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METABOLISM OF DRUGS—LVI
THE METABOLIC FATE OF PHENACETYLUREA

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Abstract—The identification of 4-hydroxyphenacetylurea and 3-methoxy-4-hydroxyphenacetylurea in the urine of rabbits fed phenacetylurea is reported together with that of unchanged phenacetylurea. In addition to these metabolites, hydrolyzed metabolites, phenaceturic acid, and phenylacetic acid were also demonstrated to be excreted into the urine. However, unlike some drugs that possess an amide group in the molecule, the excretion of phenacetylurea *N*-glucuronide could not be recognized.

It is considered from the above results that the metabolic fate of phenacetylurea consists of two different pathways: one is hydrolysis of the ureide group, and the other is successive hydroxylation and methylation of the benzene nucleus.

The methyl acetyl derivative of phenacetylurea *N*-glucuronide was synthesized by acylation of methyl 1-deoxy-1-thioureido-2,3,4-tri-o-acetyl-β-D-glucopyranosiduronate with phenylacetylchloride, and subsequent desulfurization with silver nitrate.

4-Hydroxyphenacetylurea and 3-methoxy-4-hydroxyphenacetylurea were synthesized by condensation of the corresponding acid esters with urea.

PHENACETYLUREA (phenurone) is a synthetic anticonvulsant, effective in most types of seizure. It was synthesized by Spielman *et al.*[1] and its anticonvulsant properties were investigated by Everett[2] and by Everett and Richards.[3]

In the study on the metabolism of this drug in rats, Asher, Taylor and Richards[4] showed that the ureide group was initially hydrolyzed and the acid product was subsequently conjugated, but no other metabolic pathways have been reported so far.

It was previously shown in this laboratory that carbamate *N*-glucuronides were excreted in considerable amounts together with other metabolites in the urine of rabbits after dosing with meprobamate and ethinamate.[5, 6]

It was also reported by other workers in recent years that arylureas[7–9] and sulfonamides[10–12] administered were excreted as amide-type *N*-glucuronides. However, no report concerning the formation of ureide *N*-glucuronide *in vivo* has been made.

The present investigation was initiated in order to examine whether phenacetylurea could form ureide *N*-glucuronide. In addition, investigation of other metabolic pathways was also undertaken. The mixture of metabolites extracted continuously with AcOEt* from urine of the rabbit fed phenacetylurea was separated by means of silica gel chromatography, and four metabolites besides unchanged phenacetylurea were obtained as crystalline materials.

On the basis of the characterization of these metabolites, it seemed that phenacetylurea was metabolized along two different routes.

* The abbreviations used in this paper are: AcOEt, ethyl acetate; MeOH, methanol; EtOH, ethanol; EtONa, sodium ethylate; MeONa, sodium methylate; HIO_4, metaperiodate; BuOH, butanol; AcOH, acetic acid.

This paper presents isolation, characterization, and identification of these metabolites, and also discusses the possible pathways *in vivo*.

METHODS

Material. Phenacetylurea was donated by Dainippon Pharmaceutical Inc.

Administration of phenacetylurea. Phenacetylurea (200 mg/kg) was suspended in 10% gum arabic and given by stomach tube to male albino rabbits weighing about 3 kg.

Extraction of metabolites from urine. A 24-hr urine from 16 rabbits which were given a total of 9·6 g phenacetylurea was continuously extracted for 20 hr with AcOEt at pH 2·5.

Paper and thin-layer chromatography. Thin-layer chromatography was carried out by use of silica gel plates ('Silica-Rider', Daiichi Pure Chemicals Co., Tokyo), 0·25 mm thick, activated at 100° for 1 hr. The solvent system used was $CHCl_3$–AcOEt (1:1). The chromatograms were visualized by spraying with H_2SO_4–$K_2Cr_2O_7$ solution followed by heating. Paper chromatography was carried out in the ascending technique with Toyoroshi no. 50 filter paper (Toyoroshi Co., Tokyo).

