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141. Tsutomu Furuya: Studies on the Metabolism of Naturally Occurring Coumarins. V.* Urinary Metabolites of Coumarin and Dihydrocoumarin.

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Coumarin and its derivatives are extensively employed as essence for foodstuffs, while some of them are known to have anticoagulant action and powerful inhibitory action against germination of plant seeds.

The metabolism of tromexan[1,2] and 4-methylcoumarin[3] has already been reported, but nothing is known of the urinary excretion products of coumarin itself ingested by animals.

When the present work was almost completed, the author became aware that Williams, *et al.*,[4] in the latest arriving journal, reported on the metabolism of coumarin and *o*-coumaric acid. Some difference, however, could be seen between the present result and that obtained by the British workers.

In the present paper investigation on metabolism of coumarin and dihydrocoumarin using paper chromatography is described and interrelation between the metabolic process of coumarins is also elucidated.

The author expresses his gratitude to Prof. S. Shibata and Assist. Prof. M. Fujita of the University of Tokyo for their kind advices and encouragement.

Experimental

Materials—Commercial specimens of coumarin and hippuric acid were used after careful purification. *o*-Coumaroylglycine was synthesized as follows:

o-Coumaric acid (5 g.) was dissolved in 70 cc. of 5% Na_2CO_3 and the cooled solution was treated with 4 g. of Cl·COOEt, under mechanical stirring for 30 mins. The crude *o*-coumaric acid ethylcarbonate was recrystallized from dil. Me_2CO; m.p. 152°. To 1 g. of this acid a very slightly excess of PCl_5 was added and the mixture was warmed until the evolution of HCl ceased. The oily residue obtained after removal of $POCl_3$ was taken up in a small amount of dry Et_2O and the solution was added under stirring to a cold solution of glycine (1 g.) in 5% Na_2CO_3 solution (30 cc.). The stirring was continued for another 30 mins., 5 cc. of 10% NaOH solution was added, and the mixture was warmed on a boiling water bath for 15 mins. On acidification *o*-coumaroylglycine was separated and it was recrystallized from water to colorless needles, m.p. 227~228°. *Anal.* Calcd. for $C_{11}H_{11}O_4N$: C, 59.72; H, 5.01: N, 6.33. Found: C, 59.68; H, 5.00; N, 6.08.

Melilotoylglycine was obtained by the reduction of *o*-coumaroylglycine with 2.5% Na-amalgam. Colorless needles (from water), m.p. 123~125°. *Anal.* Calcd. for $C_{11}H_{13}O_4N$: C, 59.18; H, 5.87; N, 6.28. Found: C, 59.16; H, 5.36; N, 6.20.

Salicyluric acid was obtained by the same synthetic method as described in the case of *o*-coumaroylglycine starting from acetylsalicylic acid. Colorless needles (from water), m.p. 169~170°.

The synthesis of hydroxycoumarins and other related compounds used has been described in Part I of this series.[5]

Animal, Diet, and Dosage—Rabbits kept on a definite diet were used for the present investigation. Coumarin (200 mg./kg.) was emulsified with 5 drops of Tween 80 and 10 cc. of distilled water, and administered by a stomach tube.

Identification of Metabolites—The urine excreted during 48 hrs. after oral administration of 540 mg. of coumarin was acidified with dil. H_2SO_4 and extracted continuously with Et_2O for 6 hrs..

The F and C fractions extracted with Et_2O before and after hydrolysis were developed by paper chro-

* Part IV: This Bulletin, **6**, 696 (1958).

** Hongo, Tokyo (古谷 力)

1) R. Pulver, K. N. von Kaulla: Schweiz. med. Wochschr., **78**, 956 (1948).
2) J. J. Burns, S. Wexler, B. B. Brodie: J. Am. Chem. Soc., **75**, 2345 (1953).
3) H. Kitagawa: Yakugaku Zasshi, **76**, 582 (1956).
4) J.A.R. Mead, J. N. Smith, R. T. Williams: Biochem. J., **68**, 67 (1958).
5) M. Fujita, T. Furuya: This Bulletin, **6**, 511 (1958).

matography. The results obtained are shown in Table II, and the color reaction and Rf values of glycine conjugates are listed in Table I.

