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## 154. Masuo Akagi, Yoneshiro Oketani, and Takayoshi Uematsu : Studies on Food Additives. IX.[*1] Estimation of *p*-Ethoxyphenylurea in Biological Materials and Physiological Disposition in the Animals.

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In the preceding paper[1] it was demonstrated that *p*-ethoxyphenylurea was excreted as N-glucuronide and some other forms in the urine when *p*-ethoxyphenylurea was administered to rabbit orally. The isolated N-glucuronide appears to be a new type, for ureido compounds have not yet been reported to form N-glucuronides in any animal bodies. There seems to be no information concerning the physiological disposition of *p*-ethoxyphenylurea and it was desirable, therefore, to determine its distribution in the biological materials, which would be significant to elucidate the origin of its toxic actions.

Methods for the detection and determination of *p*-ethoxyphenylurea in food include the following: ultraviolet spectrum,[2] infrared spectrum,[3] anisylaldehyde,[4] Ekkert reaction,[5,6] Jorissen test,[7~11] nitric acid,[12~15] xanthydrol,[16] bromination,[17] Suganuma method,[18] Nessler's reagent,[19] Ehrlich reagent,[20,21] diazotization,[22~25] and quinhydrone.[26]

However, the methods mentioned above are not applicable to metabolic study owing to their low sensitivity or non-specificity. In this paper a sensitive method for determining a small amount of *p*-ethoxyphenylurea in biological fluids is described and physiological disposition of *p*-ethoxyphenylurea is also reported.

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[*1] Part VIII. M. Akagi, I. Aoki: This Bulletin, **10**, 200 (1962).

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## Experimental[*3]

**Materials**——*p*-Ethoxyphenylurea of commercial origin (Kanda Bussan Co., Ltd.) was purified by recrystallization from EtOH, m.p. 172°(decomp.). *p*-Dimethylaminobenzaldehyde (Ehrlich reagent) (Wako Pure Chemical Industries, Co., Ltd.), m.p. 73~75°. Dimethylformamide was distilled after treating with dehyd. $CuSO_4$. Ethylenedichloride was purified by successive washings with *N* NaOH, *N* HCl, and twice with $H_2O$, followed by distillation. EtOAc was purified by distillation. Benzene was distilled, followed by washing with *N* HCl, *N* NaOH, and $H_2O$. EtOAc-benzene (7:3) used as the extraction solvent was saturated with $H_2O$.

**Methanol-Hydrochloric Acid**——MeOH was saturated with HCl gas at 0° and diluted with equal volume of MeOH.

**Ehrlich Reagent Solution**——Three vols. of 5% (w/v) *p*-dimethylaminobenzaldehyde in dimethylformamide (valid for 1 week) were mixed with one vol. of MeOH-HCl. This solution was freshly prepared for each determination.

**Sample Solution**——An accurately weighed quantity of *p*-ethoxyphenylurea was dissolved in EtOAc or other solvent to prepare a sample solution.

**Coloration Procedure**——The sample or test solution (1~10 ml.) containing 2.5~25 γ *p*-ethoxyphenylurea in a 50 ml. distillation flask was evaporated to dryness *in vacuo* at 50°, and the residue was dissolved in 1 ml. of Ehrlich reagent solution by standing at room temperature for 5 min. Five ml. of ethylenedichloride was added to the solution, shaken thoroughly, and absorption of the solution was measured at 450 mμ after 30 min. The solution containing the same volume of Ehrlich reagent was used as the blank.

**Procedure for Biological Material**——Animal tissue (1 g. liver, kidny, lung, fat, testicle, and brain) was added to 5 ml. of $H_2O$ and grounded to an emulsion in a homogenizer. To 4 ml. of the homogenate in a 50 ml. glass-stoppered bottle were added 1 ml. of *N* NaOH and 25 ml. of the extraction solvent, and the mixture was shaken mechanically for 30 min. The bottle was centrifuged at 3000 r.p.m. for 5 min. and 20 ml. of the organic phase was transferred to a 50 ml. glass-stoppered bottle containing 2 ml. of *N* NaOH, the mixture was shaken thoronghyl for 30 min., and centrifuged. Fifteen ml. of the solvent phase was transferred to a 50 ml. glass-stoppered flask, 3 ml. of *N* HCl added, shaken for 30 min., and centrifuged. One to thirteen ml. of the organic phase was pipetted and the coloration procedure processed as described above.

