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The Metabolism of [3-^{14}C]Coumarin

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In an earlier paper[1] we gave an account of a qualitative investigation of the fate of coumarin in the animal body. 3- and 7-Hydroxycoumarin were proved by isolation to be metabolites of coumarin in rabbits. We also showed by paper chromatography that these two compounds were metabolites of coumarin in the ferret, guinea pig, mouse and rat. Furthermore, evidence was obtained that 5- and 8-hydroxycoumarin were metabolites, albeit minor ones, in most of these animals. We did not find 4- and 6-hydroxycoumarin, 6,7-dihydroxycoumarin, *o*-coumaric acid (*o*-hydroxy-*trans*-cinnamic acid), melilotic acid (*o*-hydroxyphenylpropionic acid) or salicylic acid in the urine of animals dosed with coumarin.

Since the above paper was published and while the present work was in progress, two papers have appeared on the metabolism of coumarin. Furuya[2] has confirmed that 3- and 7-hydroxycoumarin are metabolites of coumarin in rabbits; traces of 8-hydroxycoumarin, but no 4-, 5-, or 6-hydroxycoumarin, were found. Booth, Masri, Robbins, Emerson, Jones and DeEds[3] have isolated 3- and 7-hydroxycoumarin and a new major metabolite, *o*-hydroxyphenylacetic acid, from the urine of coumarin-fed rabbits. They also report the detection by paper chromatography of melilotic acid and melilotoylglycine; *o*-coumaric acid was detected after acid hydrolysis of the urine, and the precursor of this acid was regarded as being *o*-hydroxyphenylhydracrylic acid. They[3] claimed that, when coumarin was fed to rats, 3- and 7-hydroxycoumarin were not detectable in the urine. They isolated *o*-hydroxyphenylacetic acid, which was a major metabolite in rats, and claimed, on chromatographic evidence, that melilotic acid and *o*-coumaric acid were present in the urine. The presence

in the urine of *o*-hydroxyphenyllactic acid and of *o*-hydroxyphenylhydracrylic acid was tentatively suggested, the latter as a precursor of *o*-coumaric acid.

To obtain a more quantitative idea of the metabolism of coumarin, we have now synthesized [3-^{14}C]coumarin and have reinvestigated the metabolism of coumarin in rats and rabbits. There are, apparently, considerable differences between these two species with regard to their ability to metabolize coumarin. We have found that all six of the possible hydroxycoumarins are formed in the rabbit and that *o*-hydroxyphenylacetic acid, as reported by Booth *et al.*,[3] is also a major metabolite accounting for about 20 per cent of the dose. Furthermore, *o*-hydroxyphenyllactic acid is a urinary metabolite in rabbits. In rats, hydroxycoumarins are only minor urinary metabolites, and it appears that coumarin is more extensively degraded in rats than in rabbits.

Experimental

[3-^{14}C]Coumarin. Diethyl [2-^{14}C]malonate (1·05 g; 0·5 mc; Radiochemical Centre, Amersham) was warmed at 50° in a 250-ml flask with N NaOH (13·4 ml) until it dissolved (2–3 min). After keeping the mixture at room temperature for 2·5 h, the solution was neutralized with 6·2 ml of 2N HCl and then evaporated over a small flame until it began to solidify. The flask was then kept in an evacuated desiccator over P_2O_5 for 24 h. To the dry mixture of malonic acid and sodium chloride in the flask, salicylaldehyde (0·85 ml) and aniline hydrochloride (0·1 g) were added. The ingredients were thoroughly mixed and kept on a water-bath at 50–60° for 6 h and then overnight at 37°. After this time the mixture had become pasty but still smelt of salicylaldehyde. It was therefore heated at 55–60° for a further 1·5 h. The hard cake which then formed was triturated with 5 ml of water, drained, and then washed successively with 2 ml of absolute ethanol and 2 ml of ether. The yield of coumarin-3-carboxylic acid was 0·94 g (75 per cent). The acid was mixed with sodium metabisulphite (0·94 g) and water (5 ml) and warmed to 40–50° until all the solid had dissolved. The temperature was then raised to 60–70° until the evolution of CO_2 had ceased. The pasty mass of

sodium hydrocoumarinsulphonate was dried *in vacuo* over P_2O_5 (yield, 1·73 g). The dry salt was then heated under reflux at 190° for 6 h. After cooling, the residue was extracted with ether and from these extracts 0·6 g of ^{14}C-coumarin, m.p. 61°, was obtained by evaporation (recovery of ^{14}C was 387·6 μc or 77 per cent). After recrystallization from *n*-hexane, the coumarin had m.p. 69–70° with an activity of 690 μc/g. By heating the residual sodium hydrocoumarinsulphonate at 190° for 1·5 h, a further 23 mg of coumarin, m.p. 63–65°, was obtained.

Reference compounds. Coumarin, *o*-coumaric acid and *o*-hydroxyphenylacetic acid were purchased and purified. 3-, 4-, 5-, 6-, 7- and 8-Hydroxycoumarins, aesculetin, melilotic acid, 3-, 4- and 7-hydroxycoumarin glucuronides and *o*-coumarylglycine were obtained according to Mead, Smith and Williams[1,4]. *o*-Hydroxyphenyllactic acid has been prepared by Plöchl and Wolfrum[5] as a colourless oil by reduction of 3-hydroxycoumarin with sodium amalgam in alkaline solution. Its calcium salt, containing $6H_2O$, was also prepared but few preparative details were given. We have prepared this calcium salt in a similar way from 3-hydroxycoumarin, as a white microcrystalline powder, but found it to be a hemihydrate.

