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.

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Studies in Detoxication

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

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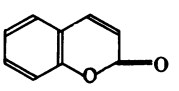
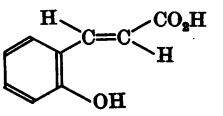
Coumarin (2-oxo-1:2-benzopyran) is the sweet-smelling constituent of white clover, and it has been found in a large variety of plants. Many of its derivatives are pharmacologically active and they include anticoagulant drugs (see Hunter & Shepherd, 1955), rodenticides and insecticides. Coumarin itself 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)

of interest to know something about the metabolism of the *trans*-isomer, *o*-coumaric acid (*o*-hydroxy-*trans*-cinnamic acid, II), since this acid could be a metabolite of coumarin.

A study of the metabolism of the hydroxycoumarins has been described in the preceding paper, which contains data relevant to this work (Mead, Smith & Williams, 1958).

EXPERIMENTAL

Animals. For most of the quantitative data and the isolation of metabolites, chinchilla rabbits kept on a constant diet were used. Other species are referred to in the text.

Reference compounds. Coumarin, m.p. 69–70°, and *o*-coumaric acid monohydrate, m.p. 208–209°, were purchased and purified. *o*-Methoxy-*trans*-cinnamic acid, m.p. 185–186°, was prepared by methylation of *o*-coumaric acid with dimethyl sulphate and NaOH in the usual manner, and melilotic acid (*β*-*o*-hydroxyphenylpropionic acid), m.p. 83–84°, by the action of alkali on dihydrocoumarin (Light and Co.).

Acetyl *o*-coumaryl chloride, m.p. 54° (5 g., Houben & Pfankuch, 1926) and an excess of glycine (10 g.) were shaken with aq. 10% (w/v) Na_2CO_3 soln. (100 ml.) until the chloride had dissolved. The yellow solution was treated with charcoal, filtered and acidified with conc. HCl. A pasty precipitate separated, which was triturated in water until it solidified and then recrystallized from a large volume of hot water. This crude acetyl coumaryl glycine was dissolved in a slight excess of 2N-NaOH and the solution boiled for 5 min. After cooling, and acidifying the solution with conc. HCl, *o-hydroxy*-trans-*cinnamoylglycine* (1 g.) was obtained as colourless prisms, m.p. 226–227° after recrystallization from methanol (Found: C, 59·6; H, 5·0; N, 6·0. $C_{11}H_{11}O_4N$ requires C, 59·7; H, 5·0, N, 6·3%).

Analytical methods. Glucuronic acid and ethereal sulphates were determined as in the preceding paper (Mead *et al.* 1958). Absorption spectra were measured with a Unicam SP. 500 spectrophotometer.

Umbelliferone (7-hydroxycoumarin) in urine was determined fluorimetrically (with an H 553 Spekker fluorimeter) as described by Mead, Smith & Williams (1955).

To determine conjugated umbelliferone in urine, the conditions of hydrolysis of the ethereal sulphate and glucuronide of umbelliferone had first to be investigated. Potassium umbelliferone sulphate added to urine was completely hydrolysed by heating on the water bath for 30 min. with 0·1N-HCl, 1 ml. of urine and 2 ml. of 0·15N-HCl being used. The recovery of umbelliferone, estimated fluorimetrically, was quantitative. Under these conditions only 2% of the glucuronide is hydrolysed. With umbelliferone glucuronide stronger acid and longer heating were necessary. When 1 ml. of urine containing about 0·5 mg. of the glucuronide was heated under reflux for 3 hr. with 2 ml. of 9N-HCl, the recovery of umbelliferone was 80±2%. When umbelliferone was added to normal urine and the solution subjected to the above hydrolysis conditions, it could be recovered quantitatively. The estimation of conjugated umbelliferone in urine was therefore carried out as follows. The urine (1 ml.) was mixed with 0·15N-HCl (2 ml.) and heated on the water bath for 30 min. After cooling, 2 ml. of 0·1N-NaOH was added and the solution was made up to 100 ml. with 0·1M-glycine buffer, pH 10·3. The fluorescence of this solution was measured as such or after further dilution with buffer if necessary. This gave an estimate of umbelliferone conjugated as sulphate. For the estimation of total umbelliferone, 1 ml. of urine and 2 ml. of 9N-HCl were heated under reflux for 3 hr. The solution was neutralized with 3 ml. of 5N-NaOH and diluted to 100 ml. with water. Then 1 ml. of this solution was diluted to 25 ml. with glycine buffer and the fluorescence measured. In calculating the umbelliferone conjugated with glucuronic acid, a correction was made to allow for the fact that only 80% of the glucuronide is hydrolysed under these conditions.

