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).

TABLE I. Identification of Metabolites in Urine by Paper Chromatography

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

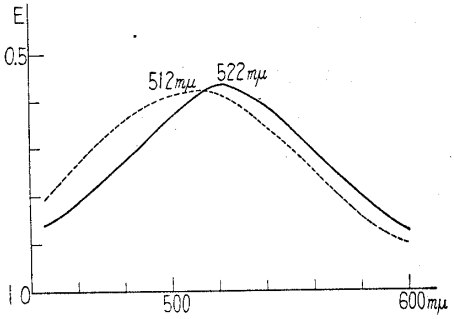

Fig. 1. Absorption Spectra of *o*-Coumaroylglycine (80 γ) and Melilotic Acid (80 γ) colored by Emerson's Reagent

——— *o*-Coumaroylglycine
····· Melilotic acid

Using the absorption max. at 522 mμ for *o*-coumaroylglycine and 512 mμ for melilotic acid (Fig. 1), regression equations between concentration and optical extinction were prepared as follows:
o-Coumaroylglycine: n=40, $y=0.00555x-0.0077$ or $x=180.180y+1.3874$ $\sigma=0.004$
Melilotic acid: n=40, $y=0.00507z+0.0093$ or $x=197.239y-1.8264$ $\sigma=0.004$

Quantitative Determination of Metabolites in the Urine—*o*-Coumaroylglycine, *o*-coumaric acid, melilotic acid, umbelliferone, and coumarin were successfully separated using the organic phase of BuOH-benzene-$(NH_4)_2CO_3$ buffer as the developing solvent (Rf values are 0.12, 0.23, 0.37, 0.55, and 0.86, respectively). The spots were cut out and eluted by heating with 10 cc. of 0.5% Na_2CO_3. The concentration of metabolites was determined accurately by measuring the color intensity developed by Emerson's reagent referring to their regression equations.

The recovery of *o*-coumaroylglycine and melilotic acid by means of this quantitative method is shown in Table II and the results of quantitative determination of the metabolites are summarized in Table III～V and in Fig. 2.

TABLE II. Recovery of *o*-Coumaroylglycine and Melilotic Acid

Compound added		Found (γ)			Recovery (average %)
o-Coumaroylglycine (80 γ)	(1)	82.41	81.30	80.45	101.74
Melilotic acid (80 γ)	(1)	81.36	80.46	80.81	101.10
	(2)	82.23	82.41	81.43	102.53

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

TABLE III. Free and Conjugated Products

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	*o*-Coumaric acid				Umbelliferone			
			Free		Conjugated		Free		Conjugated	
			(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)
I	2.8	560	116.256	20.76	18.368	3.28	12.615	2.28	16.709	3.02
II	2.6	520	99.996	19.23	9.880	1.90	12.484	2.43	19.831	3.86
III	2.6	520	98.592	18.96	24.596	4.73	7.809	1.52	9.094	1.77
IV	2.7	540	112.914	20.91	19.818	3.67	8.536	1.60	10.670	2.00
V	2.7	540	114.642	21.23	19.818	3.67	9.603	1.80	13.071	2.45

Expt. No.	Rabbit wt. (kg.)	Dose (mg.)	*o*-Coumaroylglycine Free		Melilotic acid Free		Coumarin Free		Total		Total
									Free	Conjugated	
			(mg.)	(% of dose)	(mg.)	(% of dose)	(mg.)	(% of dose)	(% of dose)	(% of dose)	(% of dose)
I	2.8	560	83.311	11.02	46.549	8.23	5.881	1.18	43.47	6.30	49.77
II	2.6	520	76.237	10.86	41.281	7.86	3.193	0.69	41.07	5.76	46.83
III	2.6	520	72.657	10.35	42.541	8.10	3.702	0.80	39.73	6.50	46.23
IV	2.7	540	83.325	11.43	46.359	8.50	5.623	1.17	43.61	5.67	49.28
V	2.7	540	84.710	11.62	44.723	8.20	4.710	0.98	43.83	6.12	49.95

TABLE IV. Metabolic Products (%) of *o*-Coumaric Acid

Form	*o*-Coumaric acid	Umbelliferone	*o*-Coumaroylglycine	Melilotic acid	Coumarin	Total
Free	20.22	1.92	11.06	8.18	0.96	42.34
Conjugated	3.45	2.62	—	—	—	6.07
Total	23.67	4.54	11.06	8.18	0.96	48.41