To 1 ml. of plasma or blood in a 50 ml. glass-stoppered bottle were added 3 ml. of $H_2O$, 1 ml. of *N* NaOH, and 25 ml. of the extraction solvent, and the mixture is processed as in the case of other biological materials.

**Animals**——Rabbits (♂, approx. body weight, 2.5 kg.) were kept on a daily diet of 50 g. of oats, 100 g. of carrot, and 300 g. of cabbage. They were given orally 500 mg./kg. body wt. of *p*-ethoxyphenylurea in gelatin capsules (J. P. No. 2).

Wister rats were maintained on a solid food (Oriental Yeast Co., Ltd., Tokyo) and $H_2O$ throughout the experiments. They were administered 500 mg./kg. body wt. of *p*-ethoxyphenylurea by stomach tube as a suspension in 5% (w/v) aqueous gum arabic solution.

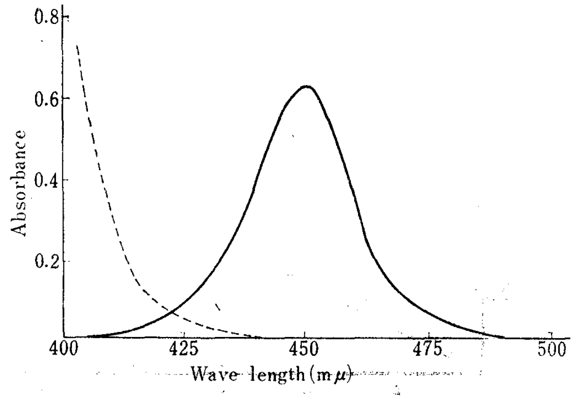

Fig. 1. Absorption Spectrum of Colored Solution

– – – – Blank solution

——— Colored solution obtained from 5 γ of *p*-ethoxyphenylurea per ml. of ethylene dichloride

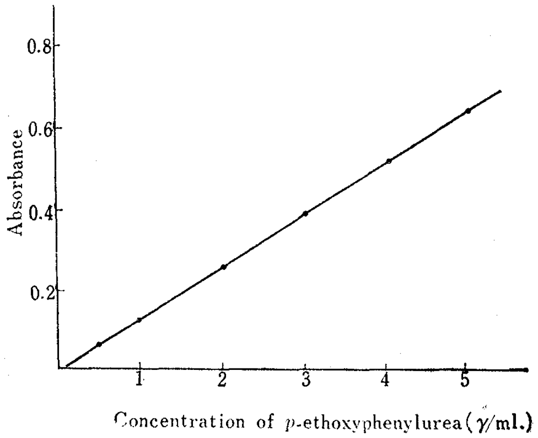

Fig. 2. Calibration Curve

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[*3] In this work Hitachi Model EPU-2A spectrophotometer and Shimadzu Model RS-27 recording spectrophotometer were used.

## Results and Discussion

The colored solution obtained by the described procedure gave an absorption spectrum, as shown in Fig. 1, with an absorption maximum at 450 mμ. The absorbance of the blank was about 0.02 at this wave length.

An example of the calibration curve is shown in Fig. 2.

### Effect of the Solvent of Ehrlich Reagent on Color Development

Results obtained are shown in Fig. 3. The solvent of the reagent was varied, and other conditions were the same as described. Color intensity produced increased in the order of water, methanol, ethylenedichloride, and dimethylformamide. In the case of ethylenedichloride, straight linear relationship was not obtained owing to evolution of hydrogen chloride gas. Therefore, dimethylformamide was chosen for the determination which also had the advantage of being a good solubilizer for *p*-ethoxyphenylurea.