TABLE I. Rf Values and Color Reactions of Glycine Derivatives

Compound	Solvent System							UV Fluorescence		Emerson's reagent	Diazotized sulfanilic acid	PDAB + Ac_2O
	A	B	C	D	E	F	G	untreated	2N-NaOH			
o-Coumaroylglycine	0.12	0.30	0.82	0.07	0.02	0.49	0.52	WB	Y	RV	O	P
Melilotoylglycine	0.19	0.68	0.81	0.21	0.05	0.67	0.82	—	—	RV	O	Y
Salicyluric acid	0.12	0.68	0.88	0.47	0.19	0.44	0.70	B	B	RV	Y	RO
Hippuric acid	0.20	0.82	0.82	0.59	0.18	0.60	0.75	—	—	—	—	YO

B: Black O: Orange P: Pink R: Red V: Violet W: White Y: Yellow

TABLE II. Identification of Metabolites in Urine by Paper Chromatography

Compound	Coumarin-urine		Dihydrocoumarin-urine	
	F	C	F	C
3-Hydroxycoumarin	++	++	++	++
4-Hydroxycoumarin	−	−	−	−
5-Hydroxycoumarin	−	−	−	−
6-Hydroxycoumarin	−	−	−	−
7-Hydroxycoumarin (umbelliferone)	+	++	++	++
8-Hydroxycoumarin	+*	+*	+*	+*
Coumarin	+	−	+	−
Dihydrocoumarin	+	−	+	−
o-Coumaric acid	+	+	+	+
Melilotic acid	+*	+*	++	+
Salicylic acid	−	−	−	−
o-Coumaroylglycine	+*	−	+	−
Melilotoylglycine	−	−	+	−
Salicyluric acid	−	−	−	−
Hippuric acid	+	−	+	−

* trace

p-Dimethylaminobenzaldehyde solution (PDAB+Ac_2O) was used as a chromogenic reagent.[6] 4% Solution of *p*-dimethylaminobenzaldehyde in Ac_2O containing a few AcONa crystals was sprayed on the paper chromatogram. The reaction was completed by heating the sprayed paper strips for 1~2 mins. at 130~150°. Yellow to red coloration was observed in the presence of acylglycine.

Isolation of Metabolites—The 48-hr. urine after dosage of 540 mg. of coumarin was acidified with 18*N* H_2SO_4 and the solution was extracted continuously with Et_2O for 6 hrs. The solvent was removed after drying over anhyd. Na_2SO_4. The residual material was dissolved in benzene. The benzene-insoluble material was collected and recrystallized from hydr. EtOH to light yellow needles, m.p. 228°. It showed no depression of m.p. on admixture with the authentic sample of umbelliferone.

After removal of benzene, the brownish residue was recrystallized from water to give yellow needles (yield: 20 mg.), m.p. and mixed m.p. with authentic 3-hydroxycoumarin, 152~153°.

The residual urine after hydrolysis was treated in the same way and 30 mg. of 3-hydroxycoumarin was obtained from benzene-soluble portion, whereas 30 mg. of umbelliferone was isolated from the benzene-insoluble portion.

Regression Equations of Concentration-Intensity of Light Absorption of Coumarin, *o*-Coumaric Acid, and 3-Hydroxycoumarin—Absorption spectra of coumarin, *o*-coumaric acid, and 3-hydroxycoumarin developed by Emerson's reagent are shown in Fig. 1.

Regression equations were obtained by the same method as was reported in Part III of this series[7] by estimation at the maximum absorptions at 518, 522, and 485 mμ for coumarin, *o*-coumaric acid, and 3-hydroxycoumarin, respectively.

6) G. W. Gaffney, K. Schreier, N. Di Ferrante, K. I. Altman: J. Biol. Chem., **206**, 695 (1954).
7) T. Furuya: This Bulletin, **6**, 520 (1958).

