REFERENCES

Blix, G. (1936). *Hoppe-Seyl. Z.* **240**, 43.

Blix, G., Gottschalk, A. & Klenk, E. (1957). *Nature, Lond.*, **179**, 1088.

Blix, G., Lindberg, E., Odin, L. & Werner, I. (1956). *Acta Soc. Med. Upsalien.* **61**, 1.

Blix, G. & Odin, L. (1955). *Acta chem. scand.* **9**, 1541.

Böhm, P. & Baumeister, L. (1956). *Hoppe-Seyl. Z.* **305**, 42.

Böhm, P., Ross, J. & Baumeister, L. (1957). *Hoppe-Seyl. Z.* **307**, 284.

Cornforth, J. W., Daines, M. E. & Gottschalk, A. (1957). *Proc. chem. Soc., Lond.* p. 25.

Gottschalk, A. (1951). *Nature, Lond.*, **167**, 845.

Gottschalk, A. (1953). *Nature, Lond.*, **172**, 808.

Gottschalk, A. (1954). *Nature, Lond.*, **174**, 652.

Gottschalk, A. (1955a). *Biochem. J.* **61**, 298.

Gottschalk, A. (1955b). *Nature, Lond.*, **176**, 881.

Gottschalk, A. (1956). *Yale J. Biol. Med.* **28**, 525.

Gottschalk, A. (1957a). *Biochim. biophys. Acta*, **23**, 645.

Gottschalk, A. (1957b). *Arch. Biochem. Biophys.* **69**, 37.

Heidelberger, C. (1953). *Biochem. Prep.* **3**, 59.

Heimer, R. & Meyer, K. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 733.

Klenk, E. (1941). *Hoppe-Seyl. Z.* **268**, 50.

Klenk, E. & Faillard, H. (1954). *Hoppe-Seyl. Z.* **298**, 230.

Klenk, E. & Faillard, H. (1955). *Hoppe-Seyl. Z.* **299**, 191.

Klenk, E. & Faillard, H. (1957). *Dtsch. Z. Verdau.-u. Stoffwechselkr.* **17**, 51.

Klenk, E., Faillard, H. & Lempfrid, H. (1955). *Hoppe-Seyl. Z.* **301**, 235.

Klenk, E. & Lempfrid, H. (1957). *Hoppe-Seyl. Z.* **307**, 278.

Klenk, E. & Stoffel, W. (1956). *Hoppe-Seyl. Z.* **303**, 78.

Klenk, E. & Uhlenbruck, G. (1956). *Hoppe-Seyl. Z.* **305**, 224.

Klenk, E. & Uhlenbruck, G. (1957). *Hoppe-Seyl. Z.* **307**, 266.

Kuhn, R. & Brossmer, R. (1956a). *Chem. Ber.* **89**, 2013.

Kuhn, R. & Brossmer, R. (1956b). *Chem. Ber.* **89**, 2471.

Kuhn, R. & Brossmer, R. (1957). *Angew. Chem.* **69**, 534.

Martinsson, A., Raal, A. & Svennerholm, L. (1957). *Biochim. biophys. Acta*, **23**, 652.

Odin, L. (1955a). *Acta chem. scand.* **9**, 714.

Odin, L. (1955b). *Acta chem. scand.* **9**, 862.

Odin, L. (1955c). *Acta chem. scand.* **9**, 1235.

Svennerholm, L. (1956). *Acta Soc. Med. Upsalien.* **61**, 75.

Svennerholm, L. (1957). *Ark. Kemi*, **10**, 577.

Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsalien.* **57**, 230.

White, T. (1940). *J. chem. Soc.* p. 428.

Yamakawa, T. & Suzuki, S. (1951). *J. Biochem., Tokyo*, **38**, 199.

Yamakawa, T. & Suzuki, S. (1952). *J. Biochem., Tokyo*, **39**, 175.

Yamakawa, T. & Suzuki, S. (1955). *J. Biochem., Tokyo*, **42**, 727.

Zilliken, F., Braun, G. A. & György, P. (1955). *Arch. Biochem. Biophys.* **54**, 564.

Zilliken, F. & Glick, M. C. (1956). *Naturwissenschaften*, **43**, 536.

Studies in Detoxication

71. THE METABOLISM OF HYDROXYCOUMARINS*

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As a preliminary to the study of the fate of coumarin in animals (Mead, Smith & Williams, 1958), it was necessary to study the metabolic behaviour of the six isomeric hydroxycoumarins and to prepare either synthetically or biosynthetically their glucuronides and ethereal sulphates, since any of these compounds could be metabolites of coumarin.