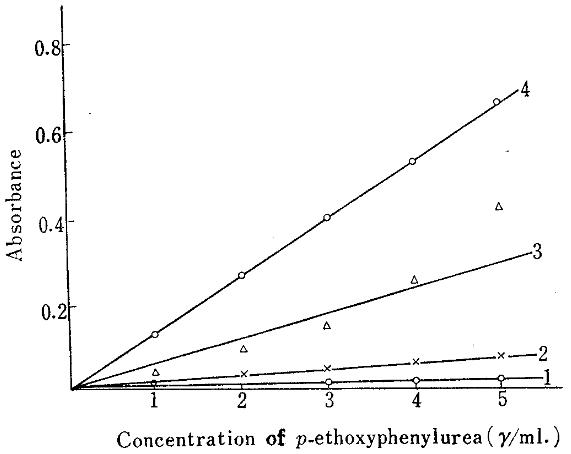

Fig. 3. Effect of the Solvent of Ehrlich Reagent on Color Development

1. In $H_2O$ 5% Ehrlich reagent
2. In MeOH 5% Ehrlich reagent
3. In Ethylene dichloride 5% Ehrlich reagent
4. In Dimethylformamide 5% Ehrlich reagent

### Effect of the Dilution Solvent on Color Development

Fig. 4 illustrates the effect of the dilution solvent on color development. Other conditions were the same as above. The values of absorbance increased in the order of diminishing polarity, that is, water, methanol, ethanol, propanol, ethyl acetate, and ethylene dichloride. Ethylene dichloride seemed to be hyperchromic effect. It was of interest that it showed only a hyperchromic effect without affecting transmission. Carbon tetrachloride, benzene, and hexane could not be used owing to turbidity or separation into two phases. Therefore, ethylene dichloride was chosen as a dilution solvent in the coloration procedure.

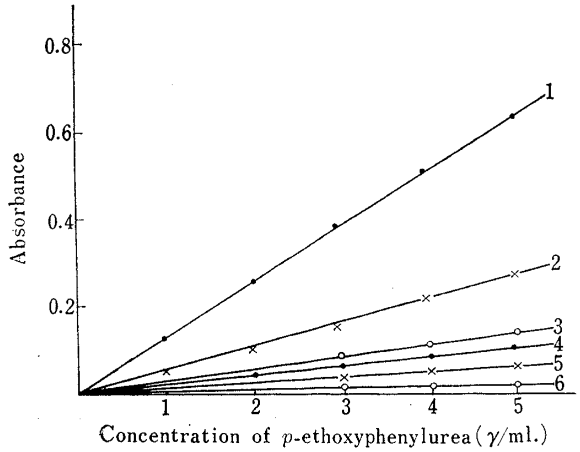

Fig. 4. Effect of the Dilution Solvent on Color Development

| | |
|---|---|
| 1. Ethylene dichloride | 4. EtOH |
| 2. EtOAc | 5. MeOH |
| 3. PrOH | 6. $H_2O$ |

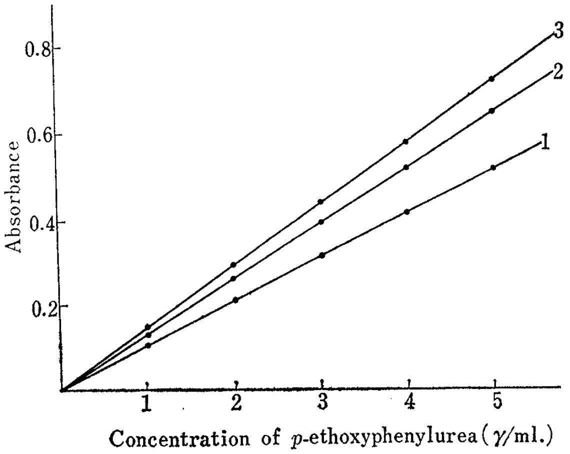

Fig. 5. Effect of the Concentration of Ehrlich Reagent on Color Development

1. 3% Ehrlich reagent (Dimethylformamide soln.)
2. 5% Ehrlich reagent (Dimethylformamide soln.)
3. 6% Ehrlich reagent (Dimethylformamide soln.)

### Effect of the Concentration of the Reagent on Color Development

Relationship between concentration of the reagent and color intensity, under the same conditions as the standard procedure, is shown in Fig. 5.

