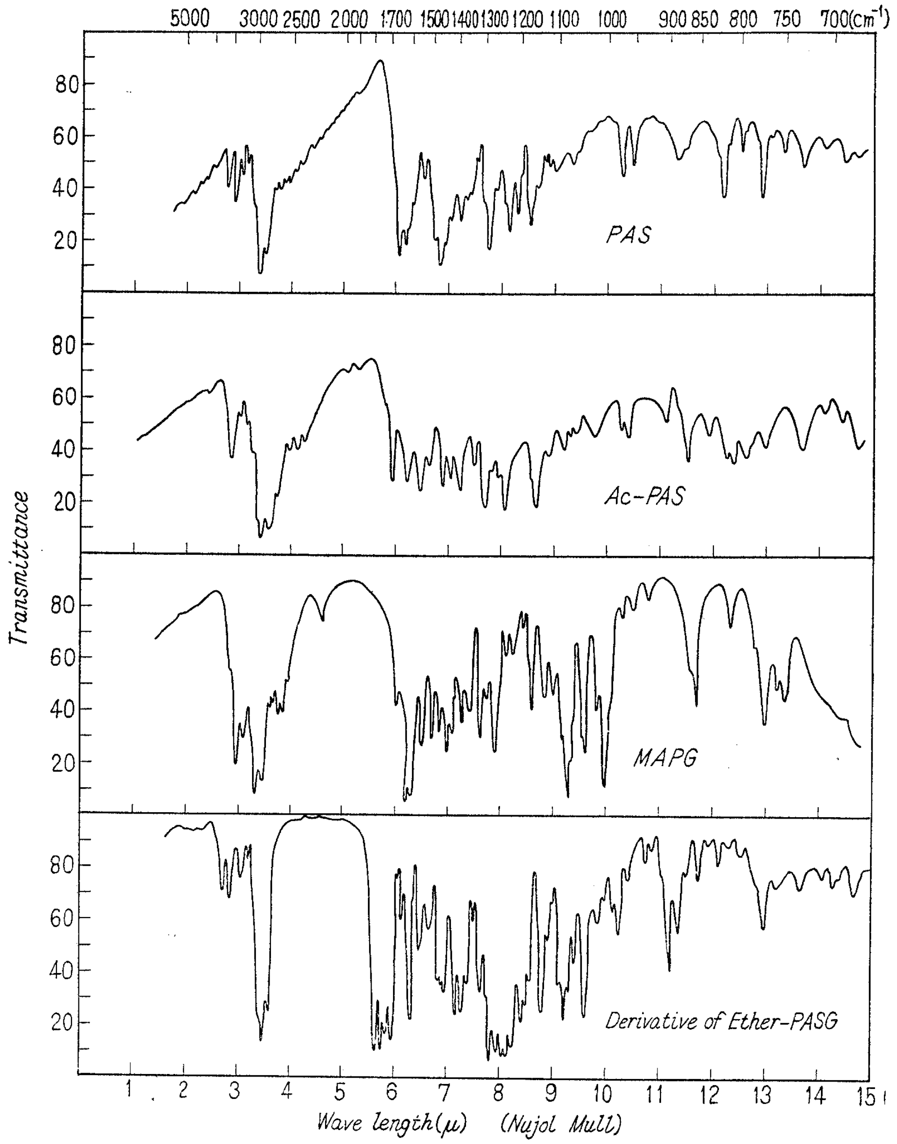

Fig. 3. Infrared Absorption Spectra of Metabolites

Enzymatic Hydrolysis of Glucuronides—There are extensive literatures on the enzymatic hydrolysis of glucuronides. Levvy and Worgan[22] concluded that β-glucuronidase hydrolyzed the ester glucuronide as the ether, and the hydrolysis was inhibited by boiled saccharate solution (saccharo-1,4-lactone).[23,24]

Tsou and Seligman,[25] and Levvy[26] concluded from their respective studies that β-glucuronidase is specific for the hydrolysis of β-D-glucopyranuronosides. Above supports the view that the glucuronides of metabolites of PAS, hydrolyzed enzymatically, were the glucuronides having the structure of β-D-configuration.