The glucuronides of 3-, 4- and 7-hydroxycoumarin have been previously described (Flatow, 1910; Roseman, Heubner, Pancratz & Link, 1954; Mead, Smith & Williams, 1955), and Sieberg (1921) has reported that umbelliferone (7-hydroxy-), aesculetin (6:7-dihydroxy-) and daphnetin (7:8-dihydroxy-coumarin) after injection into rabbits were excreted in conjugated forms which were not described.

* Part 70: McIsaac & Williams (1957).

MATERIALS AND METHODS

Reference compounds. The following hydroxycoumarins were prepared: 3-, m.p. 153° (Linch, 1912), 4-, m.p. 206° (Boyd & Robertson, 1948), 5-, m.p. 228° (Adams & Bockstahler, 1952), 6-, m.p. 250–252° after recrystallization from dioxan (Bargellini & Monti, 1915) and 8-hydroxycoumarin, m.p. 159–160° (Cingolani, 1954).

Ethereal sulphates of hydroxycoumarins. Potassium salts of the hitherto undescribed ethereal sulphates of 3-, 5- and 8-hydroxycoumarins were synthesized by the general method of Burkhardt & Lapworth (1926) (see Table 1). The sulphate of 6-hydroxycoumarin was prepared in poor yield by the Elb's persulphate oxidation of coumarin. Coumarin (73 g.) and KOH (128 g.) were dissolved in 2 l. of water containing $FeSO_4,7H_2O$ (2 g.). Potassium persulphate (132 g.) was added over 8 hr. to the stirred solution kept below 20°. The solution was then brought to pH 2 with conc. H_2SO_4 and extracted twice with ether to remove coumarin. At this stage a large precipitate, mainly K_2SO_4,

Table 1. *Properties of the ethereal sulphates of the hydroxycoumarins**

The ethereal sulphate of 4-hydroxycoumarin could not be made by the method of Burkhardt & Lapworth (1926).

Potassium	Analysis found (%)	
	K	S
2-Oxo-1:2-benzopyran-3-yl sulphate† (plates from aq. ethanol)	14·0	11·5
2-Oxo-1:2-benzopyran-5-yl sulphate† (needles from aq. 50% ethanol)	14·1	11·3
2-Oxo-1:2-benzopyran-6-yl sulphate‡ (plates from aq. 80% ethanol)	13·8	11·2
2-Oxo-1:2-benzopyran-8-yl sulphate† (prisms from water)	13·9	11·4
($C_9H_5O_6SK$ requires	13·9	11·4)

* Potassium 2-oxo-1:2-benzopyran-7-yl sulphate was described by Mead *et al.* (1955).
† Yields 80–85%, based on the weight of hydroxycoumarin taken.
‡ The light absorption of this compound in water was $\lambda\lambda_{max.}$ 273, 317 mμ with $\epsilon_{max.}$ 10 200 and 4800.

had formed. This was filtered off and extracted with 400 ml. of 66% (v/v) aq. acetone. Evaporation of the extract left a residue (3 g.) from which *potassium 2-oxo-1:2-benzopyran-6-yl sulphate* was obtained (see Table 1).

All these potassium salts were sparingly soluble in water and insoluble in ethanol; they did not melt below 300°. After a brief hydrolysis (1–5 min.) of these salts with boiling N-HCl the presence of the corresponding hydroxycoumarin could be demonstrated by paper chromatography. None of these sulphates in aqueous solution showed any fluorescence in u.v. light (360 mμ), but when exposed to u.v. light in N-NaOH they rapidly developed a blue–green or yellow–green fluorescence (photocatalytic effect of Feigl, Feigl & Goldstein, 1955).

The ethereal sulphates of 3-, 5-, 6-, 7- and 8-hydroxycoumarin and of 4-methyl-7-hydroxycoumarin were readily hydrolysed at pH 5·9 by an arylsulphatase preparation obtained from the gastric juice of Roman snails (*Helix pomatia*) (Jarrige & Henry, 1952).

Animals and dosing. Female chinchilla rabbits kept on a constant diet (see El Masry, Smith & Williams, 1956) were used and the coumarins were administered in aqueous suspension by stomach tube.

Analytical methods. Glucuronides in urine were determined by the naphtharesorcinol method as modified by Paul (1951). In this modification, 2 ml. of glucuronide solution containing 5–70 μg. of glucuronic acid was treated successively with 2 ml. of freshly prepared and filtered 0·35% (w/v) naphtharesorcinol in 0·1N-H_2SO_4, 3 ml. of 15N-H_2SO_4 and 0·2 ml. of 1% (w/v) aq. chloramine T, and the mixture heated in 6 in. × ⅝ in. test tubes for 2 hr. in a boiling-water bath. The tubes were then cooled in ice for 10 min. and extracted with 8 ml. of ethyl acetate. The ethyl acetate layer was removed, adjusted to 10 ml., the optical density measured at 565 mμ and compared with values of a standard curve prepared under the same conditions with glucurone.

