Summary

The paper chromatography of more than 30 kinds of natural and synthetic coumarin derivatives was studied using 8 different solvent systems.

The relationship between Rf values and structure of compounds in regard to the properties of the developing solvent systems was discussed.

The separation of coumarin derivatives by multibuffered chromatography was also studied.

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102. Mitiiti Fujita and Tsutomu Furuya: Studies on the Metabolism of Naturally Occurring Coumarins. II.[1] Urinary Metabolites of Umbelliferone.

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The metabolism of umbelliferone (7-hydroxycoumarin), which is one of the most widely distributed coumarins, was studied by Williams, *et al.*,[2] who isolated its crystalline glucuronide as a rabbit urinary metabolite. The paper chromatographical method was adopted for this work for the qualitative and quantitative analysis of urinary metabolites of umbelliferone using rabbit as an experimental animal.

Details of paper chromatography of coumarins were given in the preceding paper.

Experimental

Material—Umbelliferone, m. p. 230~231°, was synthetically prepared for this study.

Animals and Diet—Female rabbits (2.3~2.7 kg. body wt.) were kept on a diet of 350 g. lees of bean-curd and 350 g. cabbage per day.

Dosing—Umbelliferone emulsified with 5 drops of Tween 80 and 10 cc. of distilled water was administered by stomach tube. The urine was collected 24 and 48 hrs. after administration of umbelliferone, and covered with toluene to prevent bacterial contamination.

Identification of Urinary Metabolites—Urine excreted during 48 hrs. after oral administration of 520 mg. of umbelliferone was acidified with dil. H_2SO_4, and then continuously extracted with ether for 6 hrs. After drying over anhyd. Na_2SO_4, the brownish extract was evaporated to dryness at a low temperature. The residue was taken up in 30 cc. of acetone (Fraction F, Table II). After the addition of 100 cc. of 18*N* H_2SO_4, the residual urine was hydrolysed by boiling for 2 hrs. and then extracted with ether. The residue after removal of the solvent was taken up in 30 cc. of acetone (Fraction C, Table II).

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

Compound	Solvent system A	D	G	H	UV-Fluorescence Untreated	2*N* NaOH	Emerson's reagent	Diazotized sulfanilic acid
Resacetophenone	0.59	0.91	0.43	0.56	—	—	V	Br
β-Resorcylic acid	0.17	0.50	0.42	0.52	—	—	RV	Br-Y

TABLE II. Identification of Urinary Metabolites by Paper Chromatography

Compound	F	C
Umbelliferone	+	++
4, 7-Dihydroxycoumarin	—	—
5, 7-Dihydroxycoumarin	—	—
6, 7-Dihydroxycoumarin (esculetin)	—	—
7, 8-Dihydroxycoumarin (daphnetin)	—	—
Umbellic acid	—	—
Resacetophenone	—	—
β-Resorcylic acid	—	—

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1) Part I: This Bulletin, **6**, 511(1958).

2) J. A. R. Mead, J. N. Smith, R. T. Wiliams: Biochem. J., **61**, 569(1955).

Fractions F and C were submitted to paper chromatography using BuOH-benzene-$(NH_4)_2CO_3$ buffer, $CHCl_3$-AcOH-H_2O, 20% KCl, or 2% AcOH as the developing solvent system.

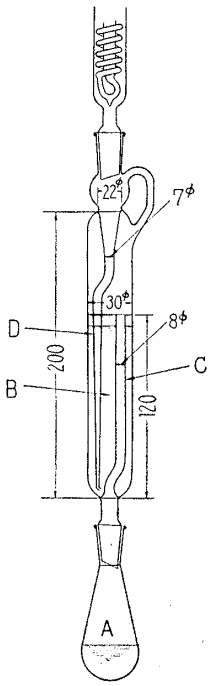
Resacetophenone and β-resorcylic acid which were presumed to be excreted in urine were used as the reference compounds for paper chromatographical identification of urinary metabolites.

The color reaction and Rf values obtained by the present study are shown in Table I.