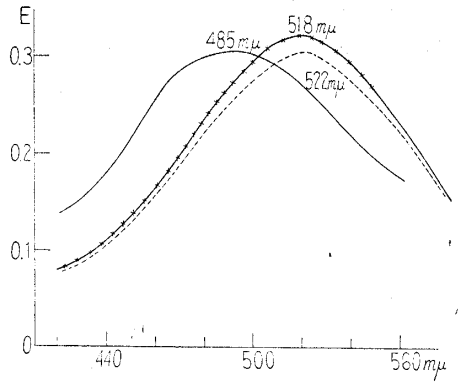

Fig. 1. Absorption Spectra of Coumarin (80 γ), *o*-Coumaric Acid (80 γ), and 3-Hydroxycoumarin (160 γ) colored by Emerson's Reagent

-×-×- coumarin
---------- *o*-coumarin acid
——— 3-hydroxycoumarin

Coumarin n=40, y=0.0075882x+0.0116 or x=131.78y−1.5287 σ=0.006. *o*-Coumaric acid n=40, y=0.0084208x−0.006 or x=118.75y+0.0713. 3-Hydroxycoumarin n=40, y=0.00086556x−0.001667 or x=1155.30y+1.9259 σ=0.0011. x: Concentration (γ) y: Optical density.

Quantitative Determination of Metabolites in the Urine—The quantitative determination of urinary metabolites was performed as previously described in the case of umbelliferone. After development with organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer, the spots were cut out and then eluted with 10 cc. of 0.5% Na_2CO_3 solution by boiling for 30 mins. Coumarin, *o*-coumaric acid, and 3-hydroxycoumarin colored with Emerson's reagent were determined colorimetrically. The spots of coumarin, *o*-coumaric acid, and 3-hydroxycoumarin were separated from each other giving Rf values of 0.23, 0.55, and 0.44, respectively.

The recovery rate is listed in Table III, and the quantitative results are summarized in Tables IV~VI and in Fig. 2.

TABLE III. Recovery of Coumarin, *o*-Coumaric Acid, and 3-Hydroxycoumarin

Compd. added		Found (γ)			Recovery (average %)
Coumarin (40γ)	(1)	38.47	40.01	41.23	99.75
o-Coumaric acid (40γ)	(1)	39.06	41.43	38.27	98.98
	(2)	39.89	42.36	41.36	103.00
3-Hydroxycoumarin (80γ)	(1)	82.08	83.24	84.03	103.90
	(2)	84.35	85.21	84.31	105.78

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

TABLE IV. Free and Conjugated Products

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	3-Hydroxycoumarin Free (mg.)	3-Hydroxycoumarin Free (% of dose)	3-Hydroxycoumarin Conjugated (mg.)	3-Hydroxycoumarin Conjugated (% of dose)	Umbelliferone Free (mg.)	Umbelliferone Free (% of dose)	Umbelliferone Conjugated (mg.)	Umbelliferone Conjugated (% of dose)
I	2.8	560	72.168	11.61	105.858	17.03	23.061	3.71	61.974	9.97
II	2.5	500	61.716	11.12	89.688	16.16	15.540	2.80	43.346	7.81
III	2.5	500	55.001	9.91	106.728	19.23	24.975	4.50	44.622	8.04
IV	2.7	540	84.396	14.08	84.396	14.08	21.698	3.62	44.715	7.46
V	2.7	540	72.228	12.05	93.027	15.52	22.777	3.80	44.416	7.41

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	*o*-Coumaric acid Free (mg.)	*o*-Coumaric acid Free (% of dose)	*o*-Coumaric acid Conjugated (mg.)	*o*-Coumaric acid Conjugated (% of dose)	Coumarin Free (mg.)	Coumarin Free (% of dose)	Total Free (% of dose)	Total Conjugated (% of dose)	Total (% of dose)
I	2.8	560	7.840	1.25	8.969	1.43	4.940	0.90	17.47	28.43	45.90
II	2.5	500	9.184	1.64	2.296	0.41	2.200	0.44	16.00	24.38	40.38
III	2.5	500	8.848	1.58	6.720	1.20	1.900	0.38	16.37	28.47	44.84
IV	2.7	540	7.318	1.21	5.201	0.86	11.124	2.06	20.97	22.40	43.37
V	2.7	540	11.249	1.86	1.270	0.21	6.588	1.22	18.93	23.14	42.07

