poured into ice water containing 4.0 cc. of conc. HCl. The separated crystals were collected, washed with water, and dissolved in 50 cc. of $CHCl_3$. The $CHCl_3$ solution was washed with dil. $NaHCO_3$ solution and water, dried over $MgSO_4$, and the solvent was removed. The residue was recrystallized from AcOEt-$CHCl_3$ to 221 mg. of colorless plates, m.p. 241~242°. *Anal.* Calcd. for $C_{27}H_{38}O_7$ (Diacetylgitoxigenin): C, 68.33; H, 8.07; $COCH_3$, 18.14. Found: C, 68.30; H, 8.02; $COCH_3$, 17.76. $[\alpha]_D^{17}$ −6° (c=1.0%, $CHCl_3$), λ_{max}^{EtOH} 216 mμ (log ε 4.14).

Summary

Rhodexin-B, a cardiac glycoside of *Rhodea japonica* ROTH., was subjected to the Mannich hydrolysis and oleandrigenin (16-acetylgitoxigenin) was obtained as the main product, together with gitoxigenin, dianhydrogitoxigenin, and rhamnose. From these results the existing formulation for the structure of rhodexin-B and -C were corrected to 16-acetylgitoxigenin rhamnoside (II) and 16-acetylgitoxigenin rhamnosido-glucoside (III), respectively. This report constitutes the first example of the Mannich hydrolysis of the glycosides of oleandrigenin linking with a 2-hydroxy sugar.

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101 Mitiiti Fujita and Tsutomu Furuya: Studies on the Metabolism of Naturally Occurring Coumarins. I. Separation and Identification of Coumarin Derivatives by Paper Chromatography.

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Studies on the metabolism of drugs are usefully applied for finding a drug which is stable in human body keeping its direct effectivity. As a general method for such studies, several workers have carried out chemical investigation on metabolic products excreted in urine after administration of a certain drug in animals. The knowledge induced from the studies concerning the fate of a drug in body and the maintenance period of effectiveness would play an important part in improvement of medicaments.

A number of metabolic studies on chemicals have been reported, whereas constituents of crude drugs has not been sufficiently studied.

The present authors commenced studies on the metabolism of coumarin derivatives which frequently occur in plants to examine their mode of action and the mechanism of detoxication. Difficulties of this study arise from the coëxistence of similar constituents in plants and the poor recovery of excreted product. In such a case paper chromatography was usefully applied to analyse the excreted substances.

As a first step in this study, separation and identification of more than 30 kinds of natural and synthetic coumarins were studied by paper chromatography.

The following works have already been published to deal with the paper chromatography of coumarins:

Swendsen[1] separated and identified coumarins from *Pimpinella saxifraga* L., *P. magna* L., and *Angelica* spp. using petroleum ether-benzene-methanol (25 : 20 : 10) as a developing solvent system, but the result was not so satisfactory with variable Rf values.

Riedl and Neugebauer[2] employed the filter paper impregnated with ethylene glycol or propylene glycol, and benzine as a mobile phase. Although this system gives fairly good result for the separation of furocoumarins, it is not so easy to get a uniformly saturated

* Hongo, Tokyo (藤田路一, 古谷 力).

1) A. B. Swendsen: Pharm. Acta Helv., **27,** 44(1952).
2) K. Riedl, L. Neugebauer: Monatsh., **83,** 1083(1952).

stationary phase on the filter paper and to keep the treated filter paper under a constant state.

Mitchell[3] reported that coumarin and dihydrocoumarin were separated paper chromatographically using a mixture of dimethylcyanamide and formamide as a stationary phase and petroleum ether as a mobile phase.

A buffered filter paper was first employed by Swain[4] for the separation of various coumarin derivatives and then by Reppel[5] for hydroxycoumarin derivatives.

The present authors examined various solvent systems for the paper chromatography of mono-, di-, and tri-hydroxycoumarins and their closely related cinnamic acid derivatives to obtain satisfactory results.