RESULTS

Separation of metabolites from the AcOEt *extracts*

Separation of metabolites from the AcOEt extracts described under Methods was performed by a procedure shown in Fig. 1.

Chromatography of substance A

A portion (6·3 per cent of total) of substance A (2·05 g) was dissolved in $CHCl_3$ containing a small volume of MeOH and submitted to column chromatography (6·5 g silica gel was packed). The column was eluted stepwise with $CHCl_3$, $CHCl_3$–MeOH (99:1) and $CHCl_3$–MeOH (98:2). The results are shown in Table 1.

From fractions 1–2 and fractions 9–15, metabolite I (MI) and metabolite III (MIII) were obtained as almost pure metabolites. Both were recrystallized from EtOH to white crystals, m.p. 212–216° and 190–195°, respectively. The i.r. absorption spectrum of the crystals from fractions 3–8 suggested that they consisted mainly of MIII, but were contaminated with MI. Therefore, it was again submitted to silica gel chromatography as described above. From the $CHCl_3$–eluate a little more MI (18 mg) was obtained and from the fraction of $CHCl_3$–MeOH (99:1) 50 mg of MIII was obtained.

Chromatography of substance B

Substance B was dissolved in $CHCl_3$ and chromatographed through a silica gel (200 g) column in which effluents were fractionated by stepwise elution with $CHCl_3$ and the solvent mixtures of $CHCl_3$ and increasing amounts of MeOH. The result is summarized in Table 2.

Metabolite V (MV) obtained from fractions 16–30 was recrystallized from petroleum ether to plates, m.p. 76·5°. Metabolite III (MIII) obtained from fractions 37–42 was recrystallized from EtOH to white crystals, m.p. 190–195°. Crystals from fractions 46–69 and from fractions 93–134 were recrystallized from benzene–$CHCl_3$ to give the

same colorless needles, m.p. 143° (MIV). Metabolite II (MII) obtained from fractions 70–78 was recrystallized from EtOH to white crystals, m.p. 189–192°. Crystals from fractions 135–149 were thought to be mostly inorganic.