It was found that the values of absorbance through each run varied linearly with the concentration of the reagent. However higher concentration of the reagent gave larger absorbance of the blank. In consideration of practical application, the concentration of 5% (w/v) of the reagent was adopted for the standard procedure.

### Effect of the Concentration of Hydrochloric Acid on Color Develpment

Fig. 6 shows the effect of the concentration of hydrochloric acid on comparative intensities of the colors. In the case of methanol-hydrochloric acid, higher concentration of hydrochloric acid gave less absorbance, while in the case of aqueous hydrochloric acid, 5*N* hydrochloric acid gave less absorbance than conc. hydrochloric acid. On the basis of these results it seemed that half-saturated methanol-hydrochloric acid was the most favorable concentration of hydrochloric acid for determining *p*-ethoxyphenylurea and also in the advantage of excluding loss of hydrogen chloride gas.

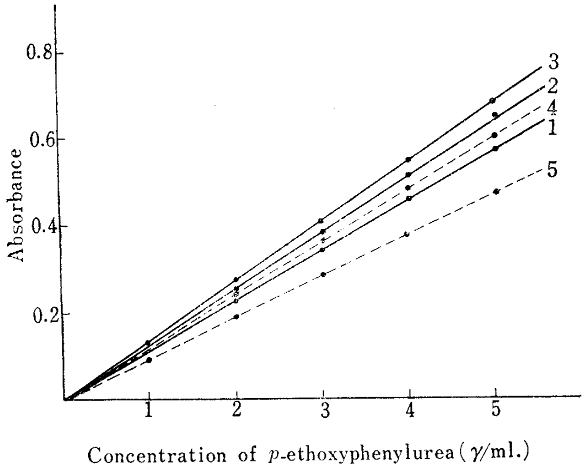

**Fig. 6.** Effect of the Concentration of Hydrochloric Acid on Color Develpment

1. Saturated MeOH-HCl
2. ½ Saturated MeOH-HCl
3. ¼ Saturated MeOH-HCl
4. Conc. HCl-Dimethylformamide=0.75:25 (v/v)
5. 5*N* HCl-Dimethylformamide=0.75:25 (v/v)

### Stability of the Color

Results is obtained when the colored solution was allowed to stand for 90 minutes after dilution with ethylenedichloride shown in Table I. Absorbance of the colored solution was constant after 15 minutes.

TABLE I. Stability of the Colored Solution

| Time after dilution (min.) | | 10 | 15 | 20 | 30 | 40 | 60 | 90 |
|---|---|---|---|---|---|---|---|---|
| Absorbance | 1.0 | 0.111 | 0.113 | 0.113 | 0.111 | 0.113 | 0.113 | 0.113 |
| γ/ml. | 2.5 | 0.347 | 0.356 | 0.351 | 0.357 | 0.357 | 0.357 | 0.356 |

### Choice of Solvent for Extraction of *p*-ethoxyphenylurea from Biological Materials

The solvent must be the one which could extract *p*-ethoxyphenylurea quantitatively with the least amount of its interfering metabolites. The distribution ratio of *p*-ethoxyphenylurea between organic solvents and water is demonstrated by dielectric constants of solvents in Table II. Ethyl acetate was better than other solvents examined. Nonpolar solvents showed lower values of distribution ratio, which seemed to be due to the presence of zwitter ion structure of *p*-ethoxyphenylurea as described by Clow.[27] There appeared to be a propotional relationship between distribution ratios and dielectric constants.[28]

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[27] A. Clow: Trans. Faraday Soc., 33, 381 (1937).

[28] T. Kuwata: "Yozai," 76 (1957). Maruzen Co., Ltd., Tokyo.

Table II. Distribution Ratio of *p*-Ethoxyphenylurea between Water and Solvents, and Dielectric Constants of These Solvents

| Solvent | Distribution ratio[a)] | Dielectric constant |
|---|---|---|
| Ethylacetate | 0.90 | 6.12 |
| Chloroform | 0.53 | 5.05 |
| Ethylenedichloride | 0.43 | — |
| Ethyl ether | 0.35 | 4.33 |
| Benzene | 0.08 | 2.28 |
| Carbon tetrachrolide | 0.02 | 2.24 |
| Petroleum ether | 0.03 | — |
| Hexane | 0.07 | 1.85 |

a) This is expressed as the ratio of *p*-ethoxyphenylurea in the organic phase to total *p*-ethoxyphenylurea after shaking water with an equal volume of the solvent at 18°.

