hydroxypropyl)-3-(5-nitro-2-furyl)acrylamide (XIX) showed a higher solubility in water and a strong antibacterial activity.

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140. **Tsutomu Furuya**: Studies on the Metabolism of Naturally Occurring Coumarins. IV.[1] Urinary Metabolites of Dimethylesculetin and Scopoletin.

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Dimethylesculetin is obtained from the immature fruits of *Artemisia capillaris* THUNB. The alcoholic extracts of this plant is being used as a remedy for jaundice in this country. In connection with its remedial effect it seemed worthwhile to study the metabolic fate of this compound on which the present study chiefly concerns.

As a reference the metabolism of scopoletin obtained from Belladonna roots and other Solanaceous plants has also been studied.

The author is indebted to Prof. S. Shibata and Assist. Prof. M. Fujita for their kind advices and suggestions in carrying out the present study. Deep gratitude is expressed for the kind gift of dimethylesculetin by Dr. K. Imai, Takamine Research Laboratory, Sankyo Co. Ltd.

Experimental

Material—Dimethylesculetin, m.p. 144°, recrystallized from hydr. EtOH, was used.

Animal, Diet, and Dosage—Same as described in the preceding paper.[1]

Identification of Metabolites—The 48-hr. urine after oral administration of 540 mg. herniarin was acidified with dil. H_2SO_4 and continuously extracted with ether for 6 hrs. The F and C fractions, which were obtained by respective extraction with ether before and after hydrolysis as indicated in the case of herniarin, were examined by paper chromatography.

Esculetin 7-methyl ether, scopoletin (esculetin 6-methyl ether), and unchanged dimethylesculetin were detected in the urinary metabolites as shown in Table I.

TABLE I. Indentification of Metabolites in Urine by Paper Chromatography

Compound	F	C
Dimethylesculetin	trace	—
Esculetin 7-methyl ether	++	++++
Scopoletin	++	+
Esculetin	trace	+

Isolation of Metabolites—The 48-hr. urine after dosage of 540 mg. of dimethylesculetin was acidified with 100 cc. of 18*N* H_2SO_4 and extracted continuously for 6 hrs. after hydrolysis by heating on a boiling water bath. The ethereal solution was shaken twice with 8.8*N* buffer solution, referring to the result of multibuffered paper chromatography.[2] The brownish residue obtained from the ethereal extract was recrystallized repeatedly from water, decolorizing with addition of charcoal. Colorless needles, m.p. 184° (160 mg.), were obtained, which showed no depression on admixture with the authentic sample of esculetin 7-methyl ether.

The alkaline solution was acidified with dil. HCl, extracted with ether using a continuous extractor, and the ethereal extract was evaporated. The pale brownish residue (40 mg.), whose solution gave an intensive fluorescence, was recrystallized from water to give scopoletin, m.p. 205°.

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1) Part III: This Bulletin, **6**, 520 (1958).

2) This Bulletin, **6**, 513 (1958).

Quantitative Determination of Metabolites—Unlike the procedure given in the previous paper, the present study was carried out to estimate the metabolic products directly on filter paper chromatogram by densitometer.

Apparatus—The densitometer (Model Kobayashi) was used for determination of paper chromatograms.

Color Reagents—Solution A: 0.6% ethanolic NaOH solution. Solution B: 2% EtOH solution of phosphomolybdenic acid (this solution should be kept in dark for an undue period.).

Standard Solution—10 mg. each of esculetin 7-methyl ether, scopoletin, and esculetin was dissolved in 10 cc. of Me_2CO.

Calibration Curves—Standard solution of esculetin 7-methyl ether (0.01 cc.) was spotted on the starting line of a filter paper (Toyo Roshi No. 51, 3×40 cm.). The chromatogram developed with the organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer was immersed in the solution A for a moment, air-dried for 5 mins., then immersed in the solution B and instantly air-dried again. The spot appeared shortly, blue for esculetin 7-methyl ether, greenish blue for scopoletin, and blue for esculetin. After a lapse of 40 mins. the paper was dipped into 30% ether-liquid paraffin solution to make the paper translucent. The color density was read by densitometer using a 2-mm. slit and a red filter.

The quantity of materials is usually estimated from the area below the curve obtained by plotting the color density, but the sum of color densities was employed in the present work. The calibration lines obtained in this way are shown in Figs. 1 and 2. Two calibration lines (1) and (2) in Figs. 1 and 2 are from different commercial preparations of phosphomolybdenic acid, and the calibration line (1) was used for quantitative analysis of metabolites.