Anal. Calcd. for $(C_9H_9O_4)_2Ca$ 0·5 H_2O: H_2O, 2·2. Found: H_2O, 2·1 (loss at 110°).

o-Hydroxyphenyllactic acid gave a single spot of R_f 0·09 in benzene–acetic acid–water (1 : 1 : 2) on chromatography on Whatman No. 4 paper.

The *benzylamine salt* of *o*-hydroxyphenylacetic acid formed minute crystals from ethyl acetate, m.p. 165°.

Anal. Calcd. for $C_{15}H_{17}NO_3$: C, 69·45; H, 6·6. Found: C, 69·7; H, 6·3.

The *benzylamine salt* of *o*-hydroxyphenylpropionic (melilotic) acid formed white woolly needles from ethyl acetate, m.p. 146–147°.

Anal. Calcd. for $C_{16}H_{19}NO_3$: C, 70·3; H, 7·0; N, 5·1. Found: C, 70·5; H, 7·2; N, 5·25.

The *benzylamine salt* of *o*-hydroxyphenyllactic acid formed a white powder from ethyl acetate, m.p. 132°.

Anal. Calcd. for $C_{16}H_{19}NO_4$: C, 66·4; H, 6·6. Found: C, 66·2; H, 6·6.

2,4-Dihydroxycinnamic acid, m.p. 210° (d.), was obtained in low yield as a yellowish powder according to Posen.[6] DL-*o*-Tyrosine, m.p. 249–250°, was prepared from α-benzamido-*o*-hydroxycinnamic acid,[7] according to Blum.[8]

o-Hydroxyphenylpyruvic acid, the anhydride of which is 3-hydroxycoumarin, does not appear to have been described (see Erlenmeyer and Stadlin[9]). Attempts were made to prepare it for chromatographic purposes. 3-Hydroxycoumarin (1 g) in 2N Na_2CO_3 (50 ml) was heated under reflux for 0·5 h. The solution was cooled to 0° and acidified to pH 2 by cautious addition of ice-cold concentrated HCl. The solution was extracted with ether (3 × 20 ml) and the extract dried (Na_2SO_4 anhyd.) for 1 h. Removal of the ether left a yellowish oil (0·32 g) which was used for paper chromatography (see Table I). This oil, which was prepared on several occasions, showed on paper a main spot which was assumed to be *o*-hydroxyphenylpyruvic acid, and a weak spot corresponding to 3-hydroxycoumarin. On keeping the oil for 2 to 3 days, it gradually crystallized to a material consisting of 3-hydroxycoumarin and the decarboxylation product, *o*-hydroxyphenylacetic acid. In aqueous solution the oil gave a violet-red colour with aqueous 1 per cent ferric chloride and a green colour with 2,6-dichloroquinonechloroimide (Gibbs's reagent). 3-Hydroxycoumarin gives a green colour and *o*-hydroxyphenylacetic acid no colour with ferric chloride (see Table I for colours with Gibbs's reagent).

Animals. Chinchilla doe rabbits and female albino rats, maintained on a constant diet, were used. Coumarin, suspended in water containing a little bile salt, was administered orally to the animals. Urine was collected daily and the faeces usually at the end of the experiment (2–4 days). One rat was kept in a 'perspex' tank with an arrangement for collecting expired air; the other animals were kept in metabolism cages for collection of urine and faeces only.

Measurement of radioactivity. These measurements were carried out on urine, faeces and tissues as described earlier.[10]

R_f values and colour reactions. These are given in Table I. Others used in this paper are quoted in Mead, Smith and Williams.[4]

Table I. R_f values and colour reactions of coumarin and its possible metabolites. R_f values for descending chromatography are on Whatman No. 4 paper. Solvent mixtures: *A*, benzene–acetic acid–water (1 : 1 : 2) run for 2 h; *B*, *n*-butanol–acetic acid–water (4 : 1 : 5) run for 6 h; *C*, *n*-propanol–ammonia solution (7 : 3), sp. gr. 0·88, run for 6 h; *D*, ethyl methyl ketone saturated with 2N ammonia solution, run for 2 h; *E*, *n*-butanol–ammonia solution (sp. gr. 0·88)–water (8 : 1 : 11) run for 4 h. The proportions of solvents are by volume. Sprays used: Gibbs's reagent, 0·1 per cent 2, 6-dichloroquinonechloroimide in ethanol followed by saturated aqueous $NaHCO_3$; Brentamine, 0·01 per cent Brentamine fast blue B salt (I.C.I. Ltd.) in water followed by saturated aqueous $NaHCO_3$. Fluorescence; paper illuminated by light of 270 mμ or 360 mμ. Q means quenching of background fluorescence of the paper.

