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1. Masuo Akagi, Isamu Aoki, and Takayoshi Uematsu : Studies on Food Additives. X.*1 The Metabolism of *p*-Ethoxyphenylurea in the Rabbit.

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It appears that *p*-ethoxyphenylurea (Dulcin) is not permitted to use as a sweetening agent for foods except in Japan. Numerous papers[1~10] have dealt with the toxicity of this compound, some for its inhibition to digestive enzymes and some for its methemoglobin formation[7,8,10] and carcinogenicity,[1~5] although the carcinogenicity of *p*-ethoxyphenylurea seems to be controversial.

It is desirable to investigate the metabolic fate of *p*-ethoxyphenylurea in animals, since there appears to be few information[11] on the biotransformation of this compound. *p*-Ethoxyphenylurea has a ureido group directly attached to the aromatic ring and it is especially interesting to study how the ureido group behaves in animals, whether it is stable or easily liberated.

Morinaka[12] isolated cinnamic acid and O-glucuronide of *p*-hydroxyphenylurea from the urine of rabbits dosed with *p*-cinnamoyloxyphenylurea, and Tsunoo[13] isolated furyl-2-acrylic acid and O-glucuronide of *p*-hydroxyphenylurea as metabolites of *p*-(2-furylacryloyloxy)phenylurea in dogs and rabbits. Bray[14] studied the metabolism of tolylureides in rabbits and suggested that the ureido group was relatively stable *in vivo*.

Williams[15~19] fed rabbits with monoarylthioureas and showed it to be converted into the corresponding cyanamides, ureides, and carbamic acids by loss of sulfur.

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*1 Part Ⅸ : This Bulletin, **13**, 1200 (1965).

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1) O.G. Fitzhugh, *et al.* : J. Am. Pharm. Assoc. Sci. Ed., **60**, 583 (1951).
2) F. Griepentrog : Arzneimittel-Forsch., **9**, 123 (1959).
3) Y. Ikeda, *et al.* : J. Food Hyg. Soc. Japan, **1**, 62 (1960).
4) F. Bar, *et al.* : Naturwiss., **45**, 390 (1958).
5) H. Letter, *et al.* : *Ibid.*, **42**, 217 (1956).
6) H. Bekemeiser, *et al.* : Arzneimittel-Forsch., 8, 150 (1958).
7) E. Rost, A. Braun : Arb. Reichsges. Amt., **57**, 212 (1926).
8) W.A. Uglow : Arch. Hyg., **95**, 89 (1925) [Chem. Zentr., Ⅱ, 99 (1925)].
9) A.J. Lehman : Assoc. Food Drug Officials U.S., Quart. Bull., **14**, 82 (1950).
10) N. Iorlov : Gigiena i Sanit., **13**, 36 (1948).
11) Y. Terada, *et al.* : Mie Med. J., **4**, 1 (1954).
12) K. Morinaka : Z. Physiol. Chem., **124**, 247 (1922).
13) S. Tsunoo : J. Biochem. (Tokyo), **22**, 209 (1935).
14) H.G. Bray, H.J. Lake, W.V. Thorpe : Biochem. J., **44**, 136 (1949).
15) R.T. Williams, *et al.* : Biochem. J., **80**, 10 (1961).
16) R.L. Smith, R.T. Williams : J. Med. Pharm. Chem., **4**, 97 (1961).
17) *Idem* : *Ibid.*, **4**, 137 (1961). 18) *Idem* : *Ibid.*, **4**, 147 (1961). 19) *Idem* : *Ibid.*, **4**, 163 (1961).

In this paper, the metabolic fate of *p*-ethoxyphenylurea in rabbits is described, and the isolation of a new type N-glucuronide is also reported.

Experimental*3

Animals——Rabbits (approx. body weight, 2.5 kg.) were kept in metabolic cages for the separate collection of urine and feces, and fed on 50 g. of oats, 100 g. of carrots, and 300 g. of cabbage daily. They were given 0.5 g./kg. of *p*-ethoxyphenylurea in gelatin capsules (J. P. No. 2). The urine was collected every 24 hr. after dosing.