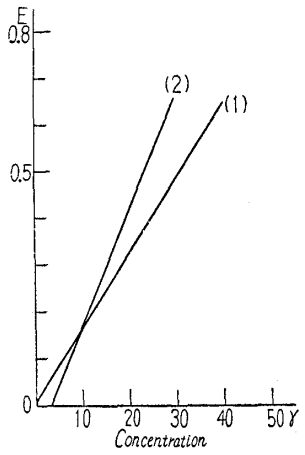

Fig. 1.
Calibration Lines of
Esculetin 7-Methyl Ether

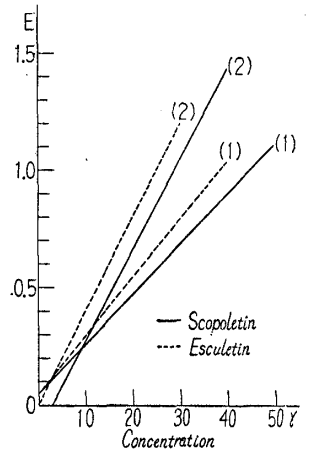

Fig. 2.
Calibration Lines of
Scopoletin and Esculetin

The recovery of esculetin 7-methyl ether, scopoletin, and esculetin from the urinary metabolites is listed in Table II.

TABLE II. Recovery of Esculetin and its Methyl Ethers

Compd. added	Found (γ)	Recovery (average %)
Esculetin 7-methyl ether (10γ)	(1) 10.5, 11.0	108
	(2) 11.0, 12.0	110
Scopoletin (10γ)	(1) 10.5, 10.5	105
	(2) 11.0, 10.5	108
Esculetin (10γ)	(2) 10.0, 10.5	103

(1) Added to normal urine
(2) Added to hydrolyzed urine after removal of acidic constituents

Stability of the Color developed by Reagents—The relationship between extinction of coloration and time after the addition of coloring reagents to the alkaline solution containing 20 γ of esculetin 7-methyl ether is shown in Table III.

TABLE III. Correlation between Extinction of Coloration and Time

Time (min.)	40	60	90	120
Extinction	0.320	0.322	0.320	0.324

Procedure of Determination—The 48-hr. urine after oral administration was acidified with dil. H_2SO_4 and extracted with Et_2O for 2 hrs., using a continuous extractor devised by the present author.

The ether solution was evaporated and the residue was dissolved in 10 cc. of Me_2CO. The acetone solution (0.01 cc.) was used for the paper chromatographical separation, employing the organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer as a developing solvent. The developed paper was immersed in solutions A and B, and treated as indicated in the section on preparation of calibration line. Thus the paper chromatograms of F and C fractions of metabolites were determined using a densitometer. The results of quantitative determination are summarized in Tables IV and V, and in Fig. 3.

TABLE IV. Free and Conjugated Products

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	Esculetin 7-methyl ether				Scopoletin			
			Free		Conjugated		Free		Conjugated	
			(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)
I	2.7	540	51.78	10.29	239.46	47.58	36.80	7.31	3.72	0.74
II	2.7	540	46.80	9.30	203.28	40.39	43.20	8.58	17.87	3.51
III	2.7	540	126.40	25.11	156.74	31.15	49.46	9.83	5.63	1.12

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	Esculetin				Total		
			Free		Conjugated		Free	Conjugated	Total
			(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	(% of dose)	(% of dose)
I	2.7	540	—	—	4.44	1.03	17.60	49.35	66.95
II	2.7	540	—	—	15.60	3.34	17.88	47.24	65.12
III	2.7	540	—	—	17.36	3.72	34.94	35.99	70.93

TABLE V. Metabolic Products (%) of Dimethylesculetin

Form	Esculetin 7-methyl ether	Scopoletin	Esculetin	Total
Free	14.90	8.57	—	23.47
Conjugated	39.71	1.79	2.70	44.20
Total	54.61	10.36	2.70	67.67

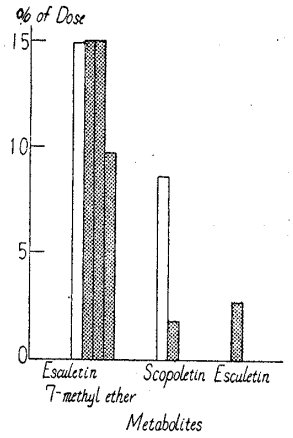

Fig. 3. Metabolic Products of Dimethylesculetin
White: Free form
Shaded: Conjugated form

TABLE VI. Conjugation of Dimethylesculetin

Expt. No.	Glucuronide		Ethereal sulfate		Total
	Glucuronic acid excreted (mg.)	Monohydroxy-monomethoxy-coumarin equivalent to glucuronic acid (% of dose)	(mg. SO_3)	(% of dose)	(% of dose)
I	230.051	45.32	28.16	13.27	49.35
II	204.480	40.28	28.16	13.27	47.24
III	155.533	30.64	17.35	8.17	35.99
Average	196.688	38.75	24.56	11.57	44.20

The estimation of total glucuronide and ethereal sulfate was performed by the method described previously and the result is listed in Table VI.

Identification and Determination of Metabolites of Scopoletin—Scopoletin and esculetin in the urinary metabolites were identified and determined by the foregoing method. The results are shown in Tables VII and VIII. Scopoletin was determined only once owing to shortage of the sample.

