The amount of glucuronide and ethereal sulfate of umbelliferone excreted in the urine was estimated as shown in Table X.

For the determination of glucuronide, the naphthoresorcinol method of Fishman and Green,[3] and for ethereal sulfate, Bray's method[4] were employed.

The results of present investigation are summarized as follows:

1) Umbelliferone administered orally to rabbits was excreted in urine almost quantitatively within 48 hours after the dosage.

2) Colorimetric determination using Emerson's reagent showed that in the case of dosage of 200 mg./kg., umbelliferone was recovered from the excreted urine 20.23% as free form and 66.21% in conjugated form. The conjugated form consisted of glucuronide (44.32%) and ethereal sulfate (19.10%).

3) No obvious change in the amount of metabolites recovered was observed in using different kind of emulsifying agents.

4) The proportion of free and combined form of umbelliferone (1 : 3) was almost constant, irrespective of individual difference in animals.

5) No metabolic change in the structure of umbelliferone, caused by biological hydroxylation or cleavage of lactone ring, was observed.

Summary

The metabolic fate of umbelliferone was studied by the quantitative analysis of urinary excrete of rabbits.

The orally administered umbelliferone was converted into glucuronide (44.32%) and ethereal sulfate (19.10%) accompanying unchanged umbelliferone (20.23%).

An extractor of high efficiency was designed to be employed for the continuous extraction of urinary metabolites.

(Received May 12, 1958)

3) W. H. Fishman, S. Green: J. Biol. Chem. **215,** 527(1955).
4) H. G. Bray, *et al.*: Biochem. J. **52,** 412(1952).

UDC 547.587.52 : 591.05

103 Mitiiti Fujita and Tsutomu Furuya: Studies on the Metabolism of Naturally Occurring Coumarins. III.[1] Urinary Metabolites of Herniarin.

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Following the preceding report[1] on the metabolism of umbelliferone, studies were extended to its methyl ether, herniarin. Herniarin (7-methoxycoumarin), obtained first by Barth and Herzig from the leaves of herniaria, is also a constituent of lavender oil and the flowers of chamomile. It gives a pleasant coumarin-like odor and is a hemostatic agent.

This paper deals with metabolic fate of herniarin ingested in rabbits.

Experimental

Material—Herniarin was easily prepared by methylation of umbelliferone as colorless plates, m.p. 117~118°.

Animal, Diet, and Dosage—Female rabbits kept on a constant diet as previously described[2] were used, and 200 mg./kg. of herniarin was administered as aqueous emulsion by a stomach tube.

* Hongo, Tokyo. (藤田路一, 古谷 力).
1) Part II: M. Fujita, T. Furuya: This Bulletin, **6**, 517(1958).
2) M. Fujita, T. Furuya: *Ibid.*, **6**, 517(1958).

Identification of Metabolites by Paper Chromatography—The urine excreted during 48 hrs. after the oral administration of 540 mg./kg. of herniarin was acidified with dil. H_2SO_4, and then extracted continuously for 6 hrs. with ether. The residue after evaporation of ether was dissolved in a small amount of acetone (F fraction), and another fraction (C), obtained by hydrolysis after the removal of F was adjusted by the same method as that of umbelliferone urine,[2] and both F and C were examined by paper chromatography. β-Resorcylic acid 4-methyl ether was used as reference compound, whose Rf value and color reaction are listed in Table I.

TABLE I. Rf Values and Color Reactions of β-Resorcylic Acid Methyl Ether

Compound	Solvent system				UV-fluorescence		Emerson's	Diazotized
	A	D	G	H	Untreated	2*N* NaOH	reagent	sulfanilic acid
β-Resorcylic acid 4-methyl ether	0.34	0.95	0.0~0.49	0.42	—	—	RV	Br

TABLE II. Identification of Urinary Metabolites by Paper Chromatography

Compound	F	C
Herniarin	+	−
Umbelliferone	++	+
4-Hydroxy-7-methoxycoumarin	−	−
Umbellic acid	−	−
Umbellic acid 4-methyl ether	−	−
β-Resorcylic acid 4-methyl ether	−	−

As the result of paper chromatography shows (Table II), free and conjugated umbelliferone and herniarin were identified.

Regression Equation between Concentration and Extinction of Herniarin—Two mg. of pure crystals of herniarin was dissolved in 50 cc. of 0.5% Na_2CO_3 and the solution was hydrolysed for 20 mins. on a boiling water bath. After cool, it was divided into five portions and adjusted to contain 200 γ to 12.5 γ per 10 cc. by multiple dilution method, and Emerson's reagent was added to each.