Paper Chromatography——Toyo Roshi No. 51 chromatography paper (2×40 and 40×40 cm.) was employed for descending development in the following solvent systems : (A) iso-PrOH-NH_4OH-H_2O (20 : 1:2), (B) AmOH-H_2O-MeOH-benzene (2:1:4:2), (C) BuOH-AcOH-H_2O (4:1:5), (D) BuOH-PrOH-H_2O (3:2:5). For the detection of compounds on paper, the following reagents were used : (1) For detection of amino and ureido groups, *p*-dimethylaminobenzaldehyde (5 g. in 100 ml. of EtOH and 33 ml. of conc. HCl); (2) for detection of glucuronic acid, naphthoresorcinol (2% in aqueous 33% trichloroacetic acid); (3) for the same purpose, aniline hydrochloride (1.0 g. in 100 ml. of MeOH); (4) for detection of phenols and *p*-phenetidine, $FeCl_3$ (2% in H_2O), (5) for detection of phenols, 2,6-dibromoquinone chloride (0.3%) in MeOH followed by 10% Na_2CO_3; (6) for the same purpose, phosphomolybdic acid (2%) followed by NH_3 gas; (7) for detection of aryl compounds, diazobenzene sulfonic acid (0.1 g. in 20 ml. of 10% Na_2CO_3).

Materials——*p*-Ethoxyphenylurea, *p*-aminophenol, and *p*-phenetidine were commercial products. *p*-Hydroxyphenylurea was prepared from *p*-aminophenol-HCl and KCNO as described by Kalckhoff.[20] Calf liver β-glucuronidase was obtained from Tokyo Zoki Kagaku Co., Ltd.

1-(*p*-Acetoxyphenyl)-3-acetylurea was prepared by the method of Heiderberger,[21] m.p. 208°. *Anal.* Calcd. for $C_{11}H_{12}O_4N_2$: C, 56.04; H, 5.12; N, 11.86. Found : C, 56.18; H, 5.25; N, 12.01.

1-Acetyl-3-(*p*-ethoxyphenyl) urea was prepared as follows : *p*-Ethoxyphenylurea (3 g.) was added with stirring to 15 ml. of pyridine and 15 ml. of Ac_2O. After 0.5 hr., the solution was poured into cracked ice, the precipitate was collected by filtration in 54% yield, and recrystallized from EtOAc to colorless needles, m.p. 220~221° (reported[22] 220°). *Anal.* Calcd. for $C_{11}H_{14}O_3N_2$: C, 59.46; H, 6.31; N, 12.61. Found : C, 59.58; H, 6.31; N, 12.70.

Potassium N-(*p*-ethoxyphenyl)urea-N'-sulfate : *p*-Ethoxyphenylurea (18 g.) was dissolved in 150 ml. of pyridine, 13 g. of chlorosulfonic acid was added in small portions to the mixture with stirring and the temperature was kept below 30°. After about 30 min., yellowish precipitates began to separate out. After stirring for further 30 min., the reaction mixture was left to stand overnight at room temperature. The mixture was neutralized with 18 g. of KOH in 100 ml. of H_2O and a white powder was obtained in 27 g. yield by filtration. The separated solid was recrystallized from H_2O to colorless needles, m.p. 245~250° (decomp.). *Anal.* Calcd. for $C_9H_{11}O_5N_2SK$: C, 36.12; H, 3.67; N, 9.36. Found : C, 36.32; H, 3.81; N, 9.45.

Potassium N-(*p*-hydroxyphenyl)urea O-sulfate was prepared by heating *p*-hydroxyphenylurea (9 g.) with sulfamic acid (13.2 g.) in pyridine (19.2 ml.) at 95~110° with stirring for 30~40 min. MeOH (30 ml.) was poured into this solution and the separated precipitate was filtered off. The filtrate was neutralized by adding 48 ml. of 10% K_2CO_3 and evaporated to dryness *in vacuo* below 60°. The residue was extracted with 120 ml. of hot MeOH. The MeOH extract was concentrated *in vacuo* to 60 ml. and cooled in an ice bath to afford 10 g. of a crude powder in 62% yield. Repeated recrystallization from MeOH containing a small amount of AcOH gave colorless needles, m.p. 252~253° (decomp.), *Anal.* Calcd. for $C_7H_7O_5N_2SK$: C, 31.11; H, 2.60; N, 10.37. Found : C, 30.83; H, 2.71; N, 10.53.