The multibuffered paper chromatography which had chiefly been used for the separation of alkaloids[6] was also applied for hydroxycoumarins.

The authors wish to thank Prof. S. Shibata of this laboratory for his kind advice. They are also indebted to Prof. Ty. Ukita of this Faculty and Mr. T. Nakabayashi of Kochi University for their kind supply of valuable samples.

Experimental

Materials—The following hydroxycoumarin derivatives were prepared for the present study: 3-Hydroxy-[7] (m.p. 153°), 4-hydroxy-(m. p. 206°),[8] 5-hydroxy-(m. p. 228°),[9] 6-hydroxy-(m.p. 250~252°),[10] 7-hydroxy-(umbelliferone) (m. p. 230~231°),[11] 8-hydroxy-(m. p. 159~160°),[12] 4, 7-dihydroxy-(m. p. 265°),[13] 5,7-dihydroxy-(m.p. 272~275° (decomp.)),[14] dihydrocoumarin ($b.p_{10}$140~146°),[15] *o*-coumaric acid (m.p. 208~209°),[16] melilotic acid (m. p. 80°),[17] herniarin (m.p. 117~118°),[18] umbellic acid (m. p. 216° (decomp.)),[19] and umbellic acid 4-methyl ether (m. p. 180~185° (decomp.)).[20] Other materials used were those kept in this laboratory. All the compounds were recrystallized, if necessary, to get chromatographically pure samples.

The EtOH solution of the samples was prepared for paper chromatography. A glass chamber (31×22×50 cm.) with a glass lid was used for developing paper chromatography of 35 sheets of paper (Toyo Roshi No. 51, size 2×40 cm.).

Solvent System—In the case of two-phase solvent systems, the organic solvent layer was used as the mobile phase.

i) *n*-BuOH-benzene-$(NH_4)_2CO_3$ buffer (80 : 5 : 15); ii) H_2O-saturated *n*-BuOH; iii) *n*-BuOH-AcOH-H_2O (4 : 1 : 5); iv) $CHCl_3$-AcOH-H_2O (2 : 1 : 1); v) benzene-AcOH-H_2O (2 : 2 : 1); vi) EtOH-NH_4OH-H_2O (80 : 4 : 16); vii) 20% KCl and viii) 2% AcOH.

Procedure—All the compounds indicated above were developed by the ascending method, using umbelliferone as a standard substance. The temperature was kept at 18°±1°, and the time of development as given in Table I.

Detection of Spots—i) Fluorescence: In almost all cases, the position of spots on the developed paper was revealed by their fluorescence observed under ultraviolet light. In some cases where the fluorescence was not observed at all or scarcely observed, 2*N* NaOH solution was sprayed to intensify the fluorescence.

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19) E. Posen: *Ibid.*, **14,** 2745(1881).
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21) M. Fujita, T. Furuya, H. Itokawa: Yakugaku Zasshi, **78,** 395(1958).