Compound	R_f values in solvent					Colour reactions			
								Fluorescence	
	A	*B*	*C*	*D*	*E*	Gibbs	Brentamine	270 mμ	360 mμ
Coumarin	0·88	0·92	0·96	0·96	0·93	none	none	Q	blue-green in NaOH
3-Hydroxycoumarin	0·72	0·91	0·86	0·44	0·53	red → green	blue	—	—
4-Hydroxycoumarin	0·15	0·91	0·72	0·18	0·46	red-purple	red	purple	weak purple
o-Hydroxyphenylacetic acid	0·28	0·89	0·87	0·35	0·47	blue	weak orange	Q	—
o-Hydroxyphenyllactic acid	0·09	0·88	0·76	0·18	0·32	blue	pale red	Q	—
o-Hydroxyphenylpyruvic acid	0·08	0·80	0·60			yellow-green	pale yellow	—	purple, green → yellow in NH_3 fumes
o-Hydroxyphenylpropionic acid	0·43	0·92	0·67	0·27	0·46	blue	pink	Q	—
2,4-Dihydroxycinnamic acid	0·00	0·81	0·31	0·02	0·05	crimson	purple-red	—	purple-blue, intense blue in NH_3 fumes
o-Tyrosine[a]	0·00	0·26	0·15	0·00	0·00	red-purple	none	—	—
Phenol	0·87	0·93	0·95	0·97	0·94	blue	orange → pink	—	—
Salicylic acid	0·79	0·92	0·77	0·38	0·51	blue	—	purple	—

[a] Violet colour with 0·1% ninhydrin in alcohol.

Isotope Dilution Methods

3-, 5-, 6-, 7-, and 8-Hydroxycoumarins. (*a*) *Total.* The carrier hydroxycoumarin (100–500 mg) was added to the urine (1–5 ml, according to its activity) which was then mixed with an equal volume of concentrated HCl and heated under reflux for 3 h. After cooling the solution, the hydroxycoumarin was extracted either by shaking with several portions of ether or by continuous extraction with ether. The ethereal extract was dried (Na_2SO_4 anhyd.), evaporated, and the residue was then recrystallized from an appropriate solvent to constant activity. 3-Hydroxycoumarin (m.p. 152–3°) was recrystallized from benzene, then ethanol and finally from water, and counted as such; it was also counted as 2-oxo-3-phenylhydrazonochroman (m.p. 173–4°) formed by treatment with N NaOH and phenylhydrazine hydrochloride[9] and recrystallized from water. 5-, 6-, 7-, and 8-Hydroxycoumarins were counted as such and after conversion into the corresponding acetoxycoumarins (5-, m.p. 85°; 6-, m.p. 145–6°; 7-, m.p. 140°; and 8-, m.p. 133°, after recrystallization from water).

(*b*) *Free.* Unconjugated hydroxycoumarins were determined by adding the hydroxycoumarins to the urine as above and then diluting with 5–20 ml of water and boiling the solution to dissolve the carrier. On cooling 3-, 6-, and 7-hydroxycoumarins separated and were recrystallized to constant activity as above. 8-Hydroxycoumarin was extracted with ether and then recrystallized and counted as above.

4-Hydroxycoumarin. On heating with acids, 4-hydroxycoumarin and it conjugates are converted into *o*-hydroxyacetophenone. Carrier 4-hydroxycoumarin was added and the urine acidified with concentrated HCl as described in the preceding paragraph. The mixture was heated under reflux for 6 h to complete the conversion of 4-hydroxycoumarin to the ketone. After cooling, the ketone was separated by continuous extraction with ether for 8 h. The extract was evaporated, taken up in a little ether, and washed with 5 per cent $NaHCO_3$ and then water. The ethereal solution was dried (Na_2SO_4 anhyd.) and evaporated to leave an oil which was dried to constant weight *in vacuo*. The oil was then treated with the calculated amount of 2,4-dinitrophenylhydrazine in concentrated HCl (1–2 ml), and the mixture

heated at 100° for 1 h. The 2,4-dinitrophenylhydrazone of *o*-hydroxyacetophenone,[11] m.p. 216°, was recrystallized from ethanol to constant activity.

6,7-Dihydroxycoumarin (*aesculetin*). This compound decomposes on heating with acid. The urine (5 ml) was mixed with the gastric juice of *Helix pomatia* (1 ml) containing β-glucuronidase, and acetate buffer (5 ml, pH 4·6), and incubated for 1 h at 37°. Aesculetin (0·4 g) was added to the mixture which was then diluted with water (100 ml) and boiled to dissolve the aesculetin. The mixture was filtered (charcoal) and on cooling aesculetin (m.p. 269°) separated and was recrystallized to constant activity from water. It was also converted to 6,7-diacetoxycoumarin[12] (m.p. 133°) which was recrystallized from ethanol and counted. The aesculetin had no radioactivity.

Coumarin. (*a*) *Free.* Coumarin (0·3–0·5 g) was added to the urine (1–5 ml), and water (20 ml) and a little ethanol added. The mixture was warmed to dissolve the coumarin and kept at room temperature for 0·5 h. The solution was then kept at 0° and when the coumarin had separated out, it was filtered and recrystallized from *n*-hexane and from light petroleum (b.p. 100–120°) to constant activity (m.p. 69–70°).