Ethyl acetate–benzene (7:3) was adopted as the extraction solvent, because only ethyl acetate was interfered by *p*-hydroxyphenylurea, one of *p*-ethoxyphenylurea metabolites, even with alkaline washing as will be mentioned below.

**Recovery of Known Amounts of *p*-ethoxyphenylurea from Biological Materials**

Known amounts (20~40 $\gamma$) of *p*-ethoxyphenylurea were equilibrated with homogenates for 2 hours and estimated as described above. The recovery rate is shown in Table III.

Table III. Recovery of *p*-Ethoxyphenylurea added to Biological Materials

| Tissue | Recovery (%) | Tissue | Recovery (%) |
|---|---|---|---|
| Control ($H_2O$) | 84.5±0.26 | Testicle | 84.9±0.40 |
| Kidney | 84.7±0.30 | Lung | 84.8±0.20 |
| Liver | 84.8±0.40 | Spleen | 83.2±1.20 |
| Brain | 83.0±0.20 | Plasma | 83.8±0.70 |
| Fat | 67.7±0.90 | Blood | 84.1±0.71 |

The low recovery was found in fat tissue, as shown in Table III. This would be due to the contamination of fat in the organic phase by which the distribution ratio of *p*-ethoxyphenylurea might be lowered by its lipid insolubility.

**Blank of Biological Material**

Reagents and biological blanks run through the procedure were not found, except for liver, kidney, and spleen which usually respectively gave a reading equivalent to 1.9, 0.2, and 0.2 $\gamma$ of *p*-ethoxyphenylurea per g. of tissue.

**Specificity of the Method**

Under the described conditions, the following substances, which were considered to interfere in the color reaction with Ehrlich reagent, did not give any significant degree of interference in the amount specified. Phenetidine (80 $\gamma$), *p*-ethoxyphenylurea-N-glucuronide (100 $\gamma$), *p*-hydroxyphenylurea O-glucuronide (100 $\gamma$) and its O-sulfate (100 $\gamma$). *p*-Hydroxyphenylurea (40 $\gamma$) gave 1.7% more intense coloration than an equivalent amount of *p*-ethoxyphenylurea in the procedure of double alkaline washings and larger in that of single or extraction procedure with ethyl acetate[*4] alone. Further

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[*4] This might be due to deesterification of EtOAc and decreased alkaline intensity.

evidence was provided for the specificity of this method for the estimation of *p*-ethoxyphenylurea from a comparison of the distribution ratios of the apparent *p*-ethoxyphenylurea with those of the authentic substance in a two-phase system consisting of hexane and ethyl acetate in various proportions and water at constant conditions. The apparent *p*-ethoxyphenylurea was obtained by extraction, with benzene-ethylacetate (3:7) under the same procedure as described, of the pooled plasma from three rats dosed with the drug (500 mg./kg. of body weight) orally and sacrificed 3 hours later.

Aliquots of this solution and of *p*-ethoxyphenylurea solution were evaporated *in vacuo*, 15 ml. of water added, and shaken with an equal volume of ethyl acetate-hexane mixture in various proportions. The fraction of the compound extracted with the various solvent mixtures was expressed as the ratio of the amount of compound in the organic phase to the total amount.

Table IV. Distribution of *p*-Ethoxyphenylurea and Apparent *p*-Ethoxyphenylurea between Water and Various Mixtures of Hexane-Ethyl Acetate

| Hexane in EtOAc (%) | Authentic *p*-ethoxyphenylurea | Apparent *p*-ethoxyphenylurea from plasma |
|---|---|---|
| 0 | 0.88 | 0.88 |
| 25 | 0.66 | 0.66 |
| 50 | 0.40 | 0.37 |
| 100 | 0.00 | 0.00 |

From the results given in Table IV, it was concluded that the substance measured in biological fluid was identical with the authentic *p*-ethoxyphenylurea, without any interference by other compounds.