TABLE I. Rf Values and Color Reactions of Coumarin and Related Compounds

Compound	Solvent system								UV-Fluorescence		Emerson's reagent	Diazotized sulfanilic acid
	A	B	C	D	E	F	G	H	Untreated	2N NaOH		
Coumarin	0.86	0.83	0.89	0.97	0.96	0.85	0.43[a]	0.66	—	GY	— (RO)	— (BrO)
Dihydrocoumarin	0.90	0.88	0.90	0.97	0.96	0.86[a]	0.60[a]	0.76[a]	—	—	— (RO)	— (BrO)
3-Hydroxycoumarin	0.44	0.81	0.85	0.95	0.89	0.64	0.42[a]	0.46[a]	—	fB	—	O
4-Hydroxycoumarin	0.38	0.84	0.90	0.85	0.44	0.75	0.40	0.25[a]	—	—	V	Y
5-Hydroxycoumarin	0.62	0.85	0.89	0.84	0.30	0.67	0.42	0.53	—	—[b]	RV	lO
6-Hydroxycoumarin	0.75	0.82	0.83	0.74	0.30	0.73	0.38[a]	0.44[a]	fB	—[b]	RV	BrR
7-Hydroxycoumarin (umbelliferone)	0.55	0.83	0.88	0.77	0.31	0.72	0.37	0.46	bB	bB	—	lY
8-Hydroxycoumarin	0.58	0.83	0.87	0.95	0	0.64	0.26	0.52	—	—	RV	lO
4,7-Dihydroxycoumarin	0.10	0.58	0.88	0.04	0.01	0.43	0.18	0.29	fB	V	V	BrY
5,7-Dihydroxycoumarin	0.24	0.85	0.88	0.06	0.01	0.48	0.17	0.26	B	—[b]	V	BrY
6,7-Dihydroxycoumarin (esculetin)	0.20[a]	0.54[a]	0.79	0.14	0.02	0.50[a]	0.21[a]	0.30	B	GY	Br	—
7,8-Dihydroxycoumarin (daphnetin)	0.21[a]	0.74	0.79	0.34	0.17	0.45[a]	0.33	0.42	—	—[b]	Br	lO
4,5,7-Trihydroxycoumarin	0.18	0.27[a]	0.68	0.02	0	0.49	0.08	0.25	lB	B	V	YO
7-Methoxycoumarin (herniarin)	0.81	0.85	0.89	0.99	0.96	0.85	0.43[a]	0.51[a]	V	V	— (RV)	— (O)
4-Hydroxy-7-methoxycoumarin	0.35	0.80	0.89	0.78	0.89	0.73	0.20	0	—	fB	V	Y
5,7-Dimethoxycoumarin (citropten)	0.84	0.87	0.88	0.98	0.96	0.78	0.29[a]	0.39[a]	B	B	— (RO)	— (BrY)
6-Hydroxy-7-methoxycoumarin (esculetin 7-methyl ether)	0.62	0.73	0.77	0.95	0.78	0.60	0.32	0.34	fB	—[b]	P	P
6-Methoxy-7-hydroxycoumarin (scopoletin)	0.34	0.78	0.78	0.92	0.40	0.68	0.24[a]	0.31[a]	bB	bB	V	RV
6,7-Dimethoxycoumarin (dimethylesculetin)	0.77	0.78	0.80	0.97	0.90	0.75	0.39[a]	0.49[a]	B	B	— (RV)	— (O)
5,7-Dimethoxy-6-hydroxycoumarin (fraxinol)	0.73	0.80	0.81	0.94	0.80	0.63	0.38[a]	0.53[a]	fB	—	RV	Y
7,8-Dihydroxy-6-methoxycoumarin (fraxetin)	0.07	0.63	0.68	0.36	0.07	0.37[a]	0.17	0.32	fB	BG	Br	Y
Esculin	0.04	0.43	0.59	0	0	0.45	0.58	0.61	B	bB	—	—
Daphnin	0.03	0.34	0.53	0	0	0.31	0.68	0.71	—	—[b]	RV	RO
Fraxin	0.04	0.32	0.43	0.03	0	0.36	0.63	0.66	GB	B	V	V
o-Coumaric acid	0.23	0.83	0.91	0.67[a]	0.52	0.54	0.44[a]	0.51[a]	fB	GY	RV	O
Melilotic acid	0.37	0.85	0.88	0.86	0.63	0.74	0.17	0.79	—	—	RV	O
Umbellic acid	0.07[a]	0.73[a]	0.83	0.03	0	0.35[a]	0.25[a]	0.24[a]	B	bB	RV	O
Umbellic acid 4-methyl ether	0.15	0.79	0.91	0.62[a]	0.47[a]	0.42	0.16[a]	0.22	fB	B	RV	O
Salicylic acid	0.34	0.84	0.90	0.95	0.93	0.68	0.63	0.67	V	V	RV	lY
Gentisic acid	0.25	0.78	0.83	0.24	0.13	0.54	0.50[a]	0.62[a]	B	GY	lV	—
Time taken for solvent front to reach 30 cm. (hr.)	18	18	18	8	5.30	9	3.30	3				