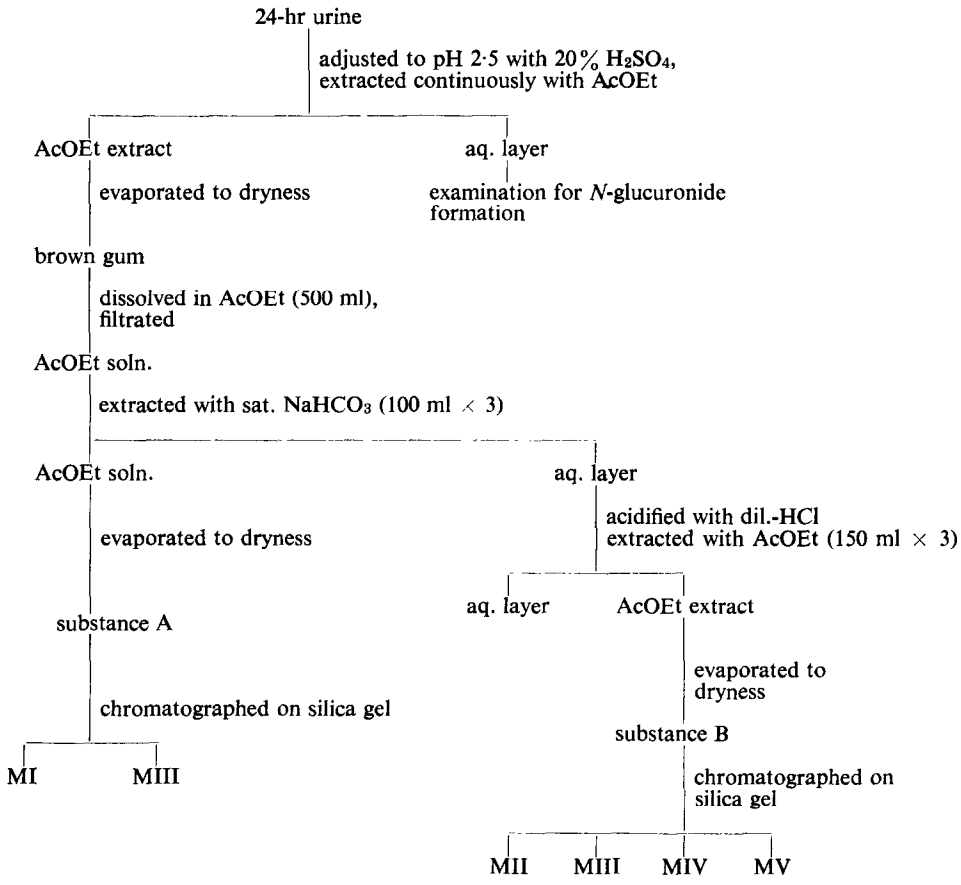

FIG. 1

TABLE 1. CHROMATOGRAPHY OF THE SUBSTANCE A

Fraction no.	Solvent	Eluted product (mg)	Note
1– 2	$CHCl_3$	26	crystals (MI)
3– 8	$CHCl_3$–MeOH (99:1)	80	crystals (MI), (MIII)
9–15	$CHCl_3$–MeOH (98:2)	20	crystals (MIII)

* Each fraction = 20 ml.

Characterization and identification of metabolites

Metabolite I (MI). It was identified with unchanged phenacetylurea by mixed m.p. test and by u.v. and i.r. absorption spectra. It developed no color with diazotized sulfanilic acid.[13]

Metabolite II (*MII*). When sprayed with diazotized sulfanilic acid reagent, metabolite II showed red-orange on filter paper. Found: C, 55·46; H, 5·34; N, 14·46. $C_9H_{10}O_3N_2$ requires C, 55·66; H, 5·19; N, 14·43. From these results, this metabolite was considered to be monohydroxyphenacetylurea which possesses a hydroxyl group on the benzene nucleus. It showed no depression of m.p. when mixed with the synthesized 4-hydroxyphenacetylurea and the u.v. and i.r. absorption spectra were also identical with those of the authentic sample.

TABLE 2. CHROMATOGRAPHY OF SUBSTANCE B

Fraction no.	Solvent	Eluted product (mg)	Note
1–15	$CHCl_3$	20	Oily substance
16–30	$CHCl_3$–MeOH (99:1)	520	Crystals (MV)
31–36	$CHCl_3$–MeOH (99:1)	50	Oily substance
37–42	$CHCl_3$–MeOH (98:2)	310	Crystals (MIII)
43–45	$CHCl_3$–MeOH (98:2)	400	Oily substance
46–69	$CHCl_3$–MeOH (98:2)	830	Crystals (MIV)
70–78	$CHCl_3$–MeOH (98:2)	170	Crystals (MII)
79–86	$CHCl_3$–MeOH (98:2)	Trace	
87–92	$CHCl_3$–MeOH (97:3)	Trace	
93–107	$CHCl_3$–MeOH (97:3)	200	Crystals (MIV)
108–134	$CHCl_3$–MeOH (95:5)	320	Crystals (MIV)
135–149	$CHCl_3$–MeOH (90:10)	410	Crystals (–)
150–152	MeOH	2400	Brown gum

* Each fraction = 100 ml.

Metabolite III (*MIII*). The color reaction with diazotized sulfanilic acid showed red-violet on filter paper. The elemental analysis of this metabolite agreed with methoxyhydroxyphenacetylurea. Found: C, 53·89; H, 5·42; N, 12·73. $C_{10}H_{12}O_4N_2$ requires C, 53·57; H, 5·39; N, 12·50. Alkaline hydrolysis of this methoxyhydroxy metabolite gave homovanillic acid, m.p. 140–143°, identified by m.p., mixed m.p. and analysis.