Potassium N-(*p*-hydroxyphenyl)urea O-glucosiduronate was obtained from the rabbits by the following procedure. The combined urine (0.6 L.) from three rabbits dosed with 0.5 g./kg. of *p*-hydroxyphenylurea was acidified with a few drops of glacial AcOH and treated with saturated normal lead acetate solution. The filtrate was made alkaline with NH_4OH to pH 8, treated with excess saturated basic lead acetate sobution, and the lead salt formed was decomposed with H_2S. The filtrate separated from PbS was neutralized with K_2CO_3 and concentrated *in vacuo* at 40° to yield 1.5 g. of white needles. Recrystallization from H_2O containing EtOH gave m.p. 245.5° (decomp.), $[\alpha]_D^{18}$ −67.0° (c=1.25, H_2O) (reported[12~14] m.p. 231° (decomp.), $[\alpha]_D$ −75.0°, 243°, −59.3°, and 257°, −67.8°). *Anal.* Calcd. for $C_{13}H_{15}O_8N_2K$: C, 42.61; H, 4.13; N, 7.65. Found : C, 42.77; H, 4.23; N, 7.81.

Synthesis of potassium *p*-hydroxyphenylurea O-glucosiduronate : *p*-Aminophenyl β-D-glucopyranosiduronic acid (712 mg.), as described below, was suspended in H_2O (6 ml.), KCNO (250 mg.) was added, and the

*3 The melting points are uncorrected.

20) F. Kalckhoff : Chem. Ber., **16**, 376 (1883).

21) M. Heiderberger, W. A. Jacobs : J. Am. Chem. Soc., **39**, 2441 (1917).

22) Carls Alberti : Gazz. chim. ital., **65**, 922 (1935).

mixture kept at 45° for 5 min. After standing for 2 hr. at room temperature, the mixture was evaporated to dryness *in vacuo* below 45°. The residue was recrystallized from acetone-H_2O to 550 mg. (64%) of colorless needles, m.p. 244~245° (decomp.), $[\alpha]_D^{21}$ −62.0°$_a$(c=3.44, H_2O). *Anal.* Calcd. for $C_{13}H_{15}O_8N_2K$: C, 42.61; H, 4.13; N, 7.65. Found: C, 42.30; H, 4.36; N, 7.41.

This compound was identified by mixed fusion with the metabolite from the urine as described above.

p-Aminophenol O-glucosiduronate (0.5 g.) was obtained in the same manner as described by Williams.[23] It came as white needles m.p. 213°(decomp.), $[\alpha]_D^{18}$ −86.7°(c=1.23, 0.1*N* HCl) (reported[23] 213°, −83), *Anal.* Calcd. for $C_{12}H_{15}O_7N\cdot H_2O$: C, 47.52; H, 5.61; N, 4.62. Found: C, 47.39; H, 5.72; N, 4.57.

Methods of Estimation——Total glucuronic acid was estimated by the method of Ishidate and Nambara,[24] and phenols by that of Bray, *et al.*[25] For estimation of sulfate the method of Fiske[26,27] was used. Free *p*-ethoxyphenylurea was determined as described in the previous paper of this laboratory.*1

Results

1. Estimation of Metabolites

The average values of normal metabolites excreted by individual rabbits used were as follows: Total phenol, 21.6 mg./day; free phenol 8.6 mg./day; combined phenol, 13.0 mg./day, ethereal sulfate 33.6 mg./day; total glucuronic acid, 49.6 mg./day. Table I summarizes the results obtained from the urine of rabbits dosed with 1 g./animal of *p*-ethoxyphenylurea.

Table I. Metabolism of *p*-Ethoxyphenylurea in the Rabbit

	Percentage of dose excreted (%)		Percentage of dose excreted (%)
Total phenol	41	Ethereal sulfate	23
Free phenol	7	Total glucuronic acid	38
Combined phenol	35	Free *p*-ethoxyphenylurea	3

It has been found that about 40% of the compound is de-ethylated, followed by conjugaiton with sulfuric acid (23%) and glucuronic acid (11%). The principal phenolic metabolite is *p*-hydroxyphenylurea and some, at least, is excreted as free (7%). There is, however, a marked increase (27%) of glucuronic acid excretion, except for O-glucuronide, which is likely to be N-glucuronide as will be described below.