a) tailing spot

A : BuOH-benzene-$(NH_4)_2CO_3$ buffer (80 : 5 : 15)
B : Water-saturated BuOH
C : BuOH-AcOH-H_2O (4 : 1 : 5)
D : $CHCl_3$-AcOH-H_2O (2 : 1 : 1)
E : Benzene-AcOH-H_2O (2 : 2 : 1)
F : EtOH-NH_4OH-H_2O (80 : 4 : 16)
G : 20% KCl
H : 2% AcOH

f=faint, b=bright, l=light,
B=blue, G=green, V=violet,
Y=yellow, Br=brown, R=red
O=orange, P=pink, —=colorless
( )=color produced by cleavage of lactone ring
b) Brownish yellow in visible light

ii) Emerson's reaction.[21] 0.5% Na_2CO_3, 0.9% 4-aminoantipyrine, and 5.4% $K_3Fe(CN)_6$ solution were sprayed successively.

iii) Diazotized sulfanilic acid was also used as a reagent for phenolic compounds.

The color reaction of these reagents developed on the paper chromatogram is indicated in Table I.

Multibuffered Paper Chromatography—Atkins and Pantin buffers, ranging from pH 7.4 to pH 11.0, was applied to the filter paper (Toyo Roshi No. 51, 2×40 cm.) within the 2-cm. zone by dipping a glass rod. Each buffered zone was followed by unbuffered zone, 1 cm. wide. pH Zones on the paper started with pH 7.4 and increased in pH at intervals of 0.2. The paper treated as above was then developed by

21) M. Fujita, T. Furuya, H. Itokawa: Yakugaku Zasshi, **78**, 395(1958).

descending method in a glass chamber of Toyo Roshi Co., Model C, using ether as a developing solvent for almost all the samples except dihydroxycoumarins for which BuOH was used (cf. Table II).

The spots on developed paper were detected as mentioned above.

TABLE II. pH Zone stopping Coumarin and Related Compounds on Multibuffered Paper

Compound	pH zone	Compound	pH zone
3-Hydroxycoumarin	10.6	Coumarin	>11
4-Hydroxycoumarin	—	Dihydrocoumarin	>11
5-Hydroxycoumarin	10.8	7-Methoxycoumarin (herniarin)	>11
6-Hydroxycoumarin	11.0	6-Hydroxy-7-methoxycoumarin (esculetin 7-methyl ether)	>11
7-Hydroxycoumarin (umbelliferone)	10.6	6-Methoxy-7-hydroxycoumarin (scopoletin)	8.8
8-Hydroxycoumarin	10.8	6, 7-Dimethoxycoumarin (dimethylesculetin)	>11
4, 7-Dihydroxycoumarin	7.4	*o*-Coumaric acid	8.4
5, 7-Dihydroxycoumarin	8.4	Melilotic acid	8.4
6, 7-Dihydroxycoumarin (esculetin)	7.4	Umbellic acid	7.8
7, 8-Dihydroxycoumarin (daphnetin)	7.6	Umbellic acid 4-methyl ether	8.2
4, 5, 7-Trihydroxycoumarin	—	Salicylic acid	9.0

—: not developed

Results and Discussion

The Rf values of coumarins and their related compounds obtained by the present study using various solvent systems are shown in Table I. The Rf values were taken from the average of five experimental results.

The two-phase solvent systems resulted in round or slightly elliptical spots while the one-phase solvent systems frequently gave tailing spots, especially in the case of separation of methoxycoumarins and phenolic acids.

The acidic solvents, in particular that contain butanol, showed higher Rf values. Using butanol-acetic acid-water, chloroform-acetic acid-water, and benzene-acetic acid-water, the mono- and dihydroxycoumarins were separated, giving well-defined spots.

A successful separation of phenolic acids, and glycosides and their aglycones resulted by using chloroform-acetic acid-water as the solvent. 2% Acetic acid, however, gave no agreeable result, producing tailing spots.

