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Studies on Cow's Urine. IV.[1] Determination by Gas-liquid Chromatography of an Acidic Part Obtained from Cow's Urine*[1]

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An acidic part obtained from cow's urine was examined by gas-liquid chromatography. As the major components of the acidic part, benzoic acid, phenylacetic acid, β-phenylpropionic acid, and derivatives in which hydroxyl or methoxyl groups are attached to, respectively, *para*-, *ortho*-, and 3,4-positions of the aromatic ring were identified. It is conceivable that these compounds were mainly metabolic products of the phenylalanine, tyrosine, and 3,4-dihydroxyphenylalanine (DOPA). Moreover, a new metabolic pathway of phenylalanine and its derivatives was tentatively represented.

Urine contains a vast number of substances as intermediates and end-products in the metabolic pathways of both endogenous and exogenous compounds. Many of these compounds are of great interest in the metabolism of amino acids.

In previous papers,[1,2] the phenolic part obtained from Holstein urine has been examined. In this paper, an acidic part from the urine of Nihon-ushi (*Bos taurus* Linneaus) will be examined by gas-liquid chromatography.

Experimental

The gas-liquid chromatography were performed on a Yanagimoto type GCG-3 Gas Chromatograph, equipped with a thermal-conductivity detector. Column; Silicone SE-30 (25%) on 80—100 mesh Celite 545, 2 m×5

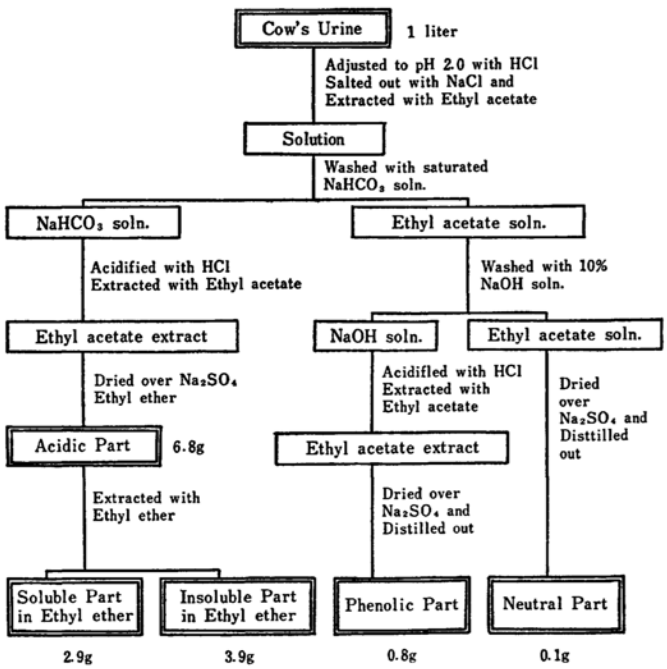

Fig. 1. Extraction process of the acidic, phenolic, and neutral parts obtained from cow's urine.

*1 Presented at the 20th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1967.

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2) R. Suemitsu and M. Hiura, *Nippon Nogei-kagaku Kaishi (J. Agr. Chem. Soc. Japan)*, **27**, 825 (1953).

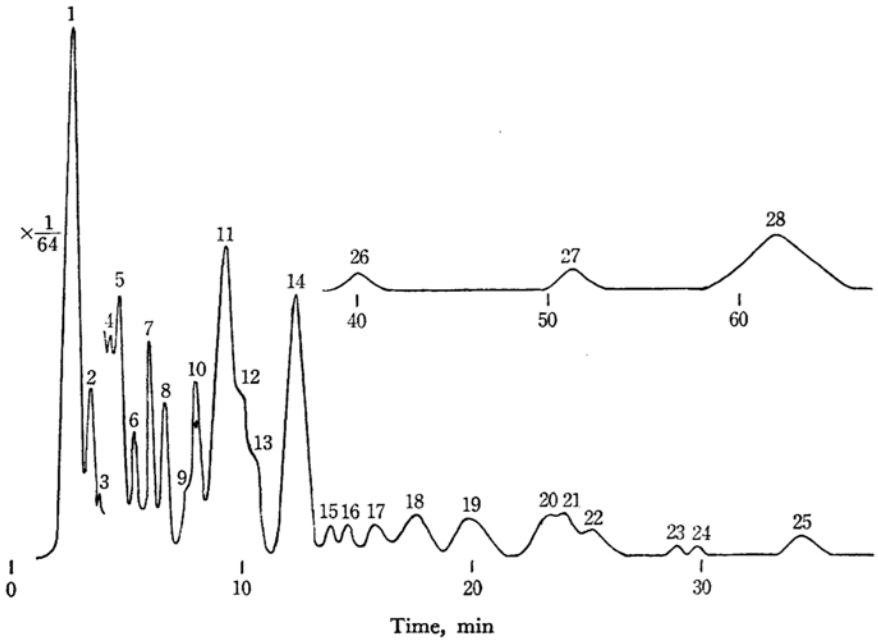

Fig. 2. Gas chromatogram of the methylesters of urinary acids (Method 1).