The final identification of this metabolite with chemically synthesized authentic 3-methoxy-4-hydroxyphenacetylurea was performed by mixed m.p. test and by comparison of the u.v. and i.r. absorption spectra.

Metabolites IV (*MIV*) and *V* (*MV*) These were identified with phenaceturic acid and phenylacetic acid, respectively, by mixed m.p. test and i.r. absorption spectra.

The structures and yields (per cent of the dose) of these metabolites are shown in Fig. 2 and the u.v. absorption spectra in Fig. 3.

Examination for phenacetylurea N-*glucuronide formation*

After continuous extraction with AcOEt for 20 hr, the aqueous layer was treated to obtain glucuronide gum by the method of Kamil, Smith and Williams.[14] The resulting gum, which was methylated and acetylated by the usual method, was submitted to a silica gel column chromatography with benzene, $CHCl_3$, $CHCl_3$–AcOEt (1:1) and AcOEt–MeOH (1:1) as effluent solvents. Aliquots of each eluate were examined by thin-layer chromatography whether or not they contained a methyl

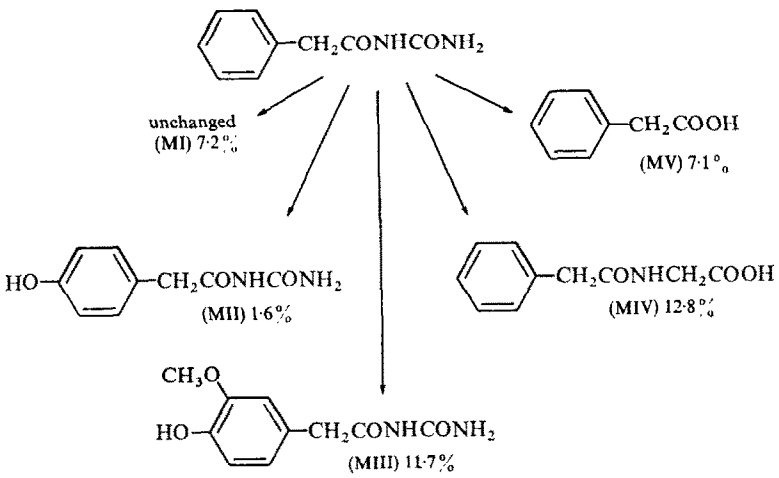

FIG. 2.

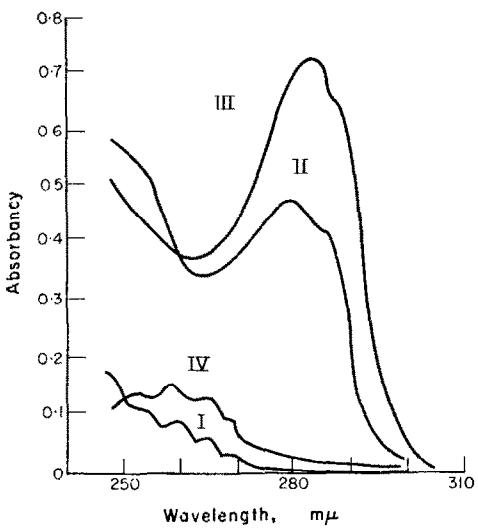

FIG. 3. Ultraviolet absorption spectra of the metabolites of phenurone. Curves I, II, III and IV represent metabolites I, II, III and IV respectively, in EtOH (50 μg/ml).

acetyl derivative of phenacetylurea *N*-glucuronide, methyl 1-deoxy-1-(3-phenylacetylureido)-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate, but no spot corresponding to the authentic sample was detected.

Chemical syntheses for identification of metabolites

Synthesis of 4-hydroxyphenacetylurea (MII). 4-Hydroxyphenylacetic acid was converted to ethyl 4-hydroxyphenylacetate (b.p.$_{760\cdot5}$ 314°)[15] by treatment with C_2H_5OH–HCl solution by the usual method.