Although most of the neutral solvent systems so far used in the present study frequently gave tailing spots, water-saturated butanol was applied successfully for the separation of dihydroxycoumarins, and coumarin glycosides and their aglycones. In this system, contrary to the two-phase the glycosides showed higher Rf value than that of the corresponding aglycones (Fig. 1).

The alkaline solvent system gave satisfactory result for the paper chromatographical separation of coumarins in general, particularly butanol-benzene-ammonium carbonate buffer, which was most usefully applied for monohydroxy- and methoxy-coumarins giving well-defined spot without tailing. This solvent system was also used for quantitative determination of coumarins.

The Relationship between Structure and Rf-Value of Coumarins—On examination of the present paper-chromatographical study, noticeable relationship between Rf values and the structures of coumarin derivatives was found, which might provide appropriate evidence for the analysis of excreted metabolic products of coumarins.

When the main constituent of the acidic solvent system is more lipophilic, as in order of butanol, chloroform, and benzene, the Rf value of mono- and dihydroxycoumarins with the solvent shows much lower value (Fig. 2). Coumarin itself which has no substituent and methoxycoumarins are not affected by the variation of lipophilic constituent of the developing solvent system (Fig. 3).

Fig. 1. One-phase Solvent System containing Water

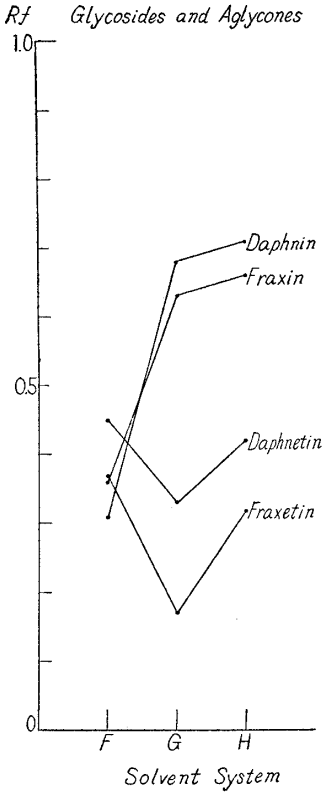
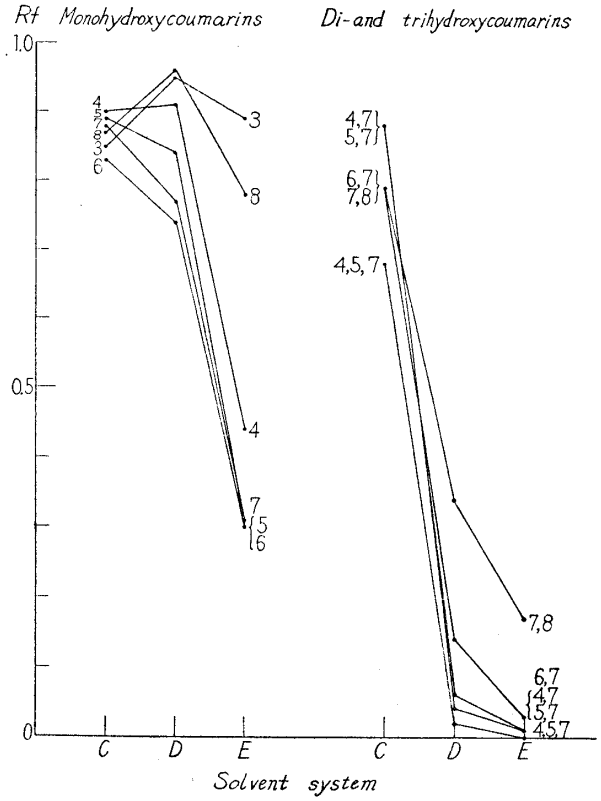