TABLE V. Metabolic Products (%) of Coumarin

Form	3-Hydroxy coumarin	Umbel-liferone	*o*-Coumaric acid	Coumarin	Total
Free	11.75	3.69	1.51	1.00	17.95
Conjugated	16.40	8.14	0.82	—	25.36
Total	28.15	11.83	2.33	1.00	43.31

Table VI. Conjugation of Coumarin

Expt. No.	Glucuronide: Glucuronic acid excreted (mg.)	Glucuronide: Monohydroxycoumarin equivalent to glucuronic acid (% of dose)	Ethereal sulfate (mg. SO_3)	Ethereal sulfate (% of dose)	Total (% of dose)
I	116.202	15.62	19.63	6.32	21.94
II	90.866	13.68	16.09	5.80	19.48
III	99.036	14.91	18.19	6.56	21.47
IV	119.082	16.60	22.22	7.42	24.02
V	107.748	15.02	21.66	7.23	22.25
Average	106.587	15.17	19.56	6.66	21.83

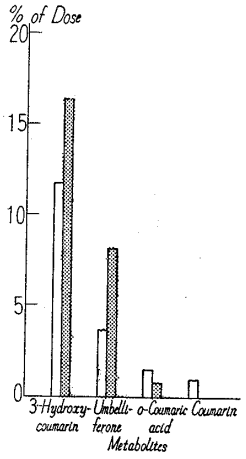

Fig. 2. Metabolic Products of Coumarin

White: Free form
Shaded: Conjugated form

Results and Discussion

The quantitative determination revealed that 43.31% of the dose (coumarin) was recovered as the urinary metabolites. The principal metabolites were 3-hydroxycoumarin and umbelliferone. 3-Hydroxycoumarin, which was recovered to 28.15% of the dose administered, consisted of 11.75% of free and 16.40% of conjugated forms, whereas umbelliferone, recovered in 11.83% of the dose, consisted of 3.69% of free and 8.14% of conjugated forms. A trace of 8-hydroxycoumarin was also detected, whereas 4-, 5-, and 6-hydroxycoumarins were not found in the urinary metabolites of coumarin.

All naturally occurring coumarins so far known possess one hydroxyl group at the 7-position, i.e. they are the derivative of the most widely distributed umbelliferone, and none which contains hydroxyl group in the 3-position is found in nature. It would therefore be suggested that there is some difference in the oxidative enzyme system of animals and plants.

Boyland[8] and Young[9] have introduced the concept that many phenolic compounds excreted in the urine following the administration of various anthracenes were derived from intermediate epoxy compounds, labile diol, or both. This mechanism of biological oxidation could also be adopted for explanation of the production of hydroxycoumarins from metabolism of coumarin. 3,4-Dihydro-diol shown in Chart 1 could readily be dehydrated as the usual

8) E. Boyland: Biochem. Soc. Symposia, **5**, 40 (1950).
9) F. G. Young: *Ibid.*, **5**, 27 (1950).