To a solution of 0·21 g urea in a mixture of 3·73 ml of 25% EtONa solution and 2 ml pyridine, 0·39 g ethyl 4-hydroxyphenylacetate was added. It was allowed to stand for 24 hr at room temperature and then poured into 30 ml of 7·3% acetic acid while stirring, and extracted with AcOEt (50 ml × 6). The AcOEt extract was successively washed with dil. HCl and H_2O, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure, leaving yellow crystalline material. Recrystallization from EtOH gave white crystals, m.p. 189–192°. (Found: C, 55·31; H, 5·19; N, 14·08. $C_9H_{10}O_3N_2$ requires C, 55·66; H, 5·19; N, 14·43%.) u.v. λ_{max}^{EtOH} mμ (log ε): 225(3·49), 279(3·26). i.r. λ_{max}^{KBr} μ: 2·90, 2·95, 3·08, 5·83, 5·85, 5·95, 6·18, 6·23, 6.58.

Synthesis of 3-*methoxy*-4-*hydroxyphenacetylurea* (*MIII*). 3-Methoxy-4-hydroxyphenylacetic acid (homovanillic acid, m.p. 142–143°) was prepared via the oxazolone derivative, which was synthesized by condensing vanillin with acetylglycine in the presence of acetic anhydride and anhydrous sodium acetate. Alkaline hydrolysis of the oxazolone derivative gave 3-methoxy-4-hydroxyphenylpyruvic acid which was oxidized *in situ* with hydrogen peroxide to 3-methoxy-4-hydroxyphenylacetic acid according to the method of Shaw *et al.*[16]

The esterification[17] of 3-methoxy-4-hydroxyphenylacetic acid, followed by condensation with urea, was carried out by a method similar to that used for 4-hydroxyphenacetylurea, described above. After recrystallization from EtOH, white crystals, m.p. 190–196°, were obtained. (Found: C, 53·28; H, 5·64; N, 12·42. $C_{10}H_{12}O_4N_2$ requires C, 53·57; H, 5·39; N, 12·50%.) u.v. λ_{max}^{EtOH} mμ (log ε):283(3·59). i.r. λ_{max}^{KBr} μ: 2·80, 2·93, 2·99, 5·91, 5·99, 6·15, 6·20, 6·63.

Synthesis of methyl acetyl derivative of phenacetylurea N-*glucuronide*

Methyl 1-*deoxy*-1-(3-*phenylacetylthioureido*)-2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosiduronate* (*VI*). Methyl 1-deoxy-1-thioureido-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (2 g), which was prepared according to the method of Kuranari,[18] was dissolved in 2·5 ml of P_2O_5-dried $CHCl_3$, and to this solution phenylacetylchloride (0·96 g) and pyridine (0·61 g) were added. The mixture was allowed to stand for 24 hr at room temperature and 30 ml of $CHCl_3$ was then added. After successive washing with $NaHCO_3$ solution and water, the $CHCl_3$ solution was dried over anhydrous sodium sulfate and concentrated to dryness at a reduced pressure. The oily residue was crystallized from hot water. The crude product was recrystallized from AcOEt-isopropyl alcohol to give 2·14 g of VI (84 per cent yield), as white crystals, m.p. 90–99°. (Found: C, 51·85; H, 5·27; N, 5·57. $C_{21}H_{26}O_{10}N_2S$ requires C, 51·76; H, 5·10; N, 5·49%.) i.r. λ_{max}^{Nujol} μ: 2·96, 3·05, 3·10, 5·69, 5·73, 5·88, 6·44, 6·50, 6·65.