TABLE VII. Free and Conjugated Products

Rabbit wt. (kg.)	Dose (mg.)	Scopoletin				Esculetin				Total		Total
		Free		Conjugated		Free		Conjugated		Free	Conjugated	
		(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	(% of dose)	(% of dose)
2.7	540	397.93	73.69	56.37	10.44	—	—	13.20	2.64	73.69	13.08	86.77

TABLE VIII. Conjugation of Scopoletin

Glucuronide		Ethereal sulfate		Total
Glucuronic acid excreted	Scopoletin equivalent to glucuronic acid (% of dose)	(mg. SO_3)	(% of dose)	(% of dose)
47.709	8.74	1.54	1.35	10.01

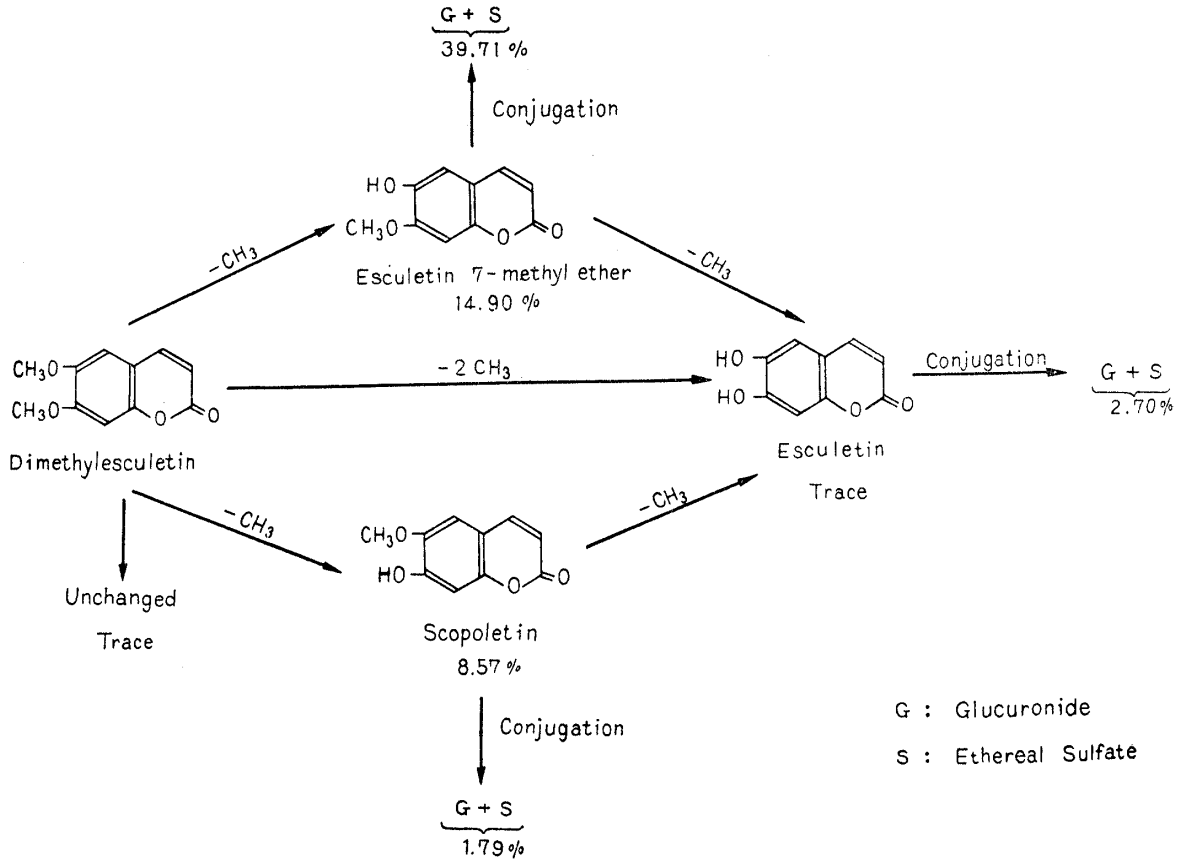

Chart 1.

Metabolic Process of Dimethylesculetin

Results and Discussion

It has been found that dimethylesculetin was demethylated in the rabbit body in accordance with the general rule for metabolism of aromatic ethers giving 54.61% of esculetin 7-methyl ether, 10.36% of scopoletin, and 2.70% of esculetin, which were excreted in the urine as glucuronide and sulfuric acid ester.

The ratio of total free and conjugated forms excreted in the urine was 1 to 2. The ratio of free and conjugated esculetin 7-methyl ether was 1 to 3, while that of scopoletin was 5 to 1. The free form of esculetin and unchanged dimethylesculetin could not be determined owing to their poor excretion in urine.

In the case of metabolism of scopoletin, a greater portion was excreted unchanged accompanying partially demethylated product, esculetin.

The present experimental results lead to a conclusion that dimethylesculetin would be demethylated preferentially at 6-methoxyl group rather than at 7-methyoxyl group, and finally esculetin formed by complete demethylation.

Summary

Dimethylesculetin was demethylated in the rabbit to give esculetin 7-methyl ether, scopoletin, and esculetin. The free and conjugated forms of each metabolite were determined directly by a densitometer applied on the paper chromatogram. The urinary metabolites obtained from scopoletin have also been examined.

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