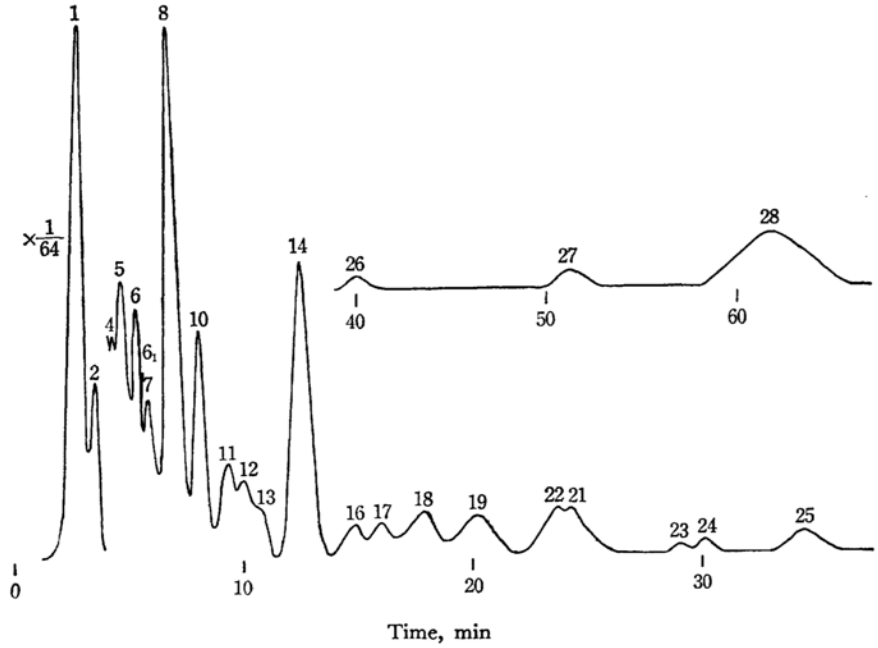

Fig. 3. Gas chromatogram of the methylesters of urinary acids (Method 2).

mmϕ: temperature 170°C; flow rate of hydrogen, 80 m*l*/min. The retention times were measured from the time of the injection to the initial emergence of the peaks.

Extraction of Urinary Compounds. Sodium chloride (200 g) was added to one liter of the fresh urine (pH 8.6) of a Nihon-ushi, and the pH was adjusted to 2.0 with 10% hydrochloric acid. The urine was extracted three times with 100 m*l* portions of ethyl acetate, and the extracts, containing acids, phenols, and neutral compounds, were washed three times with 100 m*l* portions of a saturated sodium bicarbonate solution. The sodium bicarbonate solution was then acidified with 10% hydrochloric acid and extracted with ethyl acetate. After drying over anhydrous sodium sulfate, the solution was evaporated to a brown solid with acidic compounds (6.8 g), which was then separated into an ethyl ether - soluble part (2.9 g) and

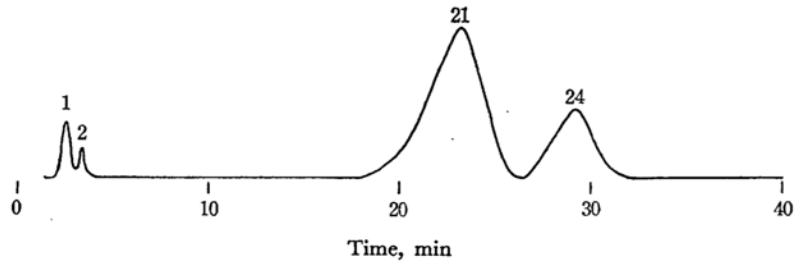

Fig. 4. Gas chromatogram of the methylesters of acidic part insoluble in ethyl ether.

an insoluble part (3.9 g). The fractionation of acidic, phenolic, and neutral part is shown in Fig. 1.

Determination of the Acidic Part Soluble in Ethyl Ether. The methylation of urinary carboxylic acids was carried out according to the following two methods: (1) The acidic part was refluxed with methanol in the presence of concentrated sulfulic acid, and (2) the acidic part was treated with ethereal diazomethane and methanol by the ordinary method. Both carboxylic groups and hydroxyl groups were also methylated by the latter method. Gas chromatograms of methylesters of the acidic part soluble in ethyl ether are presented in Fig. 2 (method 1) and Fig. 3 (method 2) respectively. The quantitative determination was carried out by measuring the peaks of Fig. 2 (triangulation method). When the peaks of Fig. 3 were compared with those of Fig. 2, it was found that there were clear differences between them. As is shown in Fig. 3, the 3, 9, 11, 13, 15, and 22 peaks decreased in area, while the 6, 6_1, 8, 10, 14, and 23 peaks increased; on the other hand, the 1, 2, 4, 5, 7, 12, 16, 17, 18, 19, 20, 21, 24, 25, 26, 27, and 28 peaks gave the same areas. These facts show that decreases in the areas in peaks are due to the presence of hydroxyl groups in urinary carboxylic acids.