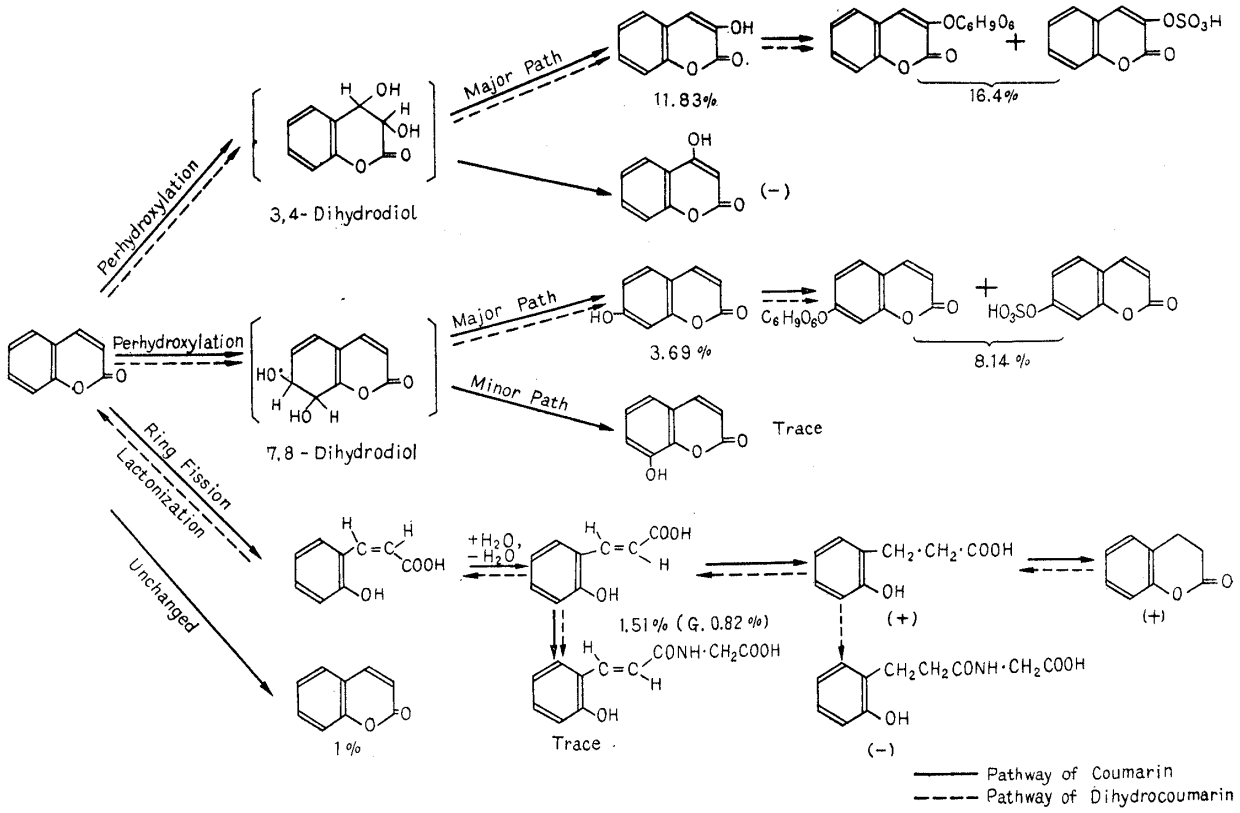

Chart 1.

Proposed Scheme for the Metabolic Transformation of Coumarin and Dihydrocoumarin.

β-hydroxycarboxylic acids to give 3-hydroxycoumarin only, and 4-hydroxycoumarin would not be formed.

Under a similar reaction mechanism, 7,8-dihydro-diol could also be converted into umbelliferone in a greater portion, and to a lesser degree to 8-hydroxycoumarin. The intermediate diol, however, has not actually been isolated.

On the other hand, leuco-anthocyanidine with diol structure have been found in plants. Accordingly it might be possible to assume that the epoxidation or perhydroxylation would be the first step in biological oxidation of aromatic compounds in both animal body and living plants.

Furthermore, a small amount of *o*-coumaric acid, which was not noticed by Williams *et al.*[4] has been detected and determined to contain approx. 1.5% as free form and 1% as glucuronide. This fact showed that opening of a lactone ring in coumarin occurs *in vivo*, though to a lesser extent, and suggested the occurrence of biological transformation of *cis* into *trans* form.

o-Coumaric acid formed from coumarin was partially reduced, forming melilotic acid and cyclized again, giving dihydrocoumarin.