Assay : The enzyme was partially purified by the following method described by Talalay, *et al.*[27]: Homogenization of 1 g. of mouse liver was done in a Potter-Elvehjem glass homogenizer for 2 min. with 5 cc. of ice-cold water. The cell-free homogenate was transferred to a graduated centrifuge tube, using 3 cc. of wash water. The homogenate was adjusted to pH 5.2 with 0.3*M* citrate buffer. The tube was maintained at 37° for 30 min. The coagulated protein was separated by centrifuging

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22) G.A. Levvy, J.T. Worgan : Biochem. J., **59**, 451(1954).
23) M.C. Karunairatnam, G.A. Levvy : *Ibid.*, **44**, 599(1949).
24) G.A. Levvy : *Ibid.*, **52**, 464(1952).
25) K.C. Tsou, A.M. Seligman : J. Am. Chem. Soc., **75.**, 1042(1949).
26) G.A. Levvy, C.A. Marsh : Biochem. J., **52**, 690(1952).
27) P. Talalay, *et al.* : J. Biol. Chem., **166**, 757(1946).

for 15 min. The supernatant liquid was transferred to another graduated tube and added with an equal volume of saturated $(NH_4)_2SO_4$ solution. The tube was centrifuged for 30 min. The precipitate was dissolved in 4 cc. of water. In normal animals, 4 cc. of the solution gave an average reading value of 376 μg. phenol after enzymatic hydrolysis of phenol glucuronide by the method described by Levvy, *et al.*[28]

This volume of the enzyme solution was added to 2 cc. of $0.1M$ citrate buffer (pH 5.2), 1 cc. of substrate solution and 1 cc. of water or 1 cc. of $0.08M$ inhibitor solution (potassium hydrogen saccharate solution was adjusted to pH 3.5 and boiled for 30 min. for the formation of saccharo-1,4-lactone) in a stoppered 15 cc. centrifuge tube. Prior to addition of the enzyme, the substrate and saccharate were adjusted to pH 5.2.

After incubation at 37° for 2 hr., the mixture was adjusted to pH 3 with 10% HCl, added with 70 cc. of EtOH, the protein precipitate was removed by centrifugation, the supernatant was decanted, and evaporated to dryness *in vacuo* at 30°. The residue was extracted with 2 drops of water and chromatographed alongside known samples.

1) **M_2**—The aqueous eluate (1 cc.) of filter paper, prepared as mentioned in the estimation of ultraviolet absorption spectrum was adjusted to pH 5.2 and used as the substrate. The paper chromatography and paper electrophoresis of the hydrolysate indicated the same pattern as authentic PAS with several solvent systems by spraying several reagents. The hydrolysis was completely inhibited by boiled saccharate solution.

2) **M_4**—The eluate (1 cc.) prepared as meotioned above was added to the incubation mixture. The hydrolysate contained PAS and the boiled saccharate inhibited the hydrolysis completely.

3) **M_5**—One cc. each of $0.06M$ solutions of M_5 and MAPG, isolated from the urine of rabbit receiving MAP, were added to the incubation mixture. The hydrolysates contained MAP and unhydrolyzed M_5 and MAPG, which behaved completely the same. The hydrolysis was also inhibited by the boiled saccharate.

4) **Synthetic Free Ether-PASG**—A solution of 5 mg. of ether-PASG dissolved in 1 cc. of water was added to the incubation mixture. The hydrolysate indicated the same behavior as M_4 and the boiled saccharate inhibited the reaction.

5) **Alkaline Hydrolysate of Derivative of Synthetic Ether PASG**—A suspension of 8 mg. of ether-PASG derivative in 0.5 cc. of 1% NaOH was heated for 20 min. in a boiling water. When cool, it was adjusted to pH 5.2 and made to 1 cc., which was added to the incubation mixture. The enzymatic hydrolysis yielded Ac-PAS and was inhibited by the boiled saccharate solution. The result shows that the alkaline hydrolysate was Ac-PAS glucuronide of the ether type. It overlapped Ac-PAS in the electrolyte (D) and (E) on the electropherogram, but differed from Ac-PAS in that it is not fluorescent under ultraviolet rays, and showed Rf 0.2 in the solvent (A). These results are summarized in Fig. 4.