(*b*) *Total.* Urine (1–5 ml) containing added carrier coumarin (0·4–0·5 g) was hydrolysed exactly as described above for the estimation of total 3-hydroxycoumarin. The hydrolysate was then extracted with ether and the extract dried (Na_2SO_4 anhyd.) and evaporated. The residue of coumarin was recrystallized as above.

o-*Coumaric acid.* *o*-Coumaric (0·2–0·5 g) was added to the urine (1–5 ml) containing acetone (2–3 ml) and the procedure for the determination of free 3-hydroxycoumarin was followed. The *o*-coumaric acid (m.p. 208°), was recrystallized from acetone–water and was counted as such and after conversion to acetyl-*o*-coumaric acid (m.p. 158°). Total *o*-coumaric acid could not be estimated because on heating with acid it is converted into coumarin.

o-*Coumarylglycine.* Free *o*-coumaric acid was not found in coumarin urine. However, *o*-coumaric acid is known to conjugate with glycine.[1] *o*-Coumarylglycine (0·1–0·2 g) was added to the urine (5 ml) and an equal volume of water added. The mixture

was heated to dissolve the compound, then filtered and cooled. The *o*-coumarylglycine which separated was recrystallized from water and acetone–water until the activity disappeared.

3-, 4-, and 7-Hydroxycoumarin glucuronides. The glucuronide (0·05–0·2 g) was added to the urine (2·5 ml), the mixture (made slightly alkaline with ammonia for the 7-derivative) warmed to dissolve the solid and the solution kept at 0° overnight (acidified with HCl in the case of the 7-derivative). The crystalline glucuronide which separated was recrystallized to constant activity from water for the 3- and 4-isomers, and water followed by aqueous ethanol for the 7-isomer

o-*Hydroxyphenylpropionic (melilotic) acid.* The acid (0·3–0·5 g) was added to the urine (5 ml) and concentrated HCl (5 ml). The mixture was heated under reflux for 3 h, cooled, saturated with NaCl, and extracted with ether (5 × 20 ml). The extract was dried (Na_2SO_4 anhyd.) and evaporated to a dark coloured oil which did not crystallize. The oil was triturated with water (5–10 ml) and calcium hydroxide to form the calcium salt. The mixture was then cooled and filtered. The filtrate was evaporated to 1 ml, filtered and cooled to 0° when calcium melilotate separated. This salt was dissolved in 2N HCl (2 ml) and the free acid extracted with ether. The extract was dried (Na_2SO_4 anhyd.), evaporated, and the residue of melilotic acid (m.p. 87°) recrystallized from carbon tetrachloride–light petroleum (b.p. 100–120°) to constant activity. It was further purified as the benzylamine salt (m.p. 146–7° from ethyl acetate), and the activity fell to zero.

o-*Hydroxyphenylacetic acid.* The acid (0·2–0·4 g) was added to urine (1–5 ml) and the procedure for melilotic acid followed. On evaporation, the ether extract gave crystalline *o*-hydroxyphenylacetic acid (m.p. 145–146°) which was recrystallized from ether–light petroleum (b.p. 60–80°) and chloroform to constant activity. It was also converted into its benzylamine salt, m.p. 165°, which was crystallized to constant activity from ethyl acetate.

o-*Hydroxyphenyllactic acid.* Calcium *o*-hydroxyphenyllactate (0·4 g) was dissolved in urine (1–5 ml) and an equal volume of water added. An equal volume of concentrated HCl was added to the mixture which was heated under reflux for 3 h. After

cooling, the mixture was extracted with ether (5 × 10 ml) and the extract dried (Na_2SO_4 anhyd.) and evaporated. The residual oil was converted to the calcium salt with calcium hydroxide and the salt recrystallized from water. It was then converted to the free acid which was converted to the benzylamine salt. The latter (m.p. 130–131°) was recrystallized from ethyl acetate to constant activity.

o-*Tyrosine*. DL-*o*-Tyrosine (0·2 g) was dissolved in urine (5 ml) and the solution brought to pH 2 with concentrated HCl. After keeping 0·5 h the solution was evaporated to 1 ml. Ethanol (5 ml) was gradually added to precipitate *o*-tyrosine hydrochloride. The latter was recrystallized from ethanol and then benzoylated with benzoyl chloride and alkali. The *N*-benzoyl-*o*-tyrosine (m.p. 187°) was recrystallized from ethanol until the activity disappeared. For total *o*-tyrosine, the urine containing the carrier was heated under reflux for 3 h with an equal volume of concentrated HCl and then the procedure was the same as for free *o*-tyrosine. No *o*-tyrosine was found.

Qualitative Examination of Excreta

Rabbits. The urine of rabbits which had received coumarin (50 mg/kg) orally was slightly alkaline (pH 7–9) and fluoresced a bright blue in ultraviolet light even up to 6 days after dosing. The first 24-h urine also gave a good positive naphthoresorcinol test for glucuronic acid; later urines did not give positive tests more intense than did normal rabbit urine. Paper chromatography in solvents *A*, *B*, *C* and *D* (Table I) showed the presence of 3-, 7- and 8-hydroxycoumarins, and *o*-hydroxyphenylacetic and -lactic acids. The same compounds and coumarin were found after acid hydrolysis of the urine. The paper chromatograms did not show up 4- and 5-hydroxycoumarins, 6,7-dihydroxycoumarin, *o*-coumaric acid, melilotic acid, salicylic acid, phenol, *o*-hydroxyphenylpyruvic acid and 2,4-dihydroxycinnamic acid. The faeces of rabbits collected for 3–4 days after dosing with ^{14}C-coumarin contained very little radioactivity and paper chromatography of acetone extracts of the faeces yielded no definite results.