Ethereal sulphates were measured by the method of Sperber (1948).

Paper chromatography and colour reactions

Chromatography was carried out as previously described (Smith, Smithies & Williams, 1953). The R_F values, in various solvents, of the compounds concerned are given in Table 2. The following procedures were used for detecting the compounds on paper (see Table 2).

Photocatalytic fluorescence. The paper was sprayed with N-NaOH and exposed to u.v. light (360 mμ). Coumarin derivatives with no free hydroxyl groups and no substituent in the 4-position developed an intense fluorescence within 1 min. of exposure to u.v. light (Feigl *et al.* 1955).

Gibbs's reagent. A spray of 0·1% ethanolic 2:6-dichloroquinone chloroimide was followed by a spray of saturated $NaHCO_3$. Phenols in which the *para*-position to the hydroxyl is unsubstituted usually give blue colours with this reagent.

Brentamine fast blue B salt. Papers were sprayed first with a 0·01% aq. solution of this salt (stabilized, tetrazotized di-*o*-anisidine) and then with 2N-Na_2CO_3. Phenolic compounds give blue or purple colours.

Fluorescence. Some coumarin derivatives fluoresce under u.v. light (360 or 270 mμ), and a few quench the background fluorescence of the paper when illuminated with light of wavelength 270 mμ and showed up as dark spots on a light-purple background.

Isolation of metabolites

Optical rotations are given in Table 5.

3-Hydroxycoumarin. Urine from rabbits fed with 3-hydroxycoumarin gave a strong naphtharesorcinol reaction and reduced Benedict's solution slightly on boiling. It gave, with $FeCl_3$ solution, a weak green colour which was increased on boiling the urine with dilute acid. No precipitate was formed with 2:4-dinitrophenylhydrazine and no salicylic acid was found.

The 24 hr. urine of rabbits which had received a total of 4·2 g. of 3-hydroxycoumarin was treated with saturated normal lead acetate as described by Kamil, Smith & Williams (1951). The normal lead acetate precipitate was found to contain a glucuronide. This precipitate was suspended in water and the lead removed with H_2S. After separation of the lead sulphide, the solution deposited crystals (0·56 g.) of 3-hydroxycoumarin glucuronide (2-oxo-1:2-benzopyran-3-yl glucosiduronic acid). After recrystallization from water, it had m.p. 207–208° (Found: C, 51·4; H, 4·2; H_2O, 2·4. Calc. for $C_{15}H_{14}O_9$,0·5H_2O, C, 51·8; H, 4·4; H_2O, 2·6%). Flatow (1910) records m.p. 207° and $[\alpha]_D$ − 72° in NaOH for this glucuronide. It did not reduce Benedict's reagent, and on hydrolysis with locust-crop liquor containing β-glucuronidase it yielded

Table 2. R_F values and colour reactions of coumarin and its derivatives

All runs were of 10 in. from the origin on Whatman no. 1 paper. Q means quenching of the background fluorescence of the paper when illuminated by light of wavelength 270 mμ and — indicates no colour reaction or fluorescence.