TABLE V. Conjugation of *o*-Coumaric Acid

Expt. No.	Glucuronide		Ethereal sulfate		Total
	Glucuronic acid excreted (mg.)	Monohydroxycoumarin equivalent to glucuronic acid (% of dose)	(mg. SO_3)	(% of dose)	(% of dose)
I	29.209	4.41	0	0	6.30
II	13.469	2.19	7.70	3	5.76
III	41.882	6.81	0	0	6.50
VI	34.104	5.34	5.33	2	5.67
V	32.699	5.12	0	0	6.12
Average	30.273	4.77	2.61	1	6.07

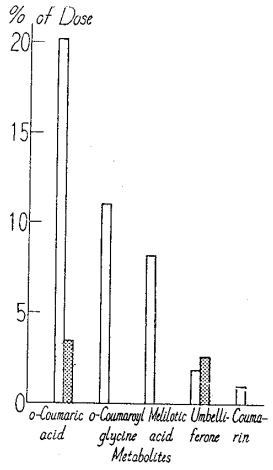

Fig. 2. Metabolic Products of *o*-Coumaric Acid

White: Free form
Shaded: Conjugated form

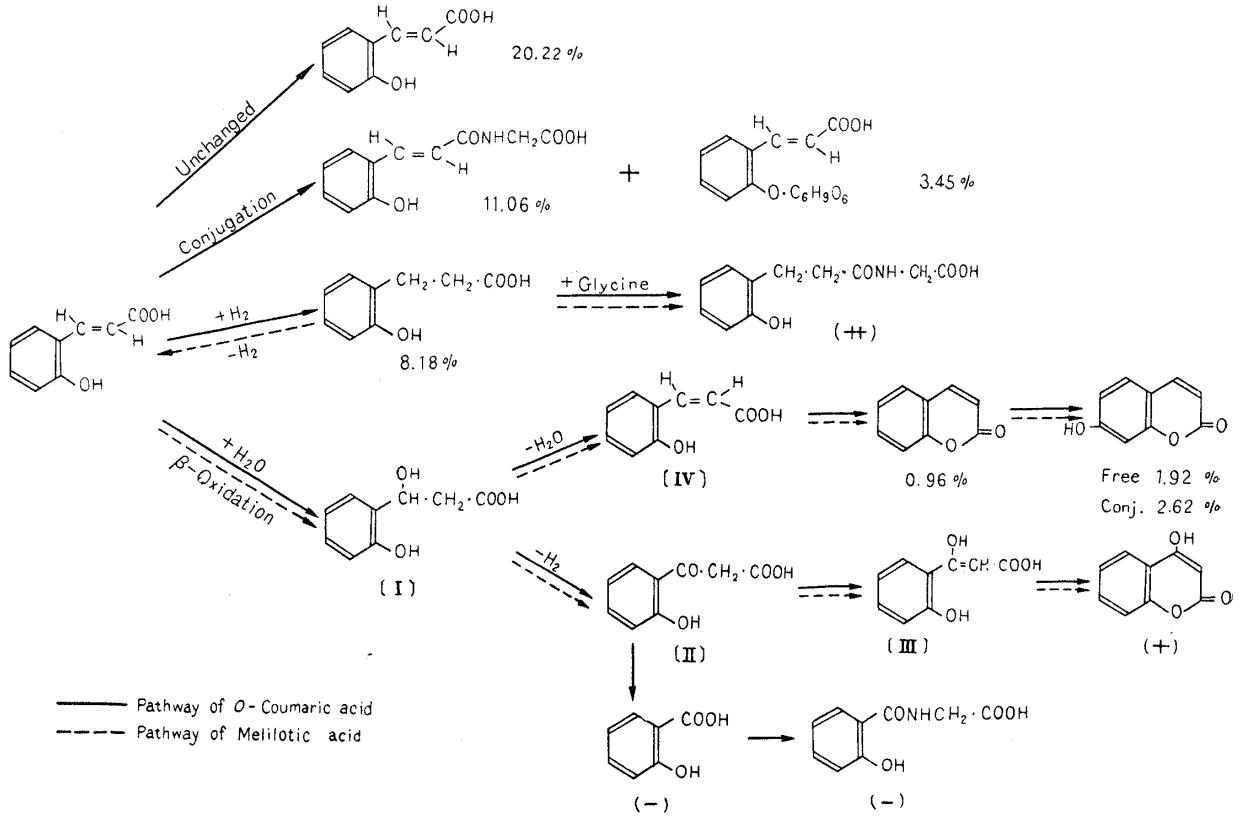

Chart 1.