Fig. 2. Acidic Solvents

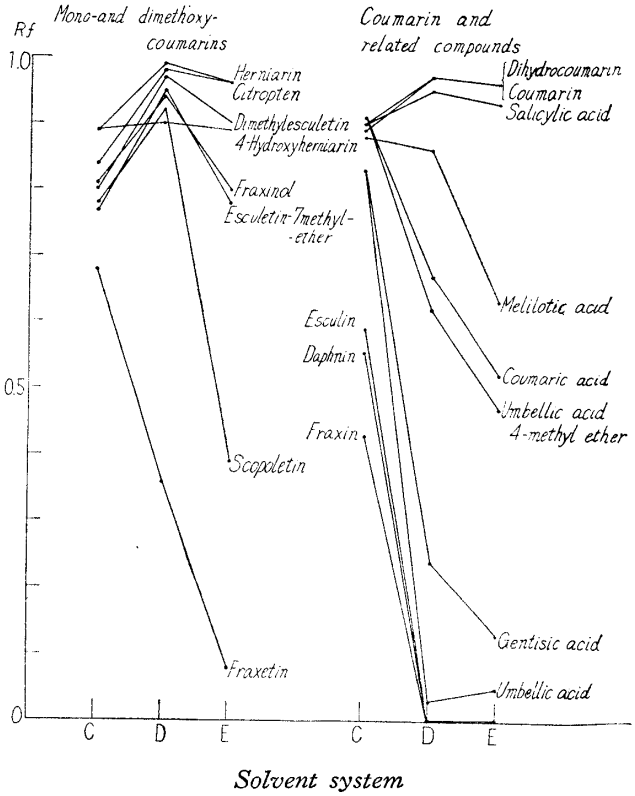


In regard to the coumarin derivatives which possess both hydroxy and methoxyl groupings in the same molecule, the effect of lipophilic constituent in the developing solvent system is not so essential in the 6-hydroxy compounds, such as esculetin 7-methyl ether, and is markedly observed in the 7-hydroxy compounds, such as scopoletin and fraxetin.

The Rf values of phenolic acids given with the solvent system of (a) chloroform-acetic acid-water or (b) benzene-acetic acid-water depend on the solubilities of the samples in the developing solvents as shown below (Fig. 3):

Umbellic acid (Rf: (a) 0.03; (b) 0.00), gentisic acid (Rf: (a) 0.24; (b) 0.13), umbellic acid 4-methyl ether (Rf: (a) 0.62; (b) 0.47), *o*-coumaric acid (Rf: (a) 0.67; (b) 0.52), and salicylic acid (Rf: (a) 0.95; (b) 0.93).

Fig. 3. Acidic Solvents



The Rf values of mono- and dihydroxycoumarins were examined using acidic, neutral, and alkaline two-phase solvent systems which contain butanol as the main

constituent. These compounds show marked decrease of Rf values as the reaction of the developing solvent changes from acidic to neutral and alkaline (Figs. 4, 5). This fact would be explicable by the salt formation of hydroxycoumarins in alkaline solvent.

The alkaline solvent system is suitable for the separation of monohydroxycoumarins whose Rf values rise as the acidity of the hydroxyl decreases: 4-Hydroxy- (Rf 0.38), 3-hydroxy- (Rf 0.44), and 6-hydroxycoumarins (Rf 0.75).

The alkaline developing solvent also gave a well-defined chromatogram of esculetin derivatives whose Rf values decrease in the order shown below: Dimethylesculetin (0.77), esculetin 7-methyl ether (0.62), scopoletin (0.34), esculetin (0.20), esculin (0.04).

The relative increase of Rf value of scopoletin in comparison with esculetin 7-methyl ether was almost to the same extent as observed in umbelliferone and 6-hydroxycoumarin. Therefore, it appears that the presence of methoxyl in the *ortho* position of hydroxyl group gives no marked effect on the Rf values.

As for multibuffered paper chromatography, 4-hydroxy- and 4, 5, 7-trihydroxycoumarins failed to develop with commonly used solvents (Table II).