Isolation of metabolites from coumarin

Rabbits were given, by stomach tube, doses of 0·1 g. of coumarin suspended in water/kg. Doses higher than this were too toxic and many of the animals died when given doses of 0·2 g./kg. Preliminary studies on the urine after coumarin dosing showed that it was non-reducing and gave a strong naphtharesorcinol reaction. When acidified, the urine showed a slight blue–green fluorescence in u.v. light, but on neutralizing or adding aq. NH_3 soln. the fluorescence became an intense blue indicating the presence of umbelliferone. Paper chromatography of the urine before and after acid hydrolysis indicated the presence of 3-, 7- and a trace of 8-hydroxycoumarins. *o*-Coumaric acid was not identified, and there were no indications of the presence of 4-, 5- or 6-hydroxycoumarins.

A total of 27 g. of coumarin was fed to several rabbits at the rate of 0·5 g./rabbit/day. The urine (5 l.) and faeces (320 g.) were collected for 5 days. The urine was worked up by systematic lead acetate precipitation as described by Kamil, Smith & Williams (1951). The precipitate from normal lead acetate contained no appreciable quantities of metabolites.

Isolation of 3-hydroxycoumarin conjugates. The basic lead acetate precipitate was freed of lead and the filtrate concentrated *in vacuo* at 40–45° to 200 ml. After keeping overnight at 0°, two kinds of crystals separated: small plates (*A*) filling the bulk of the solution and small needles (*B*) adhering to the bottom of the flask. The crystals were separated by decantation.

The crystals *A* (0·78 g.) were neutral in reaction and gave a strong test for ethereal sulphate and potassium. They were recrystallized from 80% ethanol and formed plates which did not melt on heating to 360°. They were identified as potassium 2-oxo-1:2-benzopyran-3-yl sulphate (Found: K, 14·1; S, 11·3. Calc. for $C_9H_5O_6SK$, K, 13·9; S, 11·4%). On hydrolysis with 0·3N-HCl they yielded 3-hydroxycoumarin, m.p. and mixed m.p. 153°. On paper chromatograms they behaved identically with the ethereal sulphate of 3-hydroxycoumarin (Mead *et al.* 1958).

The crystals *B* (0·46 g.) were acid in reaction, non-reducing, and gave a strong naphtharesorcinol reaction, but no ethereal sulphate test. They were recrystallized from water as colourless needles, m.p. 207–208° (decomp.) and $[\alpha]_D^{20} - 98·5°$ in N-NaOH (*c*, 1). They were identified as 2-oxo-1:2-benzopyran-3-yl β-D-glucosiduronic acid (mixed m.p. 207–208°, decomp.) and their chromatographic behaviour was identical with that of authentic samples of this glucuronide (Mead *et al.* 1958). Concentration of the mother liquor from *A* and *B* gave a further yield (1·06 g.) of this glucuronide (m.p. 207–208° and $[\alpha]_D^{20} - 98°$ in N-NaOH).