| Compound | Solvent system* | | | | Photocatalytic fluorescence | Gibb's reagent | Brentamine fast blue B salt | Colour of fluorescence excited by 360 mμ in NH₃ vapour |
	A	B	C	D				
Coumarin	0·94	0·87	0·94	0·9	Blue-green	—	—	—
3-Hydroxycoumarin	0·85	0·50	0·27	0·8	—	Weak green	Blue	—
4-Hydroxycoumarin	0·90	0·63	0·15	0·21	—	Weak blue	Red	Weak blue
5-Hydroxycoumarin	0·85	0·72	0·58	0·17	—	Blue	Purple	Weak yellow
6-Hydroxycoumarin	0·82	0·70	0·84	0·06	—	—	Weak brown	Weak yellow
7-Hydroxycoumarin (umbelliferone)	0·88	0·52	0·42	0·14	—	—	—	Blue
8-Hydroxycoumarin	0·85	0·60	0·56	0·56	—	Blue	Purple	Q
6:7-Dihydroxycoumarin (aesculetin)	0·76	0·20	0·16	0·00	—	Weak brown	Brown	Weak blue-green
o-Coumaric acid	0·90	0·30	0·05	0·26	—	Blue	Purple	Blue-green
Salicylic acid	0·90	0·85	0·32	0·85	—	Blue	Orange	Blue
Melilotic acid	0·90	0·52	0·15	0·41	—	Blue	Purple	—
Potassium 3-hydroxycoumarin sulphate	0·52	0·78	0·66	0	Blue-green	—	—	—
Potassium 5-hydroxycoumarin sulphate	0·55	0·78	0·56	0	Blue-green	—	—	—
Potassium 6-hydroxycoumarin sulphate	0·47	0·70	0·46	0	Yellow-green	—	—	—
Potassium 7-hydroxycoumarin sulphate	0·50	0·75	0·73	0	Blue-green	—	—	—
Potassium 8-hydroxycoumarin sulphate	0·44	0·67	0·50	0	Yellow-green	—	—	—
3-Hydroxycoumarin glucuronide	0·56	0·13	0·03	0	Blue-green	—	—	—
4-Hydroxycoumarin glucuronide	0·58	0·30	0·04	0	—	—	—	Q
5-Hydroxycoumarin glucuronide	0·56	0·30	0·04	0	Blue-green	—	—	—
6-Hydroxycoumarin glucuronide	0·44	0·22	0·03	0	Yellow-green	—	—	—
7-Hydroxycoumarin glucuronide	0·45	0·27	0·03	0	Blue-green	—	—	Weak blue
8-Hydroxycoumarin glucuronide	0·48	0·15	0·03	0	Yellow-green	—	—	—
o-Coumaric acid glucuronide	0·62	0·05	0·00	0	—	—	—	Q
o-Hydroxy-trans-cinnamoyl glycine	0·86	0·45	0·03	0·12	—	Blue	Purple	Yellow-green

* Solvent A is n-butanol–acetic acid–water (4:1:5); B is propan-1-ol–aq. NH₃ soln. (sp.gr. 0·88) (7:3); C is ethyl methyl ketone saturated with aq. 2N-NH₃ soln.; D is benzene–acetic acid–water (1:1:2) (all by vol.).

3-hydroxycoumarin, which was identified by its chromatographic behaviour on paper. Methylation with diazomethane, followed by acetylation with pyridine–acetic anhydride, yielded *methyl (2-oxo-1:2-benzopyran-3-yl tri-O-acetyl-β-D-glucosid)uronate* as colourless needles, m.p. 161–162°, from ethanol (Found: C, 54·9; H, 4·3. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6 %).

The basic lead acetate fraction of the urine was now prepared, and the lead-free filtrate was concentrated to 30 ml. On keeping this overnight, an ethereal sulphate crystallized out (0·3 g.). On hydrolysing this by boiling with 2N-HCl, free SO_4^{2-} ions and 3-hydroxycoumarin (m.p. and mixed m.p. 153°) were liberated.

4-*Hydroxycoumarin*. Urine from rabbits receiving 4-hydroxycoumarin gave a strong naphtharesorcinol reaction and reduced Benedict's reagent on prolonged boiling. No salicylic acid could be detected by paper chromatography in the urine before or after hydrolysis. The 24 hr. urine from rabbits which had received 9 g. of 4-hydroxycoumarin was fractionated by the lead acetate procedure, and a glucuronide was found in the basic lead acetate fraction. The lead was removed with H_2S and on concentration of this fraction to 100 ml. and keeping at 0° overnight the glucuronide crystallized (yield 1 g.). The mother liquor was concentrated *in vacuo* to a small volume and on careful addition of ethanol a further 0·5 g. of crystals was obtained. These crystals appeared to be a sparingly soluble salt of a glucuronide which was liberated from the salt (1·1 g.) by acidification with N-HCl. The glucuronide was identified as 2-oxo-1:2-benzopyran-4-yl glucosiduronic acid, which has previously been obtained by Roseman *et al.* (1954) from the urine of dogs given 4-hydroxycoumarin. The air-dried glucuronide appeared to be a mono-hydrate, m.p. 184–185° (Found: C, 51·1; H, 4·2; loss at 110° in 6 hr., 2·3. Calc. for $C_{15}H_{14}O_9,H_2O$, C, 50·6; H, 4·5; loss of 0·5H_2O, 2·5 %), which on drying at 110° over P_2O_5 *in vacuo* left a hemihydrate (Found: C, 51·6; H, 4·1. Calc. for $C_{15}H_{14}O_9,0·5H_2O$, C, 51·8; H, 4·3 %). The glucuronide (an enol glycoside) reduced Benedict's solution on prolonged boiling. On hydrolysis with locust-crop liquor glucuronidase, or for a short time with 5N-HCl, 4-hydroxycoumarin, identified by paper chromatography, was liberated. (For the absorption spectrum of the glucuronide see the following paper, Mead *et al.* 1958).