Table II. Paper Chromatography of the Compounds related to this Metabolic Study

Compound	Solvent system			
	A	B	C	D
p-Ethoxyphenylurea	0.48	0.76	0.91	0.91
p-Ethoxyphenylurea sulfamate			0.65	0.50
p-Ethoxyphenylurea N-glucuronide		0.13	0.55	0.30
p-Hydroxyphenylurea	0.46	0.60	0.76	0.75
p-Hydroxyphenylurea O-sulfate		0.23		
p-Hydroxyphenylurea O-glucuronide		0.09	0.16	0.05
p-Phenetidine		0.81	0.96	0.94
p-Aminophenol		0.69	0.53	0.58
Urea		0.39	0.51	0.41

23) R.T. Williams: Biochem. J., **37**, 329 (1943).
24) M. Ishidate, T. Nambara: This Bulletin, **5**, 515 (1957).
25) H.G. Bray, W.V. Thorpe: Methods of Biochemical Analysis, **1**, 45 (1954).
26) O. Rosenheim, J.C. Drummond: Biochem. J., **8**, 143 (1914).
27) C.H. Fiske, H.M. School: J. Biol. Chem., **47**, 59 (1921).

Results of paper chromatography of the metabolites and the related compounds are listed in Table II. Solvent systems and reagents for detection used have been described above.

2. Detection of Free *p*-Ethoxyphenylurea

A 24 hour urine (600 ml.), pooled after the feeding of 4.5 g. *p*-ethoxyphenylurea, was extracted continuously with ether for 6 hours at pH 6. *p*-Ethoxyphenylurea was detected in the ether extract by the following methods. Paper chromatogram showed Rf 0.48 in solvent system (A), 0.76 in (B), 0.91 in (C), and 0.91 in (D), giving a yellow color by the Ehrlich reagent. These Rf values were the same as that of authentic *p*-ethoxyphenylurea.

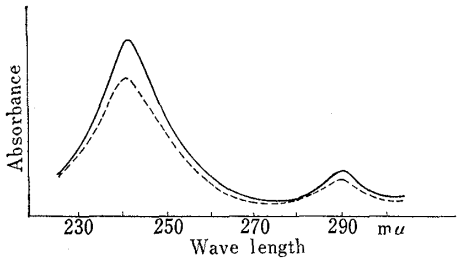


Fig. 1. Ultraviolet Absorption Spectra of synthesized *p*-Ethoxyphenylurea (solid line) and Extract (broken line) of Rf 0.76 in Solvent System B (in EtOH)

The appropriate regions of paper strips showing the presence of *p*-ethoxyphenylurea in solvent system (B) were cut off and eluted with ethanol. Ultraviolet spectrum of the ethanol eluate had λ_{max}^{EtOH} at 242 and 290 mμ, same as that of authentic *p*-ethoxyphenylurea as shown in Fig. 1.

3. Isolation of *p*-Ethoxyphenylurea

The urine, after being extracted with ether at pH 6 as described above, was adjusted to pH 1 with conc. hydrochloric acid and boiled for 30 minutes. The treated urine was adjusted to pH 6 with ammonium hydroxide and extracted with ether for 6 hours. The ether extract was evaporated to dryness and the residue was stirred with 5 ml. acetic anhydride and 5 ml. pyridine. After 30 minutes, the reaction mixture was poured on cracked ice. The separated solid (900 mg.) was recrystallized from ethyl acetate and the crystals were identified as 1′-acetyl-3-(*p*-ethoxyphenyl)urea, by m.p. 220~221° and mixed m.p. 219~220°. Found : C, 59.44; H, 6.31; N, 12.94.

The *p*-ethoxyphenylurea obtained seems to have been derived from combined *p*-ethoxyphenylurea, which would be an unstable glucuronide and would be cleaved by heating with acid.

4. Detection of *p*-Hydroxyphenylurea

It was observed that the ether extract as described in section 2 contained *p*-hydroxyphenylurea through examination by paper chromatography (Rf 0.46 in A, 0.60 in B, 0.76 in C and 0.75 in D). Also it was confirmed by ultraviolet absorption spectra as shown in Fig. 2.

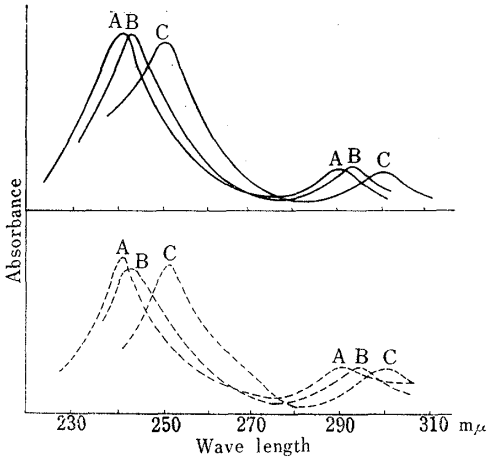


Fig. 2. Ultraviolet Absorption Spectra of Synthesized *p*-Hydroxyphenylurea (solid line) and Extract (broken line) of Rf 0.60 in Solvent System B