Isolation of 7-hydroxycoumarin. The filtrates (50 ml.) from the isolation of the 3-hydroxycoumarin conjugates were now treated with acetone (500 ml.), and on keeping overnight a gummy precipitate separated. The acetone was decanted from the precipitate, which was acid, non-reducing and gave a strong naphtharesorcinol reaction and a slight ethereal sulphate test. No crystalline product could be isolated from the precipitate. It was therefore dissolved in 5N-HCl, heated under reflux for 3 hr., cooled and extracted continuously with ether for 24 hr. The ether extract was then chromatographed on a cellulose-powder column (Hough, 1954), ethyl methyl ketone saturated with aq. 2N-NH_3 soln. being used as solvent. Umbelliferone was known to be present in this fraction and could be followed on the column by its fluorescence in u.v. light. The main fluorescent band on the column was due to this compound, although there were other minor fluorescent bands which on elution gave only minute quantities of solids. The umbelliferone eluate was concentrated to yield 157 mg. of 7-hydroxycoumarin, m.p. and mixed m.p. 230–231° after recrystallization from water (charcoal). The acetone decanted as above was evaporated to yield a gum, which on similar treatment yielded 23 mg. of 7-hydroxycoumarin.

The filtrate (5 l.) from the basic lead acetate precipitate was concentrated *in vacuo* to 500 ml. and treated with ethanol, and the bulky inorganic precipitate was filtered off. The filtrate was evaporated to a syrup which was hydrolysed, extracted with ether and chromatographed on a cellulose column as described in the preceding paragraph. This fraction yielded a further 210 mg. of 7-hydroxycoumarin (total yield, 390 mg.). The 7-hydroxycoumarin (350 mg.) was acetylated with acetic anhydride and anhydrous sodium acetate and yielded 370 mg. of 7-acetoxycoumarin, m.p. and mixed m.p. 140–141° after recrystallization from hot water (charcoal) (Found: umbelliferone, 77·1 %, determined fluorimetrically. Calc. for $C_{11}H_8O_4$, umbelliferone, 79·4 %).

Examination of the faeces. The dried faeces were extracted with ether in a Soxhlet extractor. Paper chromatography of the extract revealed no coumarin or hydroxycoumarin.

Examination of tissues. A rabbit which had received 170 mg. of coumarin/kg. died 12 hr. later. The gastric contents appeared to contain no coumarin, by the sensitive test of Feigl, Feigl & Goldstein (1955). It was, however, detected readily in the visceral fat.

Hydroxylation of coumarin in various species. Coumarin was administered to five species (see Table 3) and the 24 hr. urine collected. It was made 5N with respect to HCl by the addition of conc. HCl and then heated under reflux for 3 hr. After cooling, the hydrolysate was continuously extracted with ether for 6 hr. The extract was evaporated *in vacuo* to a gum and a small portion of this gum was chromatographed on Whatman no. 1 paper. It was placed at the origin with a platinum loop and then irrigated with the solvents (see preceding paper). The metabolites were detected as previously described (Mead *et al.* 1958) and the findings are summarized in Table 3.

Isolation of metabolites from o-coumaric acid

o-Coumaric acid (1 g./rabbit) was administered by stomach tube dissolved in aq. $NaHCO_3$ soln. The urine was slightly reducing towards Benedict's reagent and gave a strong naphtharesorcinol test. The urine as such (one drop) when chromatographed on paper in solvents *A* and *B* (Mead *et al.* 1958) appeared to contain *o*-coumaric acid, 4- and 7-hydroxycoumarins, *o*-hydroxy-*trans*-cinnamoylglycine and a substance which quenched the background fluorescence of the paper in u.v. light. This last substance appeared to be the glucuronide of *o*-coumaric acid. After hydrolysis (0·5 ml. of urine + 0·5 ml. of conc. HCl heated for 5 min.) chromatography showed increased amounts of *o*-coumaric acid, and of 4- and 7-hydroxycoumarins. The *o*-hydroxy-*trans*-cinnamoylglycine spot and the quenching spot had disappeared. Several other spots were detected on the paper by various reagents, but were not identified. 3-, 5-, 6- and 8-Hydroxycoumarins, melilotic acid and salicylic acid were not detected in either the unhydrolysed or hydrolysed urines.