A trace of *o*-coumaroylglycine and approx. 1% of unchanged coumarin were identified, while melilotoylglycine, salicylic acid, and salicyluric acid were not detected.

The ratio of free and conjugated forms in total metabolites derived from coumarin was approximately 3 : 4.

In the urine excreted after dihydrocoumarin dosing, 3-hydroxycoumarin, umbelliferone, coumarin, and *o*-coumaric acid were detected, and a considerable amount of melilotic acid and its glycine conjugates, such as melilotoyl- and *o*-coumaroyglycine were identified by paper chromatography. The formation of glycine conjugates could be explained by assuming

that dihydrocoumarin undergoes opening of the lactone ring in the same way as in the case of coumarin.

Summary

Studies on the metabolic fate of coumarin and dihydrocoumarin have been elucidated. Coumarin was mainly hydroxylated to 3-hydroxycoumarin, umbelliferone, and a trace of 8-hydroxycoumarin. *o*-Coumaric acid was subjected to opening of the lactone ring and glycine conjugates of *o*-coumaric acid and melilotic acid were detected in the metabolite. Hydroxylated compounds were conjugated with glucuronic and sulfuric acids. Various metabolites derived from dihydrocoumarin were also detected by paper chromatography. A scheme of metabolic process of coumarin and dihydrocoumarin was also proposed and discussed.

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142. Tsutomu Furuya: Studies on the Metabolism of Naturally Occurring Coumarins. VI[1]. Urinary Metabolites of *o*-Coumaric Acid and Melilotic Acid

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o-Coumaric acid and melilotic acid were extracted from the leaves of melilot in free form or as their glycosides.

Relating to the preceding work,[1] an investigation on the biological transformation of *o*-coumaric acid was carried out on which the present paper chiefly concerns.

Recently, Williams,[2] *et al.* reported a qualitative studies on the metabolism of *o*-coumaric acid and they found *o*-coumaroylglycine in the urinary metabolites but failed to detect melilotic acid. By the present investigation, however, both *o*-coumaroylglycine and melilotic acid were obtained as the metabolites accompanying unchanged *o*-coumaric acid. The amount of metabolites of *o*-coumaric acid was determined quantitatively and the metabolic fate of melilotic acid was also studied by paper chormatography.

The author expresses his deep gratitude to Prof. S. Shibata and Assist. Prof. M. Fujita for their kind advices and encouragement throughout the course of this work.

Experimental

Animal, Dosage, and Diet—*o*-Coumaric acid dissolved in 5% $NaHCO_3$ solutions was administered by a stomach tube. The procedure of experiment was the same as employed in the preceding study.[1]

Identification of Metabolites—The 48-hr. urine after oral administration of 540 mg. of *o*-coumaric acid was acidified with dil. H_2SO_4 and extracted continuously with Et_2O. The solvent was removed and a part of the residue was dissolved in 10 cc. of Me_2CO (F fraction). Another fraction of the urine was hydrolyzed and treated as mentioned above to obtain the ethereal extract (C fraction). The F and C fractions were examined by paper chromatography using the developing solvent given in the preceding paper.[1] The result of paper chromatography is shown in Table I.

Isolation of Metabolites—The 48-hr. urine after oral administration of 540 mg. of *o*-coumaric acid was hydrolyzed with dil. H_2SO_4 by refluxing for 3 hrs. and extracted continuously with Et_2O for 6 hrs. The solvent was removed and the brownish residue was recrystallized repeatedly from water with charcoal to obtain light yellow prisms, m.p. 206~207°. Yield, 30 mg. A mixed fusion with the authentic specimen of *o*-coumaric acid showed no depression of m.p.

Regression Equations of Concentration-Intensity of Light Absorption in the Determination of *o*-Coumaroylglycine and Melilotic Acid—Absorption spectra of *o*-coumaroylglycine and melilotic acid developed by Emerson's reagent are shown in Fig. 1.

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1) T. Furuya: This Bulletin, **6**, 701(1958).
2) J. A. R. Mead, J. N. Smith, R. T. Williams: Biochem. J., **68**, 67(1958).