Methyl(2-oxo-1:2-benzopyran-4-yl tri-O-acetyl-β-D-glucosid)uronate, prepared with diazomethane and pyridine–acetic anhydride, formed colourless needles of the *hemihydrate* from aq. ethanol, m.p. 124–125° (Found: C, 54·0; H, 4·4; OMe, 6·5; H_2O, 1·7. Calc. for $C_{22}H_{22}O_{12},0·5H_2O$, C, 54·0; H, 4·7; OMe, 6·4; H_2O, 1·9 %). Roseman *et al.* (1954) give

m.p. 125–126° and $[\alpha]_D^{28} - 49·4°$ (c, 2 in $CHCl_3$) for this compound and describe it as a monohydrate.

5-*Hydroxycoumarin*. Urine from rabbits receiving 5-hydroxycoumarin gave a strong naphtharesorcinol reaction and did not reduce Benedict's solution. Some unchanged 5-hydroxycoumarin, together with its ethereal sulphate and glucuronide, were detected on paper chromatograms of the urine. No other metabolites were detected. The 24 hr. urine from rabbits which had received 5·7 g. of 5-hydroxycoumarin (850 ml.) was brought to pH 4–5 with acetic acid and 100 ml. of saturated lead acetate soln. was added. The amorphous precipitate was rapidly removed by centrifuging and the supernatant liquid allowed to stand at 0° for 0·5 hr., when a lead salt of the glucuronide deposited as a mass of woolly needles. These were filtered off, suspended in 750 ml. of water at 70–80° and the solution was saturated with H_2S. After removal of PbS, the solution was cooled to 0° and the crystals (2·4 g.) of 2-oxo-1:2-benzopyran-5-yl β-D-*glucosiduronic acid* were collected and recrystallized from water. It formed sheaves of needles, m.p. 202–203° (decomp.) (Found: C, 48·1; H, 4·8. $C_{15}H_{14}O_9,2H_2O$ requires C, 48·1; H, 4·8 %). It lost water on drying at 110° but the anhydrous compound was hygroscopic and rapidly regained the lost weight when exposed to air. Hydrolysis of the glucuronide with snail gastric-juice glucuronidase or with 5N-HCl liberated 5-hydroxycoumarin, which was identified by paper chromatography.

The triacetyl methyl ester of 5-hydroxycoumarin glucuronide could not be prepared successfully by methylation with diazomethane followed by acetylation with pyridine–acetic anhydride as described by Kamil *et al.* (1951). The glucuronide had to be acetylated first with acetic anhydride, with perchloric acid as catalyst, and then methylated with methyl iodide and silver oxide. The glucuronide (100 mg.) was dissolved in acetic anhydride (0·4 ml.) and one drop of 60 % aq. $HClO_4$ added. The reaction was immediate, with evolution of heat, and in 2 min. the triacetyl glucuronide separated. A few drops of water were added to decompose excess of acetic anhydride and then an equal volume of ethanol. The triacetyl acid (80 mg.) on recrystallization from water formed needles, m.p. 220–221° (decomp.). The dry acid was dissolved in 50 ml. of $CHCl_3$ and methyl iodide (1 ml.) and silver oxide (3 g.) were added. The mixture was kept for 1 hr., then filtered and concentrated to a glassy solid. The latter crystallized from ethanol to give needles (70 mg.) of *methyl (2-oxo-1:2-benzopyran-5-yl tri-O-acetyl-β-D-glucosid)uronate*, m.p. 158–159° (Found: C, 55·5; H, 4·9. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6 %).

6-*Hydroxycoumarin*. A total of 8 g. of 6-hydroxycoumarin was fed to four rabbits. The 24 hr. urine gave an intense naphtharesorcinol reaction and did

not reduce Benedict's solution. When acidified the urine showed a dull blue–green fluorescence in u.v. light (360 mμ) and when made alkaline with a few drops of aq. NH_3 soln. the fluorescence was an intense blue. Paper chromatography of the urine showed the presence of some 6-hydroxycoumarin and its ethereal sulphate, and a substance fluorescing a bright blue. The latter substance was probably a 6-conjugated aesculetin (6:7-dihydroxycoumarin), since the blue fluorescence disappeared on hydrolysing the urine, and aesculetin as well as 6-hydroxycoumarin was detected on paper chromatograms of the acid-hydrolysed urine.