Melilotic acid (*o*-hydroxyphenylpropionic acid) also was fed to rabbits and the urine examined chromatographically. The 24 hr. urine of a rabbit which had received an oral dose of 1 g. of the acid was examined as above for *o*-coumaric acid. The urine gave a strong naphtharesorcinol reaction, was slightly reducing but gave no colour reaction with $FeCl_3$ soln. It fluoresced in u.v. light, a pale blue–green in acid and neutral conditions and an intense yellow–green when treated with NH_3 soln. Ether extracts of the acidified urine were chromatographed and spots corresponding to melilotic acid,

o-coumaric acid, *o*-hydroxy-*trans*-cinnamoylglycine and 4- and 7-hydroxycoumarins were found.

Isolation of 4-hydroxycoumarin glucuronide. A total of 6 g. of *o*-coumaric acid (as sodium salt) was fed to six rabbits. The 24 hr. urine (760 ml.) gave a strong naphtharesorcinol reaction and slightly reduced Benedict's reagent. On making strongly alkaline with aq. NH_3 soln. the urine fluoresced an intense greenish yellow owing to the presence of free *o*-coumaric acid. The glucuronide fraction of the urine was separated through basic lead acetate and the lead-free filtrate was reduced *in vacuo* to 50 ml. This solution was strongly reducing to Benedict's reagent. Excess of ethanol was added and a precipitate of inorganic material was discarded. The solution (200 ml.) was again reduced *in vacuo* to 50 ml., treated with acetone (350 ml.) and kept overnight at 0°. The acetone was decanted from the gum which had formed. This gum was now taken up in ethanol and the solution concentrated *in vacuo* to 10 ml. On keeping at 0° crystals (30 mg.) of a glucuronide separated. These were filtered off and the filtrate was examined later (see below). The crystals reduced hot Benedict's reagent and contained some inorganic material. They were therefore dissolved in 2 ml. of water and a few drops of 2N-HCl added. On keeping at 0° the glucuronide separated as long needles. Further recrystallization yielded 9 mg. of crystals, which were identified as 2-oxo-1:2-benzopyran-4-yl glucosiduronic acid, m.p. and mixed m.p. 184–185°. Its u.v. spectrum in ethanol was identical with that of the authentic glucuronide (see Table 1). On hydrolysis with 5N-HCl or locust-crop liquor containing β-glucuronidase at pH 4·6 it yielded 4-hydroxycoumarin, which was identified chromatographically in two solvents (see Mead *et al.* 1958).

Isolation of o-glucosidurono-trans-cinnamic acid. The filtrate from the separation of 4-hydroxycoumarin glucuronide described in the preceding paragraph was acidified further with a few drops of 2N-HCl, treated with charcoal and filtered. On keeping overnight at 0° a glucuronide (66 mg.) (naphtharesorcinol test) separated with m.p. 228–229° (decomp.) after recrystallization from water (charcoal). It was non-reducing and contained no free phenolic groups, but was optically active, $[\alpha]_D^{18} - 81·3°$ in 0·1N-NaOH (*c*, 0·8). It analysed as the *hemihydrate* of o-β-D-*glucosidurono*-trans-*cinnamic acid* (Found: C, 51·6; H, 4·8. $C_{15}H_{16}O_9,0·5H_2O$ requires C, 51·6; H, 4·9%). The water was not lost on heating at 110°. It titrated with 0·02N-NaOH as a dibasic acid, the equivalent weight found being 174·6, which is half the required mol.wt. 349·3. On hydrolysis with 5N-HCl or with locust-crop liquor containing β-glucuronidase it yielded *o*-coumaric acid, which was identified by its R_F value in four solvents (see Mead *et al.* 1958) and its characteristic blue–green fluorescence at alkaline pH values in u.v. light. Its u.v. absorption spectrum would be expected to be similar to that of *o*-methoxy-*trans*-cinnamic acid. The spectra of these two compounds together with those of coumarin, *o*-coumaric acid and *o*-hydroxy-*trans*-cinnamoylglycine are given in Table 1. The major band in all four compounds is at 274 mμ, and the $\epsilon_{max.}$ of the glucuronide has a *trans* value (i.e. about 18 000), whereas $\epsilon_{max.}$ for coumarin, a *cis*-derivative, is 11 000. The methyl ether of coumaric acid, however, shows a peak at 225 mμ, and this appears also in the glucuronide, which is similarly an ether. This supports the view that the compound is an ether glucuronide of *o*-coumaric acid.