A : *N* HCl-EtOH(2:1)
B : EtOH
C : *N* NaOH-EtOH(2:1)

5. Isolation of *p*-Hydroxyphenylurea

A 24 hour urine (1.2 L.), collected after receiving 6.0 g. *p*-ethoxyphenylurea, was evaporated to 200 ml. *in vacuo* at 40~50°. The concentrated urine was saturated with ammonium sulfate and then extracted with 2×200 ml. portions of acetone. The acetone extract was separated, neutralized with solid potassium carbonate, filtered, and

evaporated to 50 ml. under a reduced pressure. The residue was shaken with 500 ml. of acetone, the supernatant was separated, and reduced to 50 ml. *in vacuo*. The residue was boiled for 10 minutes with 5 ml. of conc. hydrochloric acid. The mixture was cooled and neutralized with ammonium hydroxide. It was found by paper chromatography that this sample contained *p*-hydroxyphenylurea and a small amount of *p*-ethoxyphenylurea and *p*-aminophenol. This solution (2 ml.) was chromatographed through an alumina column (40 g. of Al_2O_3), which was eluted with 1 L. of chloroform, then the column was cut into 10 fractions. Each fraction was extracted with ethanol and ultraviolet absorption of ethanol extract was measured. The third fraction from the top, which had maximum absorption bands at 242 and 292.5 mμ, was dried *in vacuo*, and 10 ml. of acetic anhydride and a drop of conc. sulfuric acid, were added to the pale brown residue. After standing overnight at room temperature, the reaction mixture was poured on cracked ice and extracted with 50 ml. of chloroform. The chloroform layer was washed with aq. sodium bicarbonate and then with water, dried over sodium sulfate, and evaporated *in vacuo* to give crystals (10 mg.). On recrystallization from ethyl acetate, it formed colorless needles, m.p. 208°. Mixed with an authentic specimen of N-(4-acetoxyphenyl)-N′-acetylurea it melted at 208°.

6. Detection of Compounds with Free Amino Group

p-Aminophenol : A 24 hour urine (500 ml.) of rabbits receiving 4.5 g. *p*-ethoxyphenyl-urea was extracted continuously with ether at pH 6, 8, and 10, respectively for 6, 6, and, 30 hours. The extracts were developed on paper, and the spot corresponding to Rf 0.69 in solvent system (B), 0.53 in (C) and 0.58 in (D) was in agreement with that of authentic *p*-aminophenol.

Attempts to isolate this substance were unsuccessful. However, when pure *p*-hydroxyphenylurea was treated as above, or especially with acid, *p*-aminophenol was detected by paper chromatography. From these extracts acetamidophenol was not detected on the paper.

Therefore *p*-aminophenol detected in the above ether extracts would be artifacts.

p-Phenetidine : From each of the ether extracts mentioned above, *p*-phenetidine and phenacetin were not detected by the diazo reagent, ferric chloride, Ehrlich reagent, and pentacyanoaquoferriate reagent, on the paper even by the modified procedure of Williams[28] unless the urine was treated with acid.

7. Detection of *p*-Hydroxyphenylurea O-Sulfate

The 24 hour urine (1 L.) after feeding 4.5 g. *p*-ethoxyphenylurea was lyophilized to a hygroscopic brown residue. About 2 g. of this gum was treated with hot methanol (20 ml.) and filtered. The filtrate was concentrated *in vacuo* to 5 ml. and the concentrate was chromatographed. A yellow spot with Rf 0.23 in solvent system (B), which was positive to Ehrlich's reagent, was in complete agreement with that of authentic *p*-hydroxyphenylurea O-sulfate. The parts of 10 strips corresponding to Rf 0.22 to 0.23 were cut out and extracted with 6 ml. of water. An aliquot of the aqueous eluate was used for measurement of ultraviolet absorption spectra, giving maxima at 237 and 280 mμ, and this was in complete agreement with the curve of authentic sample [$\lambda_{max}^{H_2O}$ 237 mμ (ε 14,900) and 280 mμ (ε 1,060)] treated by the same procedure. The remainder was adjusted to pH 1.0 with hydrochloric acid, heated at 80° for 5 minuts, cooled, neutralized with sodium bicarbonate, and then extracted with 20 ml. ethyl acetate. The ethyl acetate layer was evaporated *in vacuo* to 1 ml. to give a sample for paper partition chromatography. The sample showed one spot of *p*-hydroxyphenylurea at Rf 0.60 in (B).