The glucuronide fraction of the urine, obtained from the basic lead acetate precipitate in the usual way, was reduced to 50 ml. *in vacuo* and treated with 200 ml. of ethanol, and a gummy precipitate was formed and separated. The supernatant liquid was separated from the precipitate, concentrated to 50 ml. at 40° *in vacuo* and neutralized with solid $KHCO_3$, when plates of the potassium salt of the ethereal sulphate of 6-hydroxycoumarin were deposited (0·31 g.) and were recrystallized from water (Found: K, 13·9; S, 11·4. $C_9H_5O_6SK$ requires K, 13·9; S, 11·4%). Hydrolysis of the salt with 2 N-HCl gave a crystalline precipitate of 6-hydroxycoumarin, m.p. and mixed m.p. 225–226°. The u.v. spectrum ($\lambda_{max.}$ 273, 317 mμ; $\epsilon_{max.}$ 10 200, 4800 in water) and chromatographic behaviour of this sulphate were identical with a synthetic sample.

The above alcohol-insoluble gum was redissolved in a little water and treated with excess of ethanol, and a glucuronide separated which was eventually obtained as a dry white powder (3·5 g.). On recrystallization of the powder from a small amount of water, the ammonium salt of 6-hydroxycoumarin glucuronide was obtained as fine needles. The *ammonium (2-oxo-1:2-benzopyran-6-yl β-D-glucosid)-uronate* decomposed at 204–205° (Found: C, 49·3; H, 4·5; N, 3·8. $C_{15}H_{17}O_9N,0·5H_2O$ requires C, 49·45; H, 5·0; N, 3·8%). The NH_4^+ ion was detected by Nessler's reagent, and the salt yielded 6-hydroxycoumarin (detected chromatographically) when incubated with β-glucuronidase (snail gastric juice) at pH 4·6. The free glucosiduronic acid was not readily obtainable in a crystalline form, and the NH_4^+ ion present in the salt isolated was probably derived from the aq. NH_3 soln. usually added to the urine when preparing the glucuronide fraction with basic lead acetate. *Methyl (2-oxo-1:2-benzopyran-6-yl tri-O-acetyl-β-D-glucosid)uronate*, m.p. 174–175°, was prepared from the above salt with acetic anhydride and perchloric acid, followed by methyl iodide and silver oxide as described for the preparation of the 5-isomer (Found: C, 55·2; H, 4·4. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6%).

8-Hydroxycoumarin. Urine from rabbits receiving 0·5 g. of 8-hydroxycoumarin/kg. gave a strong naphtharesorcinol reaction and no Benedict's or $FeCl_3$ reactions. Paper chromatography of urine showed the presence of spots corresponding with 8-hydroxycoumarin, its ethereal sulphate and its glucuronide.

The basic lead acetate fraction of the 24 hr. urine of five rabbits which had received a total of 7·5 g. of 8-hydroxycoumarin was concentrated to 300 ml. and the glucuronide precipitated with excess of ethanol. The precipitate (6·3 g.) was dissolved in water (100 ml.) and the solution acidified with conc. HCl. On cooling to 0°, fine colourless needles were deposited (4·65 g.). After recrystallization from water the *2-oxo-1:2-benzopyran-8-yl β-D-glucosiduronic acid* had m.p. 158–160° (decomp.) (Found: C, 50·5; H, 4·3; H_2O, 5·2. $C_{15}H_{14}O_9,H_2O$ requires C, 50·6; H, 4·5; H_2O, 5·1%). Hydrolysis with 5 N-HCl or β-glucuronidase gave 8-hydroxycoumarin, which was identified on paper chromatograms. *Methyl (2-oxo-1:2-benzopyran-8-yl tri-O-acetyl-β-D-glucosid)uronate* (m.p. 135–136°) was prepared with diazomethane and pyridine–acetic anhydride (Found: C, 54·2; H, 5·1; H_2O, 1·6. $C_{22}H_{22}O_{12},0·5H_2O$ requires C, 54·2; H, 4·7; H_2O, 1·9%).

The ethanolic solution remaining from the precipitation of the crude glucuronide salt was concentrated to 50 ml. *in vacuo* and neutralized with $KHCO_3$, when lustrous plates of the potassium salt of the ethereal sulphate of 8-hydroxycoumarin were deposited and shown to be chromatographically identical with the synthetic material (Found: K, 13·8; S, 11·2. $C_9H_5O_6SK$ requires K, 13·9; S, 11·4%). A short acid hydrolysis yielded 8-hydroxycoumarin, m.p. and mixed m.p. 159–160°.

RESULTS AND DISCUSSION

The conjugations of the hydroxycoumarins are shown in Table 3, which also includes the results obtained with umbelliferone and 4-methyl-umbelliferone (Mead *et al.* 1955). The figures for the glucuronic acid conjugation of 5-, 6-, 7- and 8-hydroxycoumarins are probably low, since it was found that the glucuronides of these coumarins are relatively stable to acid and are not completely hydrolysed by the acid used in the Paul (1951) modification of the naphtharesorcinol method. Some results with pure aqueous solutions of glucuronides in the Paul method are given in Table 4. Glucuronides relatively stable to acid and giving low results with the naphtharesorcinol method have been mentioned previously by Hanson, Mills & Williams (1944).