Rats. These animals were given 100 mg of ^{14}C-coumarin/kg

orally. The urine had a bright blue fluorescence and reacted strongly with naphthoresorcinol. Paper chromatography as above showed the presence of small amounts of 3- and 7-hydroxycoumarin and *o*-hydroxyphenyllactic acid, and considerable amounts of *o*-hydroxyphenylacetic acid. Rat faeces were collected for 4 days after dosing and contained considerable amounts of radioactivity (see Tables II and III). Various extracts of the faeces were made, but the main bulk of this radioactivity was due to unidentified compounds. Small amounts of *o*-hydroxyphenylacetic acid and only traces of coumarin were found in the faeces.

Metabolism of 3-hydroxycoumarin. When fed to rabbits, 3-hydroxycoumarin is metabolized to 70–80 per cent by conjugation with glucuronic acid and sulphate.[4] We have now found that a small portion of it is also metabolized by rupture of the heterocyclic ring.

A rabbit (3·4 kg) was given 0·84 g of 3-hydroxycoumarin orally. Chromatography of a sample of the 24-h urine on Whatman No. 4 paper using solvents *A*, *B*, and *C* (see Table I) showed spots corresponding to 3-hydroxycoumarin and its glucuronide and ethereal sulphate,[4] and *o*-hydroxyphenylpyruvic, *o*-hydroxyphenyllactic and *o*-hydroxyphenylacetic acids. The urine of the rabbit was collected for 60 h, acidified to pH 2 with concentrated HCl, saturated with NaCl and then continuously extracted with ether for 12 h. 3-Hydroxycoumarin glucuronide (0·68 g) separated in the ether and was removed. The extract was evaporated to a gum (0·58 g) which contained glucuronide but no ethereal sulphate. Chromatography of the gum (50 μg) in the same solvents showed it to contain 3-hydroxycoumarin and *o*-hydroxyphenylacetic and -lactic acids. The labile *o*-hydroxyphenylpyruvic acid which was found in the original urine was not present and it could have decomposed to *o*-hydroxyphenylacetic acid or cyclized to 3-hydroxycoumarin, or both. The gum was now dissolved in saturated aqueous $NaHCO_3$ (5 ml) and the solution extracted with ether to remove 3-hydroxycoumarin (m.p. and mixed m.p. 152°; 10 mg). The residual solution was acidified and extracted with ether to take out the hydroxy acids. This extract on evaporation gave a gum (22·6 mg) which could not be crystallized and chromatography in three solvents showed it to

consist entirely of a mixture of *o*-hydroxyphenylacetic acid and -lactic acid. Similar results were obtained with urine which had been made 5N with respect to HCl, hydrolysed by heating under reflux for 3 h and extracted with ether.

Five rats were given 0·2 g each of 3-hydroxycoumarin by mouth. The 24-h urine was chromatographed in solvents *A*, *B* and *C*, and it was found that the main metabolite was *o*-hydroxyphenylacetic acid. A strong spot was also found for *o*-hydroxyphenyllactic acid, but 3-hydroxycoumarin was only just detectable.

Results and Discussion

The quantitative aspects of the metabolism of coumarin in rats and rabbits are shown in Table II. The dose (100 mg/kg) given to rats is twice that (50 mg/kg) given to rabbits but it is probable that this has no bearing on the main conclusions extracted from the results. If one examines the last five lines in this Table, it becomes clear that there is a considerable difference regarding route of elimination of ^{14}C in rats compared with rabbits. In rats about 50 per cent of the dose is eliminated in the urine and the rest is found in the faeces, whereas in rabbits practically all the ^{14}C is eliminated in the urine. Small amounts of ^{14}C remain in the rat tissues at the end or the experiments (up to 5 days after dosing) but there is practically none in rabbit tissues. No radioactivity was found in the expired air in the case of one rat examined and similar results were found for a rabbit not quoted in the Table. It is to be noted that the recovery of administered radioactivity is reasonably complete for this type of experiment, amounting to 81–92 per cent in rabbits and 86–100 per cent in rats.

In Table III are quoted the figures for the distribution of ^{14}C in the excreta of rats and rabbits receiving a small oral dose (1 mg/kg) of coumarin. The same pattern of excretion appears to hold as for the larger doses quoted in Table II. The recovery of ^{14}C in rabbits quoted in Table III is not quantitative and this is probably the result of attempting to count a small amount of radioactivity in large amounts of excreta. The results are almost quantitative for rats and in these animals the ^{14}C is equally divided between urine and faeces.