28) J.N. Smith, R.T. Williams : Biochem. J., **44**, 239 (1949).

The part of the strip corresponding to 0.59~0.60 was cut out and extracted with ethanol. Its ultraviolet spectra gave λ_{max}^{EtOH} 242 and 292.5 mμ, and this property was in good agreement with that of authentic *p*-hydroxyphenylurea O-sulfate.

8. Detection of *p*-Hydroxyphenylurea O-Glucuronide

The sample, same as in the section of sulfate above, or the glucuronide fraction from the lead salt, was submitted to paper chromatography. Each spot with Rf 0.09 in (A), 0.16 in (C), and 0.05 in (D), which was positive to naphthoresorcinol test for glucuronic acid and Ehrlich's reagent for ureido group, was in agreement with that of authentic *p*-hydroxyphenylurea O-glucuronide. The area of spot corresponding to Rf 0.16 in (C) was cut out and extracted with water to measure ultraviolet spectra. It was confirmed that its ultraviolet spectra corresponded to that of the authentic sample [$\lambda_{max}^{H_2O}$ 237.5 mμ (ε 13,500) and 280 mμ (ε 1,100)].

For further confirmation, the area corresponding to *p*-hydroxyphenylurea O-glucuronide, which was chromatographed on four sheets of paper (40×40 cm.) using solvent (C), was cut out and eluted with water, and the eluate was evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml. of 0.1*M* acetate buffer (pH 5.0). To the mixture was added 1 ml. of β-glucuronidase solution (10,000 units/ml.) and incubated at 38° for 3 hours. Also a mixture of authentic sample (60 mg.) dissolved in 4 ml. of 0.1*M* acetate buffer and 1 ml. of the enzyme solution was incubated at 38° for 1.5 hours. The controls contained boiled enzyme solution or water instead of the enzyme. The supernatant of the incubation mixtures was analyzed by paper chromatography with solvent (B). The chromatograms indicated the spot corresponding to *p*-hydroxyphenylurea in the case of complete enzyme systems but only that of *p*-hydroxyphenylurea O-glucuronide in the incomplete systems. Consequently, the rabbit urine dosed with either *p*-ethoxyphenylurea or *p*-hydroxyphenylurea was found to contain 4-ureidophenyl β-D-glucopyranosiduronate.

9. Major Glucuronide

a) After feeding 4.5 g. of *p*-ethoxyphenylurea to three rabbits, urine was collected for 24 hours, acidified with acetic acid to pH 4.0, and added with saturated normal lead acetate solution until no further precipitation occurred. The precipitate was filtered off, the filtrate was adjusted to pH 7.5 with ammonia, and an excess of saturated basic lead acetate was added. The precipitate was collected by filtration, washed with water, ethanol, and ethyl acetate, suspended in a mixture of 60 ml. of water and 5 ml. of conc. ammonium hydroxide, and treated with hydrogen sulfide to remove lead. The alkaline lead-free filtrate (pH 10) was dried *in vacuo* at 40~45° to a reddish gum, which was extracted with 30 ml. of methanol and the methanol solution was allowed to stand overnight. The precipitated white needles were collected in 1 g. yield. Repeated recrystallizations from methanol gave an analytical sample as colorless needles, m.p. 134~135° (decomp.), $[\alpha]_D^{18}$ −46° (c=1.00, H_2O), which formed a brown precipitate with Nessler's reagent. *Anal.* Calcd. for $C_{15}H_{23}O_8N_3$: C, 48.26; H, 6.00; N, 11.26. Found: C, 48.16; H, 6.10; N, 10.74.

b) The remainder of hygroscopic residue obtained by freeze-drying, described in the section on *p*-hydroxyphenylurea O-glucuronide, was shaken with 50 ml. of methanol and the methanol layer was left to stand for 2 weeks at room temperature. The solid (2.0 g.) was recrystallized several times from 80% methanol to form colorless needles, m.p. 185~186° (decomp.), $[\alpha]_D^{18}$ −46.8° (c=1.00, H_2O).

By flame reaction it was confirmed to be the potassium salt. *Anal.* Calcd. for $C_{15}H_{19}O_8N_2K$: C, 45.67; H, 4.68; N, 7.11. Found: C, 45.94; H, 4.92; N, 6.98.