Methyl 1-*deoxy*-1-(3-*phenylacetylureido*)-2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosiduronate* (*VII*). To a hot solution of 1·2 g of VI in 24 ml EtOH, a solution of 1·2 g $AgNO_3$ in 6 ml H_2O was added and the mixture was heated at 50–55° on a water bath. After 5 min, 10% NaOH was added to the reaction mixture in order to neutralize HNO_3 liberated from $AgNO_3$. The solution was heated until Ag_2S coagulated almost completely (about 30 min). It was then cooled and filtered. The filtrate was concentrated to dryness *in vacuo*. The residue was submitted to silica gel (50 g) column chromatography with 600 ml $CHCl_3$ and 50 ml $CHCl_3$–AcOEt (1:1) as effluent solvents. From the fraction eluted with $CHCl_3$, *N*-phenacetylurethane (m.p. 95–99°) was obtained as colorless needles.

On the other hand, the fraction eluted with $CHCl_3$–AcOEt (1:1) was evaporated to dryness *in vacuo* to yield methyl 1-deoxy-1-(3-phenylacetylureido)-2,3,4-tri-*O*-acetyl-β-D-glycopyranosiduronate (VII), 0·69 g, as white crystals. An analytical sample was obtained by recrystallization from EtOH as colorless needles, m.p. 190–196°. (Found: C, 53·36; H, 5·53; N, 5·53. $C_{22}H_{26}O_{11}N_2$ requires C, 53·44; H, 5·26; N, 5·67%). i.r. λ_{max}^{KBr} μ: 2·93, 3·00, 5·70, 5·80, 5·88, 6·49.

To a mixture of 35 ml absolute MeOH and 0·39 ml 2 N MeONa solution, 0·35 g of VII was added and the mixture was allowed to stand overnight in a refrigerator. The reaction mixture was evaporated to dryness *in vacuo*, leaving a brown crystalline mass. After addition of a small amount of water to the residue, it was extracted with AcOEt (10 ml × 3). The extract contained an oily substance which was shown to be identical with authentic methyl phenylacetate by the i.r. absorption spectrum. The aqueous solution, which was considered to contain phenacetylurea *N*-glucuronide, was then evaporated to dryness *in vacuo*. The residue was submitted to alumina (4 g) column chromatography with MeOH, MeOH–H_2O (9:1) and MeOH–H_2O (1:1) as effluent solvents. This chromatography was very effective for purification of meprobamate *N*-glucuronide,[6] however, it was unsuccessful for isolation of the phenacetylurea *N*-glucuronide, 1-deoxy-1-(3-phenylacetylureido)-β-D-glucopyranosiduronic acid. Further investigation of this aqueous layer by paper chromatography indicated that the principal product in this solution was not the expected *N*-glucuronide, but urea *N*-glucuronide, for the following reasons. (1) There was only one glucuronide spot which was positive to HIO_4-benzidine reagent at R_f 0·14 by use of the solvent system, BuOH–AcOH–H_2O (4:1:5). This R_f value seemed to be too small for phenacetylurea *N*-glucuronide. (2) This spot was also colored bright yellow by Ehrlich's reagent which is positive to urea derivatives but not to ureide derivatives. (3) The aqueous extract of the spot gave a positive naphthoresorcine test.

It seemed very likely that phenacetylurea *N*-glucuronide was quite labile in alkaline solution and it was further decomposed to methyl phenylacetate and urea *N*-glucuronide by methanolysis, as shown in Fig. 4.

DISCUSSION

Everett and Richards[3] reported that no appreciable quantities of phenacetylurea were detectable in the human urine after a single dose of 2 g of the drug and that no phenacetylurea appeared in the urine of patients taking 2–3 g/day over periods of several years. On the other hand, Asher *et al.*,[4] using radioisotope techniques, suggested that phenacetylurea was excreted presumably as conjugated phenylacetic acid and not as free acid. No work reporting other metabolites, besides the compound mentioned above, has appeared so far.

The present investigation was undertaken to establish metabolic pathways of phenacetylurea in more detail. In this study *in vivo* in rabbits, 4-hydroxyphenacetylurea and 3-methoxy-4-hydroxyphenacetylurea in addition to the unchanged compound were isolated. Another metabolite series in which the ureide group was hydrolyzed was also obtained. The products were phenylacetic acid and phenaceturic acid, some parts of which were considered to be naturally occurring.