This method was also not so suitable for separation of individual compound of mono- and dihydroxycoumarins, while it was successfully applied in the separation of monohydroxy- and dihydroxycoumarin groups.

The attempt to separate coumarin and dihydrocoumarin, and *o*-coumaric and melilotic acids failed when using multibuffered chromatography, but it gave a quite agreeable result in the separation of esculetin, scopoletin, and esculetin 7-methyl ether.

Fig. 4. Solvents containing Butanol

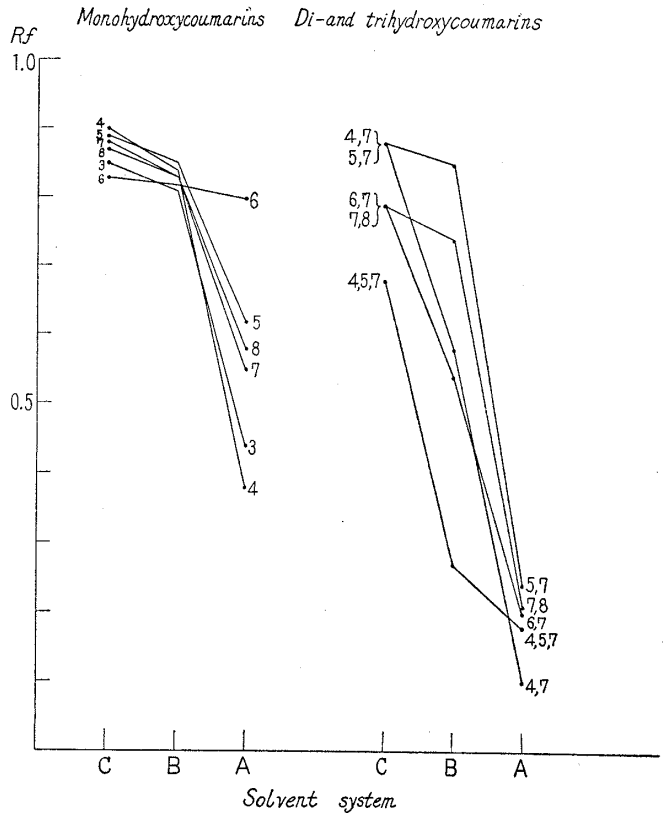
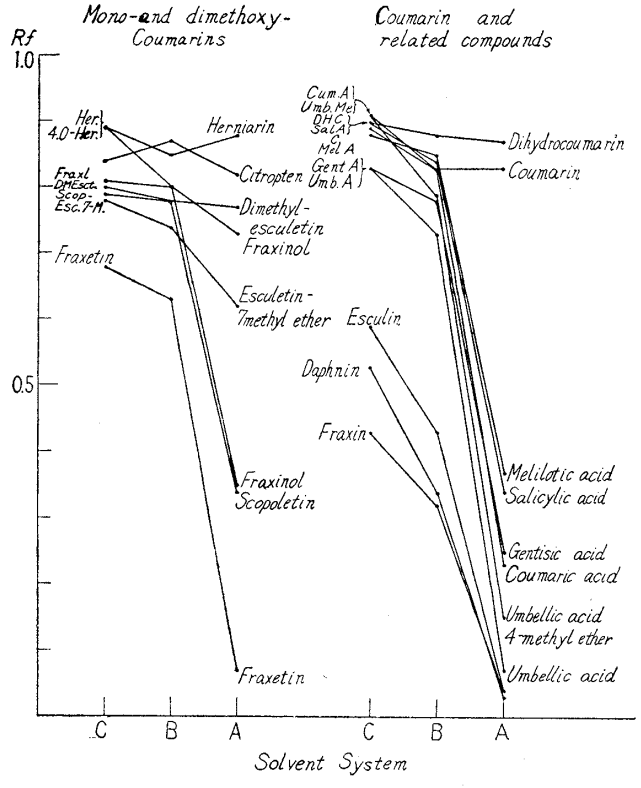


Fig. 5. Solvents containing Butanol



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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