Table II. The metabolites of [3-^{14}C]coumarin in rabbits and rats

Experiment No.	Rabbits 1	Rabbits 2	Rabbits 3	Rats 4	Rats 5	Rats 6
Dose of coumarin, mg/kg orally	40	50	50	100	100	100
Dose of ^{14}C, μc/animal	1·3	58·4	47·6	10	14·5	10
Duration of experiment, days	4	2	1	3·7	5	6·75
Metabolites examined		% of dose				
Coumarin on acid hydrolysis	12·3	14·8 (0·7)[a]	16·7 (0·4)[a]	3·1 (1·6)[a]	7·4 (0·6)[a]	—
3-Hydroxycoumarin	18·1	19·9	23·4 (2·8)[a]	1·8	1·7	—
3-Hydroxycoumarin glucuronide	—	—	[9·0][b]	—	—	—
4-Hydroxycoumarin	—	0·3	0·9	0	0·5	—
4-Hydroxycoumarin glucuronide	—	—	[0·12][b]	—	—	—
5-Hydroxycoumarin	—	0·3	0·5	—	—	—
6-Hydroxycoumarin	—	4·7	2·0 (0·2)[a]	0·3	0·3	—
7-Hydroxycoumarin	10·1	16·0	10·0 (0·7)[a]	0·26	0·45	—
7-Hydroxycoumarin glucuronide	—	—	[3·1][b]	—	—	—
8-Hydroxycoumarin	—	2·5	1·3 (0·3)[a]	0·3	0·5	—
6,7-Dihydroxycoumarin	—	0	0	—	—	—
Total coumarins		58·5%	54·8%	5·8%	10·9%	—
o-Tyrosine	—	—	—	0	0	—
o-Coumaric acid, free	—	0	0	0	0	—
o-Coumarylglycine	—	0	0	—	—	—
o-Hydroxyphenylpropionic acid	—	0[a]	0[a]	0	0	—
o-Hydroxyphenyllactic acid	—	3·5	2·6	0·6	0·9	—
o-Hydroxyphenylacetic acid	—	22·1	18·1 (12·9)[a]	12·5 (11·2)[a]	27·2	18·6 (6·5)
Total acids		25·6	20·7	13·1	28·1	—
Sum of urinary metabolites		84·1	75·5	18·9	39·0	—
Radioactivity of urine	92·4	90·2	80·3	47·0	58·9	60·5
Radioactivity of expired air	—	—	—	—	0	—
Radioactivity of faeces	—	<0·2	0·65	38·8	38·3	32·4
Radioactivity of tissues	—	0[c]	—	0·3	2·9	—
Total radioactivity	92·4%	90·2%	81·0%	86·1%	100·1%	92·9%

[a] These figures are for the free metabolite; all other figures are for metabolites obtained on acid hydrolysis.
[b] These figures are not included in the sum of urinary metabolites as glucuronides are accounted for in the total hydroxycoumarin.
[c] 4 days after dosing.

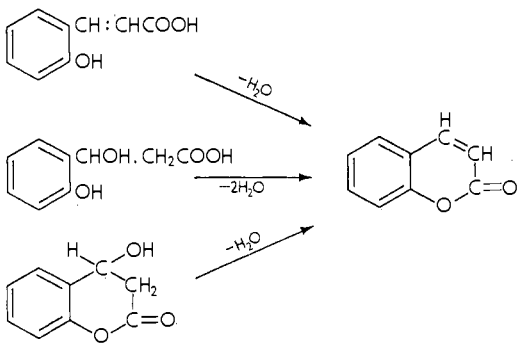
Table. III. The distribution of ^{14}C in the excreta of rabbits and rats given small oral doses (1 mg/kg) of ^{14}C-coumarin

	Rabbits	Rats
Wt. of animals, kg	2·5–3·0	0·15–0·2
Dose of ^{14}C, μc/animal	2	0·1
Duration of experiment, days	3	2
% of dose of ^{14}C in:		
Urine	51[a] (45–56)	50[a] (41–55)
Faeces	11[a] (5–23)	51[a] (45–56)
Total in excreta	62[a] (57–68)	101[a] (97–107)

[a] These figures are the average for 3 animals, the ranges are given in parentheses.

The fate of coumarin in rabbits. When ^{14}C-coumarin is given orally to rabbits at a level of 50 mg/kg, 80 per cent of the ^{14}C is excreted in the urine in 24 h and 90 per cent in 48 h. Three kinds of metabolites are excreted: (*a*) acid-labile precursor or precursors of coumarin, (*b*) hydroxycoumarins, and (*c*) *o*-hydroxyphenyl acids. Only 0·5 per cent of the coumarin is excreted unchanged.

About 15 per cent of the coumarin fed is excreted in the urine as a compound or compounds which yield coumarin on heating with acid. Possible precursors are *o*-coumaric acid and its conjugates, *o*-hydroxyphenylhydracrylic acid, and any of the hypothetical 1,2-dihydro-mono-ols of coumarin of which eight are possible (3,4-dihydro-4-hydroxycoumarin is formulated below). The following formulae show these possibilities:



On heating with 5N HCl for 3 h, *o*-coumaric acid yields coumarin, but using isotope dilution neither *o*-coumaric acid nor its glycine conjugate (see Table II) were found in the urine. *o*-Coumaric acid could therefore be eliminated as a precursor of coumarin. A solution of the labile *o*-hydroxyphenylhydracrylic acid (*β*-*o*-hydroxyphenyl-*β*-hydroxypropionic acid) was prepared by reduction of 4-hydroxycoumarin with sodium amalgam (*cf.* Booth *et al.*[3]) and on heating this solution for 3 h with 5N HCl, coumarin was formed. However, we found no evidence for the presence of *o*-hydroxyphenylhydracrylic acid in coumarin urine, although Booth *et al.*[3] had claimed, on paper chromatographic evidence, that this acid was present in coumarin urine from rabbits. At present, we have no evidence about the nature of the coumarin precursor, which also occurs in coumarin urine from rats (see Table II).