In the separation procedure of metabolites shown in Fig. 1, 3-methoxy-4-hydroxyphenacetylurea (MIII) was not separated sharply. The procedure, therefore, was not the best; however, more experiments on this separation were not performed.

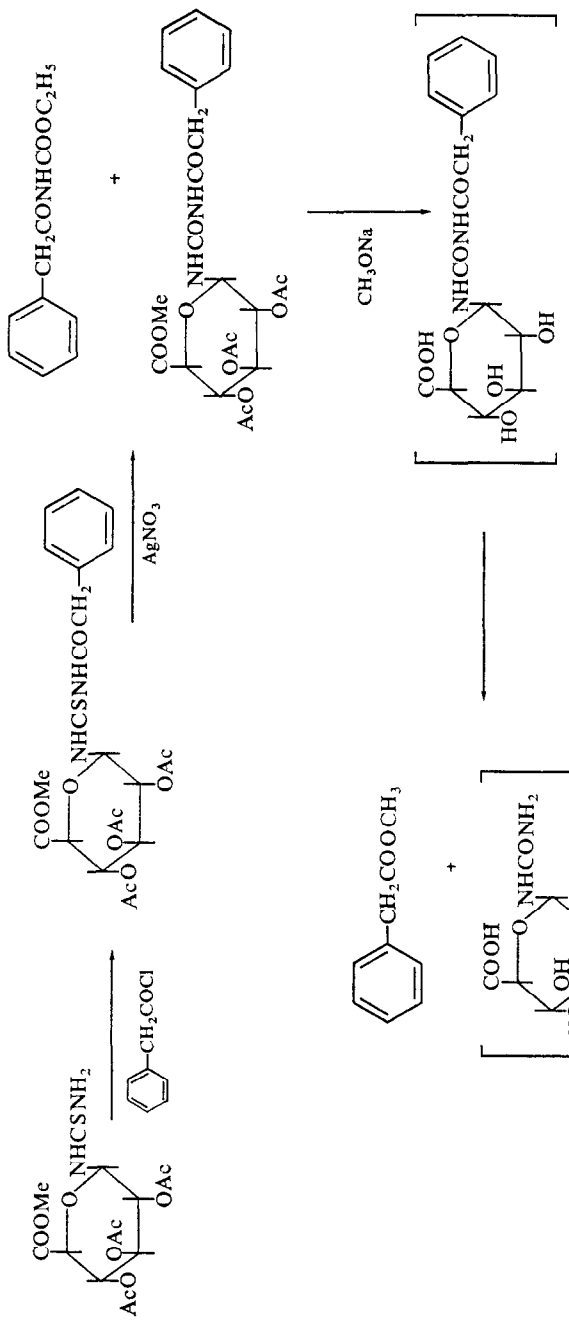

FIG. 4.