The results of paper chromatographical investigation of the urinary metabolites indicated that umbelliferone was recovered from urine, both in the free and conjugated forms.

Isolation of Metabolites—The 48-hr. urine (500 cc.) of a rabbit fed with umbelliferone (520 mg.) was acidified with 18*N* H_2SO_4 (100 cc.). After 3 hrs.' heating on a boiling bath, the solution was continuously extracted with ether for 5 hrs. The ethereal solution was evaporated to dryness and the residue was recrystallized from aq. EtOH to give colorless needles, m.p. 228~229° (200 mg.), which showed no melting point depression on admixture with umbelliferone.

Fig. 1. Continuous Extractor



A Design of Continuous Extractor—An apparatus for continuous extraction of metabolites from urine in a high efficiency was designed. (see Fig. 1).

The glass body of extractor (B) contains urine and ether in two layers. The ether in flask (A) is boiled by heating on a bath, and the ethereal vapor goes up through tube (C) into condenser. The condensed ether comes down through tube (D) and goes up through urine layer extracting metabolites to overflow at the top of tube (C) into flask (A).

Using this apparatus a rapid and efficient continuous extraction of urinary metabolites was made as shown in Table III.

TABLE III. Performance of Continuous Extractor devised by the Authors

Amount of umbelliferone added (mg.)	100	200	300
Time for extraction (mins.)	40	60	90

Determination of Umbelliferone in Urine—The 24-hr. and 48-hr. urine after dosage of umbelliferone was adjusted to a definite volume with addition of distilled water, and 40 cc. of this solution was extracted for 2 hrs. with ether, after acidification with 2 drops of 18*N* H_2SO_4, using the continuous extractor mentioned above. The residue obtained on removal of the solvent was dissolved in 10 cc. of acetone, 0.1 cc. of this solution was accurately taken with a micropipette, and spotted in 1-cm. width on the starting line marked at a distance of 7 cm. from the bottom end of a filter paper (Toyo Roshi No. 51, 10×40 cm.). After developing with the organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer the fluorescent spot of umbelliferone was located under ultraviolet light, which was cut out and eluted for 20 mins. with 10 cc. of 0.5% Na_2CO_3 with heating on a boiling water bath.

After cool, the solution was added with 0.4 cc. of 0.9% 4-aminoantipyrine and 0.2 cc. of 5.4% $K_3Fe(CN)_6$. On standing for 1 hr. the colored solution was filtered and the extinction of color was measured at 510 mμ using Beckman DU spectrophotometer.

The amount of free umbelliferone was calculated by the regression equation[3] which had been established by the present authors.

The remainder of the urine which was extracted with ether was acidified with addition of 15 cc. of 18*N* H_2SO_4, and the mixture was boiled for 2 hrs. to hydrolyse the conjugated metabolites. Extraction and colorimetric estimation were carried out as mentioned above in the case of free umbelliferone.

As a control experiment, the recovery of umbelliferone added to normal and hydrolysed urine was estimated (Table IV).

TABLE IV. Recovery of Umbelliferone added to Normal and Hydrolysed Urine

Amount of umbelliferone added		Found (γ)			Umbelliferone recovered (average %)
40 γ	(1)	39.06,	41.43,	38.27	98.98
	(2)	41.49,	41.09,	41.89	104.00
80 γ	(1)	81.02,	81.02,	77.80	99.94
	(2)	82.23,	82.23,	81.02	102.29

(1) Normal urine
(2) Hydrolyzed urine after removing acidic constituents.

Hydrolysing Time of Umbelliferone Conjugates—A mixture of 40 cc. of umbelliferone-urine and 15 g. of 18*N* H_2SO_4 was treated, changing the period of heating from 60 to 150 mins. with the interval of 30 mins. The rate of hydrolysis was colorimetrically estimated at 510 mμ as described above. The results obtained (Table V) showed no obvious variation of extinction in each case.