Both the compounds obtained above gave a purple color with naphthoresorcinol, a pink color with aniline-hydrochloric acid, and a yellow color after a few hours with

Ehrlich's reagent. Also they gave the same Rf on the paper and yielded *p*-ethoxyphenylurea after acid hydrolysis (1 *N* HCl).

Hydrolysis by β-glucuronidase was carried out as follows: The incubation mixture contained 10 mg. of the glucuronide obtained by lyophylization method, 1 ml. of β-glucuronidase solution (10,000 units/ml.), and 4 ml. of 0.1*M* acetate buffer (pH 5.0). The control for this experiment contained boiled enzyme or water instead of the enzyme. After incubation for 1.5 hours at 38°, the supernatant was submitted to paper chromatography using solvent (B) and to determination of free *p*-ethoxyphenylurea. Paper chromatogram did not indicate the spot corresponding to *p*-ethoxyphenylurea but only the starting material in each system. Also free *p*-ethoxyphenylurea was not determined by spectrophotometry. Infrared spectra of the two compounds resembled closely as shown in Fig. 3.

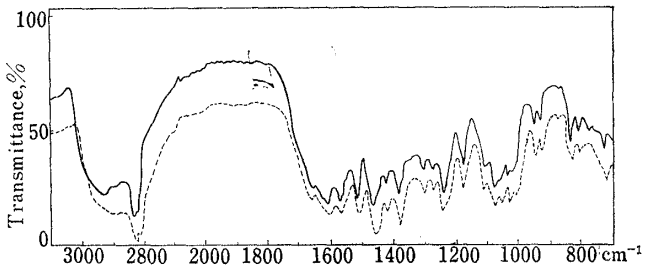


Fig. 3. Infrared Spectra of Potassium Salt (solid line) and Ammonium Salt (broken line) or *p*-Ethoxyphenylurea N-Glucuronide obtained from the Urine of Rabbits dosed with *p*-Ethoxyphenylurea (in Nujol)

From these facts, the compounds were identified as ammonium 1-[3-(*p*-ethoxyphenyl)ureido]-1-deoxy-D-glucopyranosiduronate and potassium 1-[3-(*p*-ethoxyphenyl)-ureido]-1-deoxy-D-glucopyranosiduronate respectively.

Discussion

The present study indicates that five metabolites are excreted in the urine of rabbits dosed with *p*-ethoxyphenylurea (Chart 1). The major metabolite is a de-ethylated product of *p*-ethoxyphenylurea, *p*-hydroxyphenylurea, which is excreted in the main in a conjugated form, O-sulfate and O-glucuronide.

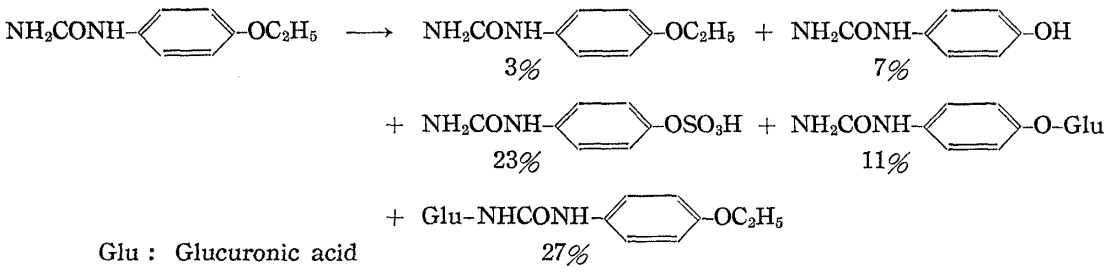


Chart 1.

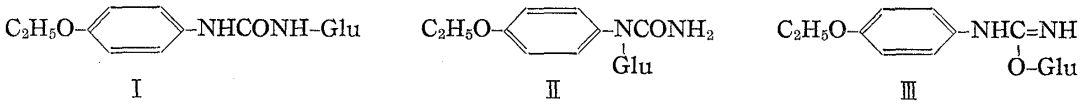
It is interesting that a relatively large amount of the dose (about 27%) is found to form N-glucuronide of *p*-ethoxyphenylurea in the animals and is isolated as its potassium and ammonium salt. This is a new type of N-glucuronide, although several amines have been found to form N-glucuronide in animals.

It is well known that the ureido group of arylurea derivatives has a tendency to undergo spontaneous decomposition *in vitro*,[14] especially in the presence of acid or alkali,[29] and *p*-phenetidine and its derivatives are de-ethylated *in vivo*.[28,30]

29) K. Täufel, C. Wagner, H. Dunwald: Z. Elektrochem., **34**, 115 (1928).
30) B.B. Brodie, J. Axelrod: J. Pharmacol., **97**, 58 (1949).