3-, 5-, 6-, 7- and 8-Hydroxycoumarins are phenolic in nature and are metabolized in rabbits mainly by direct conjugation with glucuronic and sulphuric acids. 4-Hydroxycoumarin (benzotetronic acid) differs from its isomers in that it

5

conjugates only with glucuronic acid. It formed no ethereal sulphate *in vivo* and it was the only hydroxycoumarin from which no ethereal sulphate could be obtained chemically. 4-Hydroxycoumarin is a relatively strong acid of pK 5·8, and it would not be expected to form an ethereal sulphate when fed to the intact animal. This is apparently in agreement with the suggestions of Anderton, Smith & Williams (1948) that hydroxy compounds with pK_a values less than about 7 do not form ethereal sulphates *in vivo*. The reason for this may be either that the compound does not penetrate to the site of sulphate synthesis or, if an ethereal sulphate is formed, that it is too unstable to persist. The glucuronide of 4-hydroxycoumarin also differed from the glucuronides of the other hydroxycoumarins, in that it reduced hot Benedict's reagent whereas the others did not. Both 3- and 4-hydroxycoumarin glucuronides are enol-glycosides and the reducing properties of the 4-glucuronide (II) can be attributed to the weakening of the aglycone–glucuronic acid link by the inductive effect of the carbonyl oxygen

transmitted through the 3:4 double bond of the coumarin nucleus. 3-Hydroxycoumarin glucuronide (I) is non-reducing since such electron shifts cannot readily occur.

The specific and molecular rotations of the isomeric hydroxycoumarin glucosiduronic acids and their triacetyl methyl esters are shown in Table 5. One abnormality requires comment and that is that the rotation of the triacetyl methyl ester of 8-hydroxycoumarin β-glucosiduronic acid is more negative than the free glucuronide. Normally these esters are less negative than the glucuronide. Spoke-and-ball models of this ester suggest that free rotation about the glycosidic link is hindered,
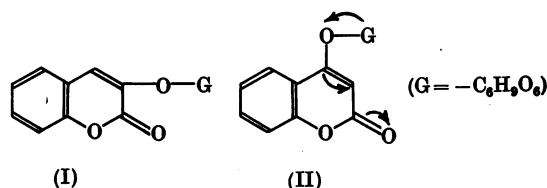

$(G = -C_6H_9O_6)$

(I)   (II)

Table 3. *Conjugation of hydroxycoumarins in the rabbit (dose 0·2 g./kg.)*

| | Conjugation as percentage of dose* | | |
Compound	Glucuronide	Ethereal sulphate	Total
3-Hydroxycoumarin	57 (55–59)	14 (12–19)	71
4-Hydroxycoumarin	37 (31–44)	1† (0–3)	37
5-Hydroxycoumarin	37‡ (28–45)	18 (15–20)	55‡
6-Hydroxycoumarin	48‡ (46–53)	28 (23–35)	76‡
7-Hydroxycoumarin	41‡ (39–42)	21 (19–22)	62‡
4-Methyl-7-hydroxycoumarin	49 (45–53)⁵	4 (2–7)⁵	53
8-Hydroxycoumarin	60‡ (50–71)	8 (4–12)	68‡

* Three animals were used unless otherwise indicated by superior figures outside parentheses.
† This figure is not significant.
‡ These figures are probably low owing to the stability of the glucuronides (see Table 4).

Table 4. *Recovery of glucuronides by the naphtharesorcinol method* as modified by Paul (1951)*

Glucuronide	Recovery (%)	Glucuronide of	Recovery (%)	Glucuronide	Recovery (%)
p-Chlorophenyl	99	3-Hydroxycoumarin	86	(−)Menthyl	107
p-Bromophenyl	99	4-Hydroxycoumarin	79	(+)Menthyl	99
p-Iodophenyl	104	5-Hydroxycoumarin	63	(+)isoMenthyl	91
p-Aminophenyl	92	6-Hydroxycoumarin	67	(+)neoMenthyl	112
p-Cyanophenyl	91	7-Hydroxycoumarin	47	2-Ethylhexanoyl	106
o-Amino-p-sulphonamidophenyl	39	4-Methyl-7-hydroxycoumarin	79	Di-isopropylmethyl	98

* See text for method.