The components were identified by comparing their retention times with those of authentic samples under the same conditions. The components of the acidic part obtained from cow's urine are shown in Table 1.

Determination of the Acidic Part Insoluble in Ethyl Ether. A gas chromatogram of the methyl esters of urinary acids insoluble in ethyl ether is shown in Fig. 4. The acidic part insoluble in ethyl ether was recrystallized from water, resulting in colorless needles with a mp of 186°C, and its infrared spectrum was perfectly identical with that of hippuric acid. Then the 21 peak was proved to be hippuric acid by comparing its gas chromatogram with that of an authentic sample.

On the hydrolysis of the same acidic part with 10% sodium hydroxide, followed by the identification of the components of the hydrolyzed products by the gas-liquid chromatographic method, benzoic acid and phenylacetic acid were proved to be the components. On the other hand, only glycine was proved to be present in the amino acid part by paper chromatography. Consequently, the 24 peak may be said to be due to phenacetyl glycine, which was proved to be another component of the acidic part.

Determination of the Phenolic Part Obtained from Cow's Urine. The determination of the phenolic part was carried out by means of a method previously reported.[1] The gas chromatogram and the components of the phenolic part are shown in Fig. 5 and Table 2 respectively.

Table 1. The components of acidic part obtained from cow's urine

Peak No.	Rt.	Component	%
1	2.1	Benzoic acid	68.4
2	2.8	Phenylacetic acid	17.4
3	3.2	*o*-Hydroxybenzoic acid	1.75
4	3.5	Unidentified	trace
5	3.8	*β*-Phenylpropionic acid	0.7
6	4.6	*o*-Methoxybenzoic acid	0.35
7	5.2	Unidentified	0.65
8	5.8	*p*-Methoxybenzoic acid	0.4
9	6.9	*m*-Hydroxybenzoic acid	trace
10	7.2	*p*-Methoxyphenylacetic acid	0.45
11	7.9	*p*-Hydroxybenzoic acid	3.5
12	8.6	Unidentified	trace
13	9.2	*p*-Hydroxyphenylacetic acid	0.95
14	11.0	*p*-Methoxyphenylpropionic acid	1.95
15	12.9	*p*-Hydroxyphenylpropionic acid	0.2
16	13.1	3,4-Dimethoxybenzoic acid	0.2
17	14.9	3,4-Dimethoxyphenylacetic acid	0.2
18	16.6	Unidentified	0.4
19	18.5	Unidentified	0.35
20	21.7	3,4-Dimethoxyphenylpropionic acid	0.55
21	22.5	Hippuric acid	trace
22	23.5	*o*-Hydroxyphenylpropionic acid	0.2
23	27.4	*o*-Methoxyphenylpropionic acid	trace
24	27.7	Phenacetylglycine	trace
25	32.4	Unidentified	0.15
26	38.7	3-Indoleacetic acid (?)	0.1
27	48.5	*β*-3-Indolepropionic acid (?)	0.15
28	57.6	Unidentified	0.95

Table 2. The components of phenolic part obtained from cow's urine

Peak No.	Rt.	Component	%
1	2.6	Guaiacol	0.9
2	4.0	Phenol	7.7
3	5.3	*p*-Cresol	85.2
4	8.4	*p*-Ethylphenol	1.4
5	12.9	Unidentified	4.8

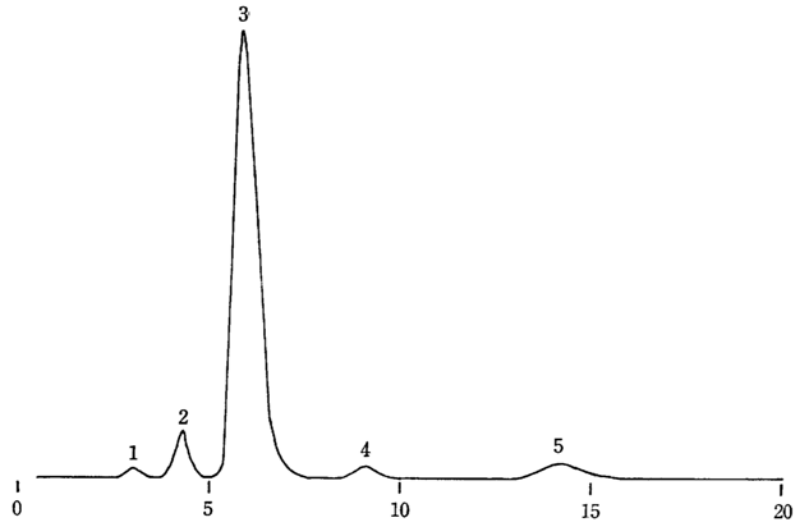

Fig. 5. Gas chromatogram of the phenolic part obtained from cow's urine. 2,4-Xylenyl phosphate (5%) 3 m×5 mmϕ, 165°C, 80 m*l*/min H_2.