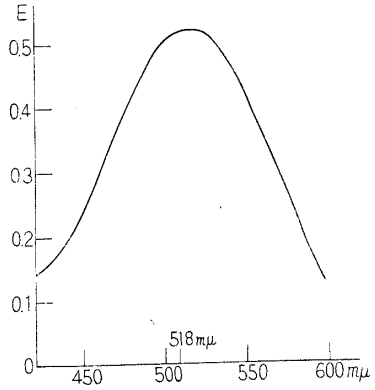
Regression equation calculated from n=35, estimation value obtained at 518 mμ, by the least squares method is as follows:

$y=0.0126+0.00490x$ or $x=204.08y-2.571$ $\sigma=0.008$

x=Concentration of umbelliferone (γ) in 10 cc. of 0.5% Na_2CO_3

y=Extinction σ=Standard deviation

Fig. 1.
Absorption Spectrum of Herniarin (100γ) colored by Emerson's Reagent



Determination of Herniarin and Umbelliferone—Forty cc. of urine 48 hrs. after dosage was acidified with 2 drops of 18*N* H_2SO_4 and extracted with ether for 2 hrs. by the continuous extractor devised by the authors. The ether solution was evaporated, the residue was dissolved in 10 cc. of acetone, and 0.1 cc. of this solution was applied to a sheet of filter paper (Toyo Roshi No. 51, 10×40 cm.). The chromatogram developed with the organic phase of $CHCl_3$-AcOH-H_2O (2 : 1 : 1) was exposed under ultraviolet light, and the fluorescent spots corresponding to coumarin derivatives were cut out. The paper strips were eluted with 10 cc. of 0.5% Na_2CO_3, the alkaline solution was hydrolysed for 20 mins. on a boiling water bath, and after cool, the color intensity developed by Emerson's reagent was read at 518 mμ by the Beckman Model DU spectrophotometer. On the other hand, for paper chromatographic separation of umbelliferone, the organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer (80 : 5 : 15) was used as the developing solvent.

The latter part of experiment was performed in the same manner as described in the preceding paper of this series.

The recovery of herniarin from the urine is listed in Table III.

TABLE III. Recovery of Herniarin added to Normal Urine

Amount of Umbelliferone added	Found (γ)					Recovery (average %)
100 γ	103.81	102.44	102.63	98.65	104.03	102.31

The Extraction Time of Metabolites—The portions of urine 24 and 48 hrs. after administration of herniarin were taken and determined by the same method as that of umbelliferone. From the result shown in Table IV and Fig. 2, the determination of urinary metabolites was carried out with the urine excreted during 48 hrs. after dosage.

Quantitative Results—The results obtained with urine collected during 48 hrs. after dosage of 200 mg./kg. herniarin are summarized in Table V. The quantitative results of conjugated form in the

metabolites are listed in Table VI. The glucuronide was determined by the method of Fishman and Green[3], and the ethereal sulfate, by that of Bray, *et al.*[4]

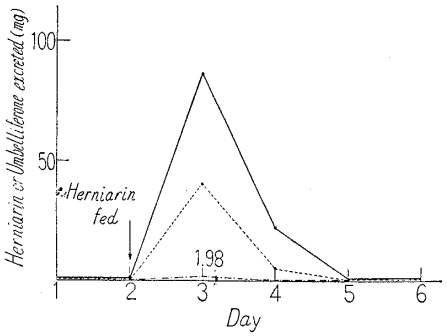


Fig. 2.
The excretion of free (solid line) and conjugated umbelliferone (dotted line), and herniarin (broken line) by rabbit (wt. 2.6 kg.) after oral dosage of 520 mg. herniarin.

TABLE IV. Relationship between Metabolites and Time for Excretion

	Herniarin		Umbelliferone						Total
			Free		Conjugated		Total		
After dosage	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)
0~24 hrs.	1.98	0.38	86.79	18.14	40.72	8.50	127.51	26.64	27.02
24~48 hrs.	—	—	22.05	3.76	4.94	1.90	26.99	5.66	5.66
Total	1.98	0.38	108.84	21.90	45.66	10.40	154.50	32.30	32.68

These experiments were carried out on the rabbit (wt. 2.6 kg.) urine after feeding of 520 mg. herniarin.

TABLE V. The Excretion of Herniarin and Umbelliferone by Rabbits receiving Herniarin orally

Expt. No.	Wt. (kg.)	Dose (mg.)	Herniarin		Umbelliferone						Total
					Free		Conjugated		Total		
			(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)
I	2.6	520	1.98	0.38	108.84	21.90	45.66	10.40	154.50	32.30	32.68
II	2.7	540	2.38	0.44	179.03	36.02	59.29	11.93	238.32	47.95	48.39
III	2.7	540	0	0	85.14	17.13	41.86	8.42	127.00	25.55	25.55
IV	2.7	540	0	0	31.53	6.35	37.92	7.63	69.45	13.98	13.98
Average			1.09	0.21	101.14	20.35	46.18	9.59	147.32	29.94	30.15

TABLE VI. Conjugated Umbelliferone

Expt. No.	Wt. (kg.)	Dose (mg.)	Glucuronide		Ethereal sulfate		Total
			Glucuronic acid excreted	Umbelliferone equiv. to glucuronic acid (% of dose)	(mg. SO_3)	(% of dose)	(% of dose)
I	2.6	520	68.51	11.91	0	0	11.91
II	2.7	540	48.43	8.14	7.88	3.21	11.35
III	2.7	540	49.78	8.36	0	0	8.36
IV	2.7	540	36.56	6.14	2.70	1.10	7.24
Average			50.82	8.64	2.65	1.08	9.72

Results and Discussion

It is known that aromatic ethers in general undergo three metabolic changes in the animal body.