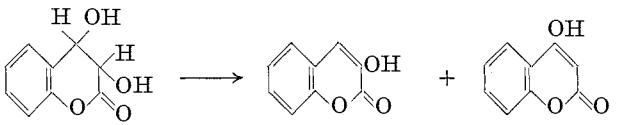
The main urinary metabolites of coumarin in the rabbit are the monohydroxycoumarins, all six of which have been found by isotope dilution methods. These hydroxycoumarins, which account for 40 per cent of the dose, are excreted mainly in conjugated forms, for not more than 4 per cent of the dose of coumarin is excreted as free hydroxycoumarins (see Table II). In the coumarin molecule the position of highest electron density[13] is 3, and this is the position which is hydroxylated to the greatest extent *in vivo*. The percentage hydroxylation and charge densities[13] of the various positions of the coumarin molecule are shown in Table IV. It is to be noted that positions 4 and 5,

Table IV. Biological hydroxylation and charge densities of the coumarin molecule

Position	Charge density[13]	% Hydroxylation in rabbit
3	1·09	21·7 (45)[a]
4	0·861	0·6[b]
5	0·989	0·4[b]
6	1·046	3·4
7	1·008	13·0
8	1·032	1·9

[a] Allowing for conversion into *o*-hydroxy acids (see text).
[b] These figures are approximately the same (see Table II).

which have the smallest charge densities, are also the least hydroxylated; the main positions of hydroxylation are 3 and 7. If the monohydroxycoumarins were formed *in vivo* by direct hydroxylation, then the extent of hydroxylation might be expected to be in the order of charge densities[13] which is $3 > 6 > 8 > 7 > 5 > 4$, but the order of biological hydroxylation in the rabbit is $3 > 7 > 6 > 8 > 5 = 4$. Thus, the extent of hydroxylation corresponds approximately to charge densities except in the case of position 7. However, the hydroxycoumarins could be formed by 'perhydroxylation' followed by dehydration. 'Perhydroxylation' could produce, theoretically, four dihydro-diols: 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-, 6,7-dihydro-6,7-dihydroxy- and 7,8-dihydro-7,8-dihydroxycoumarin. On dehydration, each of these compounds could produce two monohydroxycoumarins, probably in unequal amounts. Thus it would be expected that 3,4-dihydro-3,4-dihydroxycoumarin would dehydrate to give large amounts of 3-hydroxycoumarin and small amounts of 4-hydroxycoumarin. In the case of the 5,6-dihydrodiol, more 6-

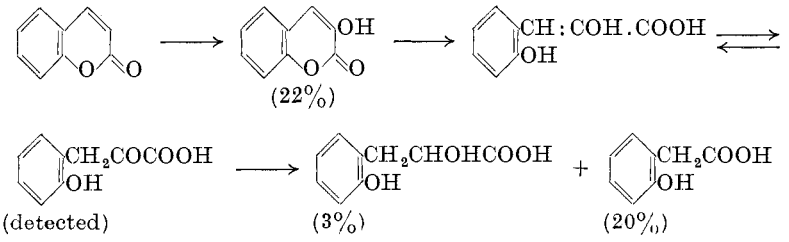


than 5-hydroxycoumarin would be expected and, similarly, with the 6,7-diol more 6- than 7-, and with the 7,8-diol more 8- than 7-hydroxycoumarin. Although 6- and 7-hydroxycoumarins could be produced from two diols each, the amount of 7-hydroxycoumarin found in the urine is much larger than would be expected from this type of reaction as it is greater than the sum of 6- and 8-hydroxycoumarins. During the dehydration of such diols, the hydroxyl group remaining on the molecule might be expected to be located mainly on the carbon atom with the higher charge density (see reference 14 for discussion).

There are 15 possible dihydroxycoumarins, but we found no evidence by paper chromatography for the formation of any one of them *in vivo*. Isotope dilution was carried out for only one of these, namely, 6,7-dihydroxycoumarin (see Table II) but the result was negative.

Nearly one quarter of the dose of coumarin is excreted as

hydroxy acids and isotope dilution was carried out for three of the possible acids. The main hydroxy acid found was *o*-hydroxyphenylacetic acid which accounted for about 20 per cent of the dose. This occurred partly conjugated and partly free (see Table II), but the nature of the conjugation was not investigated. The other hydroxy acid found was *o*-hydroxyphenyllactic acid which was present to the extent of about 3 per cent of the dose. The presence of these acids in the urine was confirmed by paper chromatography. Although Booth *et al.*[3] reported the detection by paper chromatography of both *o*-hydroxyphenylpropionic acid and its glycine conjugate in coumarin urine from rabbits, we were unable to detect the free or conjugated acid by isotope dilution (see Table II) or paper chromatography. The glycine conjugate as such, melilotoylglycine, was not looked for. The detection of *o*-hydroxyphenyllactic acid in the urine suggested that the opening of the heterocyclic ring of coumarin proceeded *via* 3-hydroxycoumarin. This latter compound therefore was fed to rabbits and the urine examined for hydroxy acids. It was found that the main metabolite was 3-hydroxycoumarin glucuronide (see Mead, Smith and Williams[4]), but paper chromatography showed definitely that *o*-hydroxyphenylpyruvic, -lactic, and -acetic acid were also being excreted. It can be concluded, therefore, that the main route of metabolism of coumarin to *o*-hydroxyphenylacetic acid is *via* 3-hydroxycoumarin. The extent of 3-hydroxylation of coumarin is much greater than that accounted for by the 3-hydroxycoumarin excreted and is probably about 45 per cent of the dose, i.e. the sum of 3-hydroxycoumarin plus *o*-hydroxyphenyllactic and -acetic acids (see Table II). The main route of metabolism of coumarin in rabbits is therefore as follows (figures in brackets are amounts found in urine):