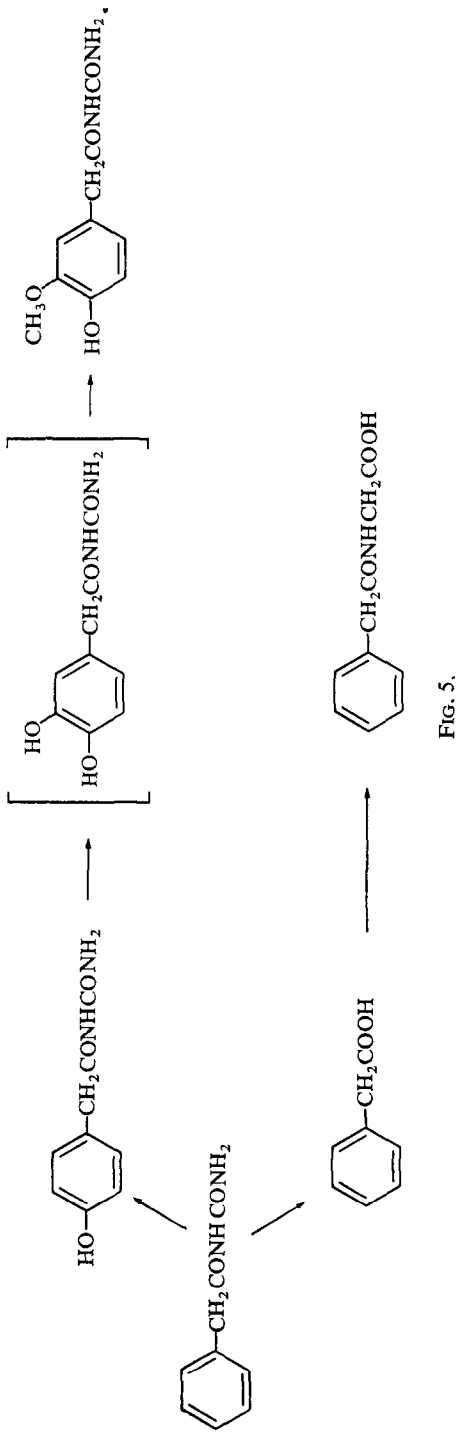
CH2CONHCONH2
CH3O
HO
CH2CONHCONH2
HO
HO
CH2CONHCONH2
HO
CH2CONH CONH2
CH2COOH
CH2CONHCH2COOH

FIG. 5.

The phenolic metabolites, 4-hydroxyphenacetylurea and 3-methoxy-4-hydroxyphenacetylurea were unequivocally synthesized by condensation of the corresponding acid esters, instead of acylhalides, with urea in a mixed solvent of EtOH–pyridine to exclude any possibility of acylation at the phenolic OH.

Next, attention was directed to whether phenacetylurea could form *N*-glucuronide. For this purpose the authors attempted, first of all, the synthesis of the authentic sample and utilized methyl 1-deoxy-1-thioureido-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate, which was unequivocally synthesized by Kuranari, as the starting material. It was converted to methyl 1-deoxy-1-phenylacetylthioureido-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (VI) by treatment with phenylacetylchloride, which was in turn desulfurized with silver nitrate to methyl 1-deoxy-1-phenylacetylureido-2,3,4-tri-*O*-acetyl-β-D-glucopyranosiduronate (VII). In this case, *N*-phenylacetylurethane was obtained as a by-product. Its formation could be interpreted by ethanolysis at the amide group, adjacent to the sugar C_1 atom, in the presence of HNO_3 liberated from the desulfurization process. When VII was treated with a solution of sodium methoxide in methanol in order to remove the protecting group, methyl phenylacetate was obtained instead of 1-deoxy-1-phenylacetylureido-β-D-glucopyranosiduronic acid. It seemed that the ureide system of this glucuronide was unstable in alkaline solution. The authors, therefore, used the methyl acetyl derivative instead of phenacetylurea *N*-glucuronide itself for examination of urinary *N*-glucuronide.

From a consideration of these facts, it seems most reasonable to conclude that the metabolic fate of phenacetylurea consists of two different pathways. The first one is successive hydroxylation at the 4 position and then the 3 position of the benzene nucleus, followed by methylation of the 3-OH group. It is very interesting that this pathway is identical with that of phenylalanine, which biotransforms to tyrosine, Dopa, and 3-methoxytyrosine successively by hydroxylation and methylation in mammals. Catecholamines and other endogenous dihydroxy compounds also undergo *m*-methylation. It must be noticed that the present finding is the first example of such a drug metabolism *in vivo*. Recently, Axelrod *et al.*[19, 20] reported a study *in vitro* on such successive hydroxylation and methylation of monohydroxy compounds.

The second pathway is hydrolysis of the ureide group and the following conjugation with glycine. The possible pathways are shown in Fig. 5.

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