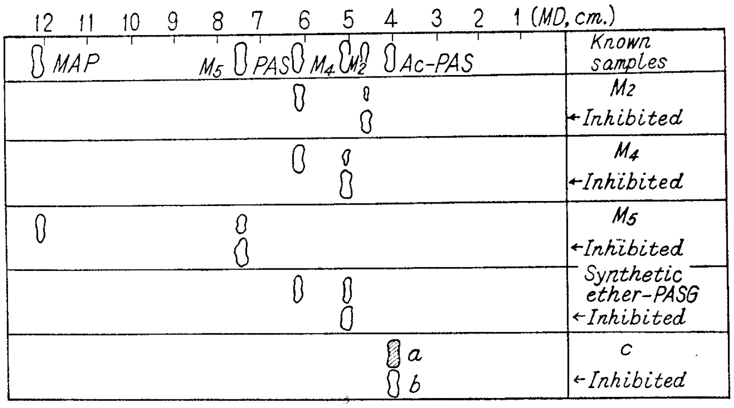

Fig. 4. Enzymatic Hydrolysis of Glucuronide

Paper electrophoresis : $5N$ AcOH, 900 V/7.5 mA/1 hr., Toyo Roshi No. 50 (24×12 cm.) a : fluorescence under UV light; b : no fluorescence; c : alkaline hydrolysate of synthetic derivative of ether-PASG

Discussion

In the previous paper, occurrence of ether-PASG in the urine of rabbit receiving PAS was suggested only through paper chromatography. Its isolation was successful in spite of its difficulties, its acid-labile properties, and small quantity of excretion. For this fact, two types of glucuronides, the ester and ether, were confirmed to exist in the urine of rabbit receiving PAS. The extracts of paper chromatogram (solvent (C)) were

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[28] G. A. levvy, *et al.* : Biochem. J., **42**, 191(1948).

determined by the naphthoresorcinol reaction[29] and the results indicated that the ester-PASG corresponds to 2.3∼3.2% of administered PAS and 0.6∼0.7% of ether-PASG. The pure derivative isolated from the urine was identified with the synthetic sample by elemental analysis, ultraviolet absorption spectra, and by admixture with the synthetic sample. Furthermore, the free ether-PASG was identified with the synthetic sample by paper chromatography, paper electrophoresis, ultraviolet absorption spectra, and behavior to chemical and enzymatic hydrolysis.

All attempts to crystallize synthetic ether-PASG was unsuccessful.

It was confirmed that ether-PASG was acid-labile and readily decarboxylated to yield MAPG. Following examinations were made to see whether the MAPG was dependent upon the isolation procedure or not.

1) Ether-PASG indicated no decomposition under all conditions used by chromatography and electrophoresis on a filter paper.

2) After oral administration of 1 g./kg. of PAS, 2-hr. urine and subsequent 1-hr. urine were collected. The later fresh urine was immediately developed with the solvent (C) for 6 hr. The paper chromatogram indicated distinct spots of MAPS and MAPG.

3) After the addition of ether-PASG to normal urine, it was incubated for 6 hr. at 37°. The urine was paper chromatographed with solvent (C), but ether-PASG did not indicate any appreciable amount of decomposition.

4) The samples of PAS-Na used contained less than 0.03% of MAP. These findings support the conclusion that urinary MAPG was not the product produced by decarboxylation of ether-PASG during the isolation procedures, but the decarboxylated product of PAS or ether-PASG occurring *in vivo*, through enzymatic or other processes.

MAPG isolated from the urine of rabbit receiving PAS was identified by means of infrared absorption spectrum with MAPG isolated from the urine of rabbit receiving MAP.

Two new metabolites were found by means of two-dimensional paper chromatography; one was a free amine having Rf 0.8 and 0.27, and the other was a conjugated amine having Rf 0.49 and 0.2 with solvent (A) and (C), respectively. Further studies are necessary to establish the identity of these two unknown metabolites.

Summary

The ether-type PAS-glucuronide was isolated as the acetyl-methyl derivative from the urine of rabbit receiving PAS and the structure was established as methyl (5-acetamido-2-methoxycarbonylphenyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate, which was chemically synthesized. The structure of *m*-aminophenyl glucuronide was identified with authentic specimen by means of infrared absorption spectra.

It was confirmed by enzymatic hydrolysis that the glucuronides of PAS had the structure of β-D-glucopyranuronoside. The relation of *m*-aminophenol, *m*-aminophenyl sulfate, *m*-aminophenyl glucuronide, ether-type PAS-glucuronide, and PAS was discussed.

(Received November 6, 1958)