### Physiological Disposition of *p*-Ethoxyphenylurea Blood Concentration (Time Curve)

Three rabbits were given 1.3 g. of *p*-ethoxyphenylurea orally, and the compound in urine and blood levels was measured at various time intervals. Its absorption was rapid, the peak blood level was achieved within 3 hours, and then declined at a slow rate (Fig. 7). Urinary excretion of *p*-ethoxyphenylurea was accounted for 2.4% of the dose in 24 hours and 2.9% in 48 hours. Terada[29] had shown that the drug was not detected in stools, and this was confirmed by us. Therefore, it seems that absorption from the gastrointestinal tract was fairly complete and the drug underwent extensive and slow metabolic change in the body.

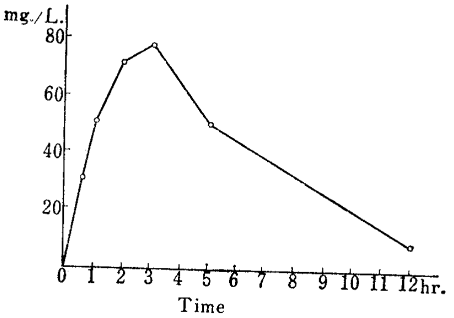

Fig. 7. Blood Level of *p*-Ethoxyphenylurea after the Oral Administration of 1.3 g. of *p*-Ethoxyphenylurea to Rabbit

### *p*-Ethoxyphenylurea found in Tissues

Distribution of *p*-ethoxyphenylurea in the tissues of rats dosed with *p*-ethoxyphenylurea orally is shown in Table V. Three and 24 hours after the drug administration, the animal was killed by decaptation and the tissues were sampled immediately. *p*-Ethoxyphenylurea was concentrated to a considerable degree in most tissues except in fat tissue. There would be hindrance to its free passage into the fat tissue and

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[29] Y. Terada, *et al.*: Mie Med. J., **4**, 1 (1951).

the low concentration on *p*-ethoxyphenylurea in the fat tissue was in agreement with its lipid-insoluble nature. The considerable amount of *p*-ethoxyphenylurea found in the rat after 24 hours suggests that its longer accumulation was due to slow biotransformation of the drug as in the rabbit.

Table V. Distribution of *p*-Ethoxyphenylurea in Rat Tissues

| Tissue | Concentration in tissues (mg./kg.) | | Ratio = $\frac{\text{concn. in tissue water}}{\text{concn. in plasma water}}$ | |
|---|---|---|---|---|
| | 3 hr. (6)[a] | 24 hr. (5) | 3 hr. | 24 hr. |
| Plasma | 46.1 ± 4.8[b] | 7.8 ± 4.5[b] | | |
| Blood | 51.0 ± 4.3 | 8.0 ± 4.6 | | |
| Liver | 96.8 ± 11.4 | 11.5 ± 6.7 | 2.1 | 1.5 |
| Kindey | 75.5 ± 9.1 | 10.5 ± 6.1 | 1.6 | 1.3 |
| Spleen | 59.1 ± 11.8 | 7.8 ± 4.4 | 1.3 | 1.0 |
| Lung | 66.1 ± 11.0 | 8.8 ± 5.1 | 1.4 | 1.1 |
| Brain | 70.9 ± 9.1 | 10.0 ± 5.9 | 1.5 | 1.3 |
| Fat | 16.6 ± 1.3 | 3.2 ± 2.0 | 0.4 | 0.5 |
| Testicle | 63.4 ± 12.0 | 7.6 ± 4.4 | 1.4 | 1.0 |

*a*) No. of rats *d*) Standard deviation

## Summary

1) Method was described for the estimation of *p*-ethoxyphenylurea in biological fluids and tissues.

2) The physiological disposition of *p*-ethoxyphenylurea was demonstrated in rabbits and rats. *p*-Ethoxyphenylurea was rapidly absorbed from the gastrointestinal tract, remained long, and was almost completely metabolized in the body.

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