In the present study, a trace of *p*-aminophenol was detected in the urine, but it was also found that *p*-hydroxyphenylurea underwent spontaneous decomposition to *p*-aminophenol in the urine. Attempts were made to detect *p*-phenetidine, *p*-acetamidophenol, and *p*-aminophenol O-glucuronide in the urine of rabbits dosed with *p*-ethoxyphenylurea but were unsuccessful. Therefore, it may be concluded that the ureido group of arylurea would be stable in the animal and *p*-aminophenol would not be a metabolite of *p*-ethoxyphenylurea but an artifact. This view is supported by other workers.[14,31~33]

Following three alternative forms may be assigned to the structure of *p*-ethoxyphenylurea N-glucuronide.



I II III

Chart 2.

Although a rigorous proof of the structure will be reported in a succeeding paper, structures (II) and (III) are not supported by the following facts. Substitution to N-1 of glucuronic acid would be more sterically hindered than to N-3. II may react rapidly with Ehrlich's reagent, and the isolated glucuronide reacts but slowly with that. As shown in Table III, N-3 substituted phenylureas exhibit strong absorption, whereas N-1 substituted phenylureas show markedly decreased intensity of absorption. These facts were suggested by Tsuzuki[34] to mean that arylureas might be partly present in the pseudo form (Ar-N=C-NH_2) and this form has greater effect than Ar-NH-CO-NH_2
OH

Table III. Ultraviolet Absorption of Substituted Phenylureas in Ethanol,[a] *p*-Ethoxyphenylurea and Metabolites in Water

Compound	$\lambda_{1\,max}$ (mμ)	$\varepsilon_{1\,max}$	$\lambda_{2\,max}$ (mμ)	$\varepsilon_{2\,max}$
-NHCONH$_2$	237	17,700	268~275	1,100
-N(C_2H_5)CONH$_2$	235~237	3,480	no absorption	
-NHCONH(C_2H_5)	240~241	19,800	275~277	1,000
-N(C_2H_5)CONH(C_2H_5)	243	3,630	no absorption	
-NHCON(C_2H_5)$_2$	239~241	18,300	270~273	1,100
-NHCONH-	256	37,200		
$(\quad)_2$=NCONH$_2$	242	11,400		
C_2H_5O- -NHCONH$_2$	236	13,700	281	1,800
Metabolites $\begin{cases}NH_4\text{ salt}\\K\text{ salt}\end{cases}$	238 238	15,700 15,900	281 281	2,100 2,000

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and Ar-NH-C=NH on the intensity of absorption. The metabolites show increased
OH
intensity of absorption as compared with *p*-ethoxyphenylurea. Therefore the formula (II), in which Ar-N=C-NH_2 form is impossible, might be excluded.
OH

As the presence of an enolic form was indicated by Clow,[35] III is possible. However, the isolated glucuronide is not hydrolysed by β-glucuronidase, because N-glucuronides are not affected by the enzyme.[36]

From these facts, the obtained *p*-ethoxyphenylurea N-glucuronide is in agreement with the structure (I). This N-glucuronide is more stable than the known N-glucuronides of amines, which are termed labile glucuronide. It was not decomposed by solvent system containing acetic acid, and did not exhibit mutarotation during 48 hours.

Tsukamoto, *et al.*[37] have shown that meprobamate N-glucuronide is very resistant to acid hydrolysis. This property is relatively in agreement with our findings.

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Summary

1. The metabolic fate of *p*-ethoxyphenylurea in the rabbit was studied.

2. About 40% of the dose was excreted as phenolic substances, presumably *p*-hydroxyphenylurea. The phenolic products were excreted as the unconjugated (7%), glucuronide (11%), and sulfate (23% of *p*-ethoxyphenylurea fed). *p*-Hydroxyphenylurea was isolated as its diacetyl derivative. The sulfate and glucuronide of *p*-hydroxyphenylurea were detected.

3. It was suggested that the main glucuronide of *p*-ethoxyphenylurea was N-glucuronide. This compound accounted for about 27% of *p*-ethoxyphenylurea fed. The N-glucuronide was isolated as crystals of both potassium and ammonium salts of *p*-ethoxyphenylurea N-glucuronide.

4. About 3% of the dose was excreted unchanged.

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