Table 5. *Optical rotations of the hydroxycoumarin β-glucosiduronic acids*

Isomer	Hydroxycoumarin glucuronide		Triacetyl methyl esters	
	$[\alpha]_D$*	$[M]_D/100$	$[\alpha]_D$* (c, 1 in CHCl₃)	$[M]_D/100$
3	−98° (c, 1 in N-NaOH)	−340°	−78°	−373°
4	−85° (c, 1 in ethanol)	−303	−50·5	−246
5	−61° (c, 0·25 in water)	−228	−50	−239
6	−86° (NH₄ salt, c, 1 in water)	−313	−28·5	−136
7	−105° (c, 1 in water)	−374	−44	−210
8	−77° (c, 1 in water)	−274	−136	−650

* t = 20–25°.

because of the space relation of the triacetyl glucuronic acid methyl ester portion of the molecule to the coumarin ring (cf. Kamil *et al.* 1951).

SUMMARY

1. The fate of 3-, 4-, 5-, 6- and 8-hydroxycoumarins in the rabbit has been studied. All five compounds are conjugated with glucuronic acid and the glucuronides have been isolated and described. With the exception of 4-hydroxycoumarin, they are also excreted in conjugation with sulphuric acid, and the ethereal sulphates of 3-, 6- and 8-hydroxycoumarins were isolated from the urine. Hydroxylation of 6-hydroxycoumarin to 6:7-dihydroxycoumarin (aesculetin) was also observed.

2. The potassium salts of the sulphuric esters of 3-, 5-, 6- and 8-hydroxycoumarins have been synthesized and described.

3. The paper-chromatographic behaviour and the colour reactions of the hydroxycoumarins and their conjugates have been described.

REFERENCES

Adams, R. & Bockstahler, T. E. (1952). *J. Amer. chem. Soc.* 74, 5346.
Anderton, J. I., Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* 43, xxxv.
Bargellini, G. & Monti, L. (1915). *Gazz. chim. ital.* 45, 90.
Boyd, J. & Robertson, A. (1948). *J. chem. Soc.* p. 174.
Burkhardt, G. N. & Lapworth, A. (1926). *J. chem. Soc.* p. 684.
Cingolani, E. (1954). *Gazz. chim. ital.* 84, 843.
El Masry, A. M., Smith, J. N. & Williams, R. T. (1956). *Biochem. J.* 64, 50.
Feigl, F., Feigl, H. E. & Goldstein, D. A. (1955). *J. Amer. chem. Soc.* 77, 4162.
Flatow, L. (1910). *Hoppe-Seyl. Z.* 64, 377.
Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* 38, 274.
Jarrige, P. & Henry, R. (1952). *Bull. Soc. Chim. biol., Paris,* 34, 872.
Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* 50, 235.
Linch, F. W. (1912). *J. chem. Soc.* 101, 1758.
McIsaac, W. M. & Williams, R. T. (1957). *Biochem. J.* 66, 369.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1955). *Biochem. J.* 61, 569.
Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* 68, 67.
Paul, J. (1951). Ph.D. Thesis, University of Glasgow.
Roseman, S., Heubner, C. F., Pancratz, R. & Link, P. K. (1954). *J. Amer. chem. Soc.* 76, 1650.
Sieberg, E. (1921). *Biochem. Z.* 113, 198.
Smith, J. N., Smithies, R. H. & Williams, R. T. (1953). *Biochem. J.* 54, 225.
Sperber, I. (1948). *J. biol. Chem.* 172, 441.

Studies in Detoxication

72. THE METABOLISM OF COUMARIN AND OF *o*-COUMARIC ACID

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Coumarin (2-oxo-1:2-benzopyran) is the sweet-smelling constituent of white clover, and it has been found in a large variety of plants. Many of its derivatives are pharmacologically active and they include anticoagulant drugs (see Hunter & Shepherd, 1955), rodenticides and insecticides. Coumarin itself has been employed for flavouring food, but its use has been discouraged owing to its damaging effect upon the liver in animals.

No previous study on the metabolic fate of coumarin has been made, except that Vasiliu, Timosencu, Zaimov & Coteleu (1938) fed it to sheep and found that it did not produce benzoic acid derivatives. However, all naturally occurring derivatives of coumarin, except dicoumarol, are either 7-hydroxy derivatives or 7-*O*-ethers of coumarin (see tables in Elderfield, 1951). This suggests that the 7-position is a possible point of biological attack on the coumarin molecule. In fact, it has already been shown that in man ethyl biscoumacetate (Tromexan) is hydroxylated in the 7-position of one of its coumarin rings (Burns, Weiner, Simson & Brodie, 1953; Burns, Wexler & Brodie, 1953).

Coumarin (I) is the lactone of *o*-hydroxy-*cis*-cinnamic acid (coumarinic acid). It was therefore

(I)   (II)