Proposed Scheme for the Metabolic Transformation of *o*-Coumaric Acid and Melilotic Acid

Results and Discussion

The recovery of *o*-coumaric acid in urinary metabolites was 48.41% of the dose. *o*-Coumaric acid was excreted 20.22% as unchanged and 3.45% in conjugated form which was found only as a glucuronide.

The formation of glucuronide is not dependent on the pKa of the compound metabolized, whereas that of ethereal sulfate occurs when the pKa of the compound is in a range of 6~11. As the pKa of *o*-coumaric acid is 4.6~4.7, the conjugation of *o*-coumaric acid with glucuronic acid is therefore explicable.

Since the glycine conjugation is known to occur in carboxylic acids with pKa 2~5, it would be reasonable that *o*-coumaric acid was excreted in conjugation with glycine (yield, 11.06%).

Furthermore, *o*-coumaric acid was reduced biologically to give a considerable amount of melilotic acid as a urinary metabolite, which was not found by Williams, *et al.*

It is notable that umbelliferone, 4-hydroxycoumarin, and coumarin were found in the urinary metabolites. These compounds would be formed by the pathway shown in Chart 1. *o*-Coumaric acid is hydrated to give 3-*o*-hydroxyphenyl-3-hydroxypropionic acid (I). By dehydrogenation of (I), 3-*o*-hydroxyphenyl-3-oxopropionic acid (II) is formed, whose enol form (III) is cyclized to give 4-hydroxycoumarin. The compound (II) is not oxidized further to give salicylic acid. On the other hand, the formation of umbelliferone could be explained by assuming that coumarin is synthesized via *o*-coumarinic acid (IV) and finally hydroxylated to hydroxycoumarin.

3- and 8-Hydroxycoumarins were found in the urine after coumarin dosing, whereas 5- and 6-hydroxycoumarins were not detected at all even by a careful examination using paper chromatography.

Dakin[3] found that the main product of cinnamic acid metabolism in the dog is hippuric acid. Booth and his co-workers[4] reported that when rats were fed with caffeic acid, *m*-hydroxyphenylpropionic acid was detected as the major metabolite, whereas *m*-hydroxyhippuric acid was the metabolic product in man. It is thus assumed that a species difference of matabolites as described above may depend on the difference of enzymatic system in metabolic pathway. The ratio of total and conjugated metabolites was 2:1 which was reverse of the case in administration of coumarin.

The metabolic fate of melilotic acid was also studied. Melilotoyl- and *o*-coumaroylglycine, *o*-coumaric acid, umbelliferone, and 4-hydroxycoumarin were detected in the urinary metabolites by means of paper chromatography. Therefore, the metabolic pathway of melilotic acid seemed to be the same as observed with *o*-coumaric acid.

Summary

A study on the metabolic fate of *o*-coumaric acid and melilotic acid has been carried out. The urinary metabolites after *o*-coumaric acid dosing were determined to consist of unchanged *o*-coumaric acid (20.22% of dose), *o*-coumaric acid glucuronide (3.45%), *o*-coumaroylglycine (11.06%), melilotic acid (8.18%), umbelliferone (1.92%), umbelliferone glucuronide (2.62%), and coumarin (0.96%).

Using paper chromatography, the presence of 4-hydroxycoumarin and melilotoylglycine was detected, but 3-hydroxycoumarin was not found.

Coumarin and umbelliferone were found in the urine after *o*-coumaric acid dosing. A *cis-trans* transformation might be involved in the metabolic process.

On dosing melilotic acid, unchanged melilotic acid, *o*-coumaric acid, melilotoylglycine, *o*-coumaroylglycine, umbelliferone, 4-hydroxycoumarin, coumarin, and dihydrocoumarin were detected in the urine.

Biochemical transformations of *o*-coumaric and melilotic acids as well as that of coumarin and dihydrocoumarin were discussed.

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3) H. D. Dakin: J. Biol. Chem., **6**, 203(1909).
4) A. N. Booth, O. H. Emerson, F. T. Jones, F. DeEds: *Ibid.*, **229**, 51(1957).