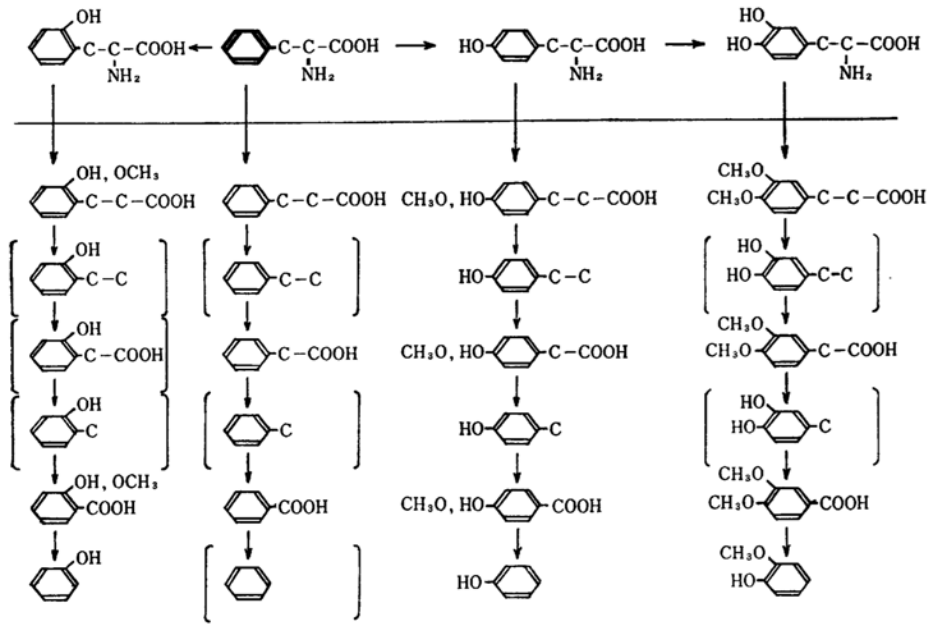

Fig. 6. Mode of formation of the metabolic products of phenylalanine.

Discussion

The following compounds were found in the acidic and phenolic parts of the cow's urine. The acidic components were benzoic acid, phenylacetic acid, β-phenylpropionic acid, *o*-hydroxybenzoic acid, *o*-methoxybenzoic acid, *p*-methoxybenzoic acid, *m*-methoxybenzoic acid, *p*-methoxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, *p*-methoxyphenylpropionic acid, *p*-hydroxyphenylpropionic acid, 3,4-dimethoxybenzoic acid, 3,4-dimethoxyphenylacetic acid, 3,4-dimethoxyphenylpropionic acid, *o*-hydroxyphenylpropionic acid, *o*-methoxyphenylpropionic acid, 3-indoleacetic acid (?), β-3-indolepropionic acid (?), hippuric acid, and phenacetyl glycine. As the phenolic components, we found phenol, guaiacol, *p*-cresol and *p*-ethylphenol.

It is well known that phenylalanine is easily converted to tyrosine, which is then further oxidized to 3,4-dihydroxy phenylalanine (DOPA); on the other hand, a part of the phenylalanine is

converted to *o*-hydroxyphenylalanine (*o*-tyrosine) *in vivo*.[3] It has also been reported that phenylalanine is converted metabolically to phenylacetic acid through phenylpyruvic acid.[4] However, the presence of the acidic and phenolic components mentioned above suggests the new metabolic pathways of phenylalanine, tyrosine, DOPA, and *o*-tyrosine shown in Fig. 6. As the pathways show, amino groups of the aromatic amino acids are deaminated to the corresponding phenylpropionic acids, which are then further converted to benzene or phenols by decarboxylation and ω-oxidation. Parentheses show the components which were not detected in this experiment, but which are very probably present. We are now trying to verify these routes; we will report on our studies in the near future.

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3) M. D. Armstrong and K. N. F. Shaw, *J. Biol. Chem.*, **213**, 805 (1955).
4) A. Meister, "Biochemistry of The Amino Acids," Academic Press, New York (1957), p. 354.