The other major hydroxycoumarin excreted is 7-hydroxycoumarin or umbelliferone, which accounted for about 13 per cent of the dose. If the heterocyclic ring of 7-hydroxycoumarin were opened *in vivo*, then 2,4-dihydroxycinnamic acid would be formed, but no evidence for the excretion of this compound was found. Salicylic acid, a possible metabolite of coumarin, was not found (see Mead *et al.*[1]).

The fate of coumarin in rats. The main differences between the fate of coumarin in rats and in rabbits were (*a*) that an appreciable proportion (nearly 40 per cent) of the dose of coumarin given to rats was eliminated in the faeces, and (*b*) that the amounts of hydroxycoumarins excreted in the urine of rats was only 3–4 per cent of the dose (or 6–8 per cent of the urinary radioactivity, see Table II) compared with nearly 40 per cent for rabbits. However, the metabolites found were qualitatively similar to those found in rabbits. The major hydroxycoumarin found (*ca.* 2 per cent of the dose) was 3-hydroxycoumarin, but small amounts of the 4-, 6-, 7- and 8-hydroxycoumarins were also found (5- was not looked for). Both *o*-hydroxyphenyllactic and -acetic acid were present in rat urine in quantities not very different from those found in rabbit urine. It appeared that rats were able to degrade the coumarin molecule *via* 3-hydroxycoumarin more extensively than rabbits. However, we were unable to account for 30–50 per cent of the urinary radioactivity of the urine of rats given ^{14}C-coumarin. Theoretically possible metabolites such as melilotic acid, 2,4-dihydroxycinnamic acid and *o*-tyrosine were looked for, but were not found.

Since rat faeces contained nearly 40 per cent of the dose of coumarin they were examined for metabolites. They were found to contain appreciable amounts, as shown by paper chromatography, of *o*-hydroxyphenylacetic acid together with an unidentified compound. Small amounts of unchanged coumarin were also present.

The toxicity of coumarin has recently been investigated in rats and dogs by Hazleton, Tusing, Zeitlin, Thiessen and Murer,[15] because coumarin has been widely used as a flavour in food and drugs. There appears to be no good data which compare rats with rabbits. In rats, the oral LD_{50} of coumarin is 0·29 g/kg when given as a 10 per cent solution in propylene glycol, and 0·52 g/kg

when given as a 6 per cent solution in corn oil.[15] During other experiments reported from this laboratory,[1] we observed that rabbits receiving 0·2 g/kg orally, suspended in water, frequently died and for most metabolic experiments the dose had to be kept down to 0·1 g/kg to avoid mortality. It would thus appear that coumarin is more toxic to rabbits than to rats. Our present experiments suggest that rats are more able to destroy the heterocyclic ring of coumarin than are rabbits and this could explain the difference in toxicity.

Summary. [3-^{14}C]Coumarin was synthesized *via* salicylaldehyde and diethyl [2-^{14}C]malonate. When fed to rabbits at a dose level of 50 mg/kg, 80 per cent of the ^{14}C of the coumarin was excreted in the urine in 24 h. By isotope dilution the metabolites were found to be coumarin (0·5 per cent), an acid-labile coumarin precursor (14·9 per cent), 3-hydroxycoumarin (21·7 per cent), 4-hydroxycoumarin (0·6 per cent), 5-hydroxycoumarin (0·4 per cent), 6-hydroxycoumarin (3·4 per cent), 7-hydroxycoumarin (12 per cent), 8-hydroxycoumarin (1·9 per cent), *o*-hydroxyphenyllactic acid (3 per cent), and *o*-hydroxyphenylacetic acid (20 per cent). These metabolites accounted for nearly 95 per cent of the excreted radioactivity. No ^{14}C was found in the expired air and very little in the faeces. The hydroxycoumarins were excreted mainly in conjugated forms. In the rabbit, 70 per cent of the excreted radioactivity was in the form of compounds containing the intact coumarin ring, and 30 per cent as compounds in which the heterocyclic ring had been opened.

In the rat, about half the ^{14}C was excreted in the urine and the other half in the faeces. About 3 per cent of the dose was excreted in the urine as hydroxycoumarins and 5 per cent as an acid-labile precursor of coumarin. The main urinary metabolite was *o*-hydroxyphenylacetic acid (20 per cent of the dose). Coumarin was more extensively metabolized by ring opening in rats than in rabbits.

The precursor of *o*-hydroxyphenylacetic acid is probably 3-hydroxycoumarin. When 3-hydroxycoumarin is fed to rabbits it is excreted mainly as conjugated 3-hydroxycoumarin, but to a lesser extent as *o*-hydroxyphenylacetic acid; in rats 3-hydroxycoumarin is mainly excreted as *o*-hydroxyphenylacetic acid.

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