(a) ArOX ⟶ *p*-HO-Ar-OX, (b) ArOX ⟶ ArOH, (c) ArOX ⟶ *p*-HO-Ar-OH

Ar=Aryl group, X=alkyl or aryl group.

Process (a) shows that hydrolytic cleavage does not take place in the ether linkage of unsubstituted alkyl phenyl ether, but hydroxylation normally occurs in the position *para* (if

3) W. H. Fishman, S. Green: J. Biol. Chem., **215**, 527(1955).
4) H. G. Bray, B. G. Humphris, W. V. Thorpe, K. White, P. B. Wood: Biochem. J., **52**, 412(1952)

the position is vacant) to the ether linkage, as in anisole,[5] phenethole,[6] and diphenyl ether.[5,7] In substituted alkyl phenyl ethers, the ether linkage is split to give a phenol, i.e. process (b), as is known by the examples of *p*-iodo-,[8] *p*-chloro-,[9] and *p*-nitro-anisoles,[9] 4-methoxy-[10] and 4, 4'-dimethoxyphenyl ethers,[11] phenetidine,[12] and phenacetin.[13]

A combination of (a) and (b) would occur in the production of dihydroxyphenol as in process (c).

According to the present conditions, it appears that the process (b) will be the case in the metabolism of herniarin. This opinion was confirmed by the fact that umbelliferone was produced *in vivo* as a result of demethylation of the herniarin administered.

From the determination of the metabolites, it was known that herniarin was converted into umbelliferone to the extent of 29.94% of the amount administered. Umbelliferone was excreted mainly in the free state (20.35%), though a small portion (9.59%) was combined. Conjugated umbelliferone was composed of 8.64% of glucuronide and 1.08% of ethereal sulfate.

It may be noted that evidences for the cleavage of lactone ring and biological hydroxylation have not been found.

From a quantitative point of view, the recovery of herniarin in the urinary metabolites was no more than 30% of the dose (200 mg./kg.). No information is available at present as to the remainder of metabolites.

Summary

The fate of herniarin in the rabbit was studied. The chief metabolite is umbelliferone but a small amount of unchanged herniarin was excreted, and both metabolites were estimated quantitatively. No evidences for biological hydroxylation or lactone-ring cleavage was obtained.

(Received May 12, 1958)

5) H. G. Bray, S. P. James, W. V. Thorpe, M. R. Wasdell: Biochem. J., **54**, 547(1953).
6) A. Kossel: Z. physiol. Chem., **4**, 296(1880); *ibid.*, **7**, 292(1883); V. Lehmann: *Ibid.*, **13**, 181(1889).
7) S. W. Stroud: J. Endocrinol. (London), **2**, 55(1940)
8) F. Röhmann: Biochem. Zentr., **3**, 688(1905).
9) H. G. Bray, V. M. Caddock, W. V. Thorpe: Biochem. J., **60**, 225(1955).
10) S. W. Stroud: Nature, **144**, 245(1939).
11) S. W. Stroud: *Ibid.*, **146**, 166(1940).
12) T. N. Smith, R. T. Williams: Biochem. J., **44**, 250(1949).
13) T. N. Smith, R. T. Williams: *Ibid.*, **44**, 239(1949).

UDC 547.854.4'861.6 : 582.284 : 545.84

104. Toru Masuda, Toyokazu Kishi, Mitsuko Asai, and Satoru Kuwada: Application of Chromatography. XXXV.* On the Biochemical Significance of 6-Methyl-7-hydroxyribolumazine in the Culture of *Eremothecium ashbyii*.

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One of the authors (Masuda) formerly isolated a green and a violet fluorescent substances from the mycelium and broth of *Eremothecium ashbyii*[1] and they were respectively named 6, 7-dimethylribolumazine and 6-methyl-7-hydroxyribolumazine.[2] As riboflavin was pro-

* Part XXXIV: This Bulletin, **6**, 291(1958).
** Juso-nishino-cho, Higashiyodogawa-ku, Osaka (増田 亨, 貴志豊和, 浅井満子, 桑田 智).
1) T. Masuda: This Bulletin, **3**, 434(1955).
2) S. Kuwada, T. Masuda, T. Kishi, M. Asai: *Ibid.*, **6**, 447(1958).