TABLE V. Correlation between Extinction and Time taken for Hydrolysis

Time (min.)	60	90	120	150
Extinction	0.180	0.186	0.186	0.187

As there are some differences in the behavior of conjugated urinary metabolites against hydrolysing agent, it is recommended that the period of hydrolysis be 2 hrs.

Excretion of Metabolites—To determine the time when the excretion of urinary metabolites is completed, the urine collected at 24-hr. intervals after administration of umbelliferone was examined by the method mentioned above.

The results (Tables VI and VII) indicated that the metabolites were almost completely excreted within 48 hrs. after dosage, and the 72-hr. urine showed no evidence of the presence of umbelliferone. Therefore, the 48-hr. urine was proved to be most suitable for further investigation.

TABLE VI. Relationship between Metabolites and Time for Excretion

Umbelliferone excreted

	Free		Conjugated		Total	
After dosage	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)
0～24 hrs.	85.53	16.41	288.24	55.43	373.57	71.84
24～48 hrs.	22.36	4.30	63.12	12.14	85.48	16.44
Total	107.69	20.71	351.36	67.57	459.05	88.28

Expt. I, Rabbit wt. 2.6 kg., Dose 540 mg.

TABLE VII. Excretion of Conjugated Umbelliferone

	Glucuronide		Ethereal sulfate		
After dosage	(mg.)	(% of dose)	(mg. SO_3)	(% of dose)	Total
0～24 hrs.	249.35	40.04	37.29	14.52	54.56
24～48 hrs.	35.50	5.70	17.25	6.72	12.42
Total	284.85	45.74	54.54	21.24	66.98

Dose—The metabolites excreted after dosage of 100, 200, or 300 mg. (per kg. rabbit) of umbelliferone were determined (Table VIII).

TABLE VIII. Relationship between Dose and Metabolites

Umbelliferone excreted in 48 hrs.

Rabbit	Dose	Free		Conjugated		Total	
wt. (kg.)	(mg./kg.)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)
2.3	100	48.39	21.04	145.02	63.05	193.41	84.09
2.7	200	371.02	68.71	56.37	10.44	427.39	79.15
2.5	300	128.25	17.10	387.23	51.63	515.48	68.73

The recovery of metabolites decreased inversely with the increasing dosage. It is proposed that 200 mg./kg. (rabbit) of umbelliferone be used for further investigation.

Results and Discussion

The 48-hr. urine after administration of 200 mg./kg. (rabbit) of umbelliferone was determined and the results are shown in Table IX.

TABLE IX. The Excretion of Free and Conjugated Umbelliferone by Rabbits receiving Umbelliferone Orally

Expt.	wt.	Dose	Umbelliferone excreted in 48 hrs.					
			Free		Conjugated		Total	
No.	(kg.)	(mg.)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)
I	2.6	520	107.69	20.71	351.36	67.57	459.05	88.28
II	2.5	500	99.90	19.98	336.90	67.38	436.80	87.36
III*	2.3	460	97.70	21.24	307.51	66.85	405.21	88.09
IV	2.6	520	98.96	18.98	327.86	63.05	426.55	82.03
	Average		101.06	20.23	330.91	66.21	431.90	86.44

TABLE X. Conjugated Umbelliferone

			Glucuronide				
Expt.	wt.	Dose	Glucuronic acid excreted	Umbelliferone equiv. to glucuronic acid	Ethereal sulfate		Total
No.	(kg.)	(mg.)	(mg.)	(% of dose)	(mg. SO_3)	(% of dose)	(% of dose)
I	2.6	520	284.85	45.74	54.54	21.24	66.98
II	2.5	500	250.38	45.44	44.28	19.47	64.92
III	2.3	460	262.80	42.20	44.58	17.36	59.56
IV	2.6	520	262.75	43.88	46.00	18.33	62.21
	Average		265.20	44.32	47.35	19.10	63.42

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.

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

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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.

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1) Part II: M. Fujita, T. Furuya: This Bulletin, **6**, 517(1958).
2) M. Fujita, T. Furuya: *Ibid.*, **6**, 517(1958).