Isolation of o-coumaric acid. This compound sometimes separated from the lead-free filtrate of the glucuronide fraction of the urine. In one experiment in which 12 g. of *o*-coumaric acid had been fed, 1 g. of the free acid, m.p. 208–209°, was isolated by continuous ether extraction of the acidified urine for 24 hr.

Action of ascorbic acid and Fenton's reagent on coumarin

Udenfriend, Clark, Axelrod & Brodie (1954) have shown that the pattern of oxidation of a number of organic compounds by a system containing ascorbic acid, Fe^{2+} ions and ethylenediaminetetra-acetic acid was similar to that found *in vivo*. Boyland & Sims (1953), using Fenton's reagent, which is regarded as a source of hydroxyl-free radicals, showed that coumarin was oxidized to 7-hydroxycoumarin. Since all the monohydroxycoumarins

Table 1. *Ultraviolet-absorption spectra in ethanol of coumarin, o-coumaric acid and some of their derivatives*

	Wavelength of absorption peaks ($\lambda_{max.}$) (mμ)			Corresponding molecular-extinction coefficients (10^{-3} $\epsilon_{max.}$)		
Coumarin	—	274	310	—	11·1	5·7
4-Hydroxycoumarin	—	268, 278	304	—	10·7, 11·6	8·5
4-Glucosiduronocoumarin	—	268, 278	304	—	10·6, 10·2	6·6
o-Hydroxy-*trans*-cinnamic acid	—	274	325–326	—	18·3	10·1
o-Hydroxy-*trans*-cinnamoylglycine	—	274	324–325	—	18·4	10·3
o-Methoxy-*trans*-cinnamic acid	225	274	318–319	13·8	16·0	8·9
o-Glucosidurono-*trans*-cinnamic acid	225	274	310	12·8	17·6	8·4

were available, we have investigated qualitatively the action of the ascorbic acid–iron system and of Fenton's reagent on coumarin.

The experiments were carried out as follows. Coumarin (2·4 m-moles) was shaken at 37° in a 500 ml. flask with 100 ml. of phosphate buffer, pH 6·7. The solution was then saturated with oxygen gas and a mixture (10 ml.) containing $FeSO_4$, $7H_2O$ (0·52 m-mole), disodium salt of ethylenediaminetetra-acetic acid (2·5 m-moles) and ascorbic acid (5·6 m-moles) was added to the solution with shaking. The flask was stoppered and kept at 37° with constant shaking for 2 hr. Small samples were removed every 15 min. and chromatographed on paper. 7-Hydroxycoumarin was detected within 15 min. and was present throughout the 2 hr. period. The reaction mixture was extracted three times with 100 ml. portions of ether. The extract was evaporated and the small gummy residue was chromatographed in solvents *B* and *C* (Mead *et al.* 1958). The results are shown in Table 3. Fluorimetric determination of 7-hydroxycoumarin showed that 0·2 % of the coumarin had been oxidized to umbelliferone in 2 hr. The major products appeared to be 5- and 7-hydroxycoumarins, with traces of 6-hydroxycoumarin and *o*-coumaric acid. No 3-, 4- or 8-hydroxycoumarin or salicylic acid was found.

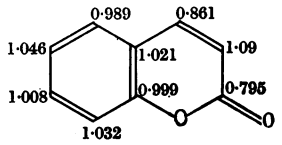
Similar experiments were carried out in which the coumarin was replaced by aniline, nitrobenzene, cyanobenzene and benzoic acid. The phenols formed were detected by colour reactions and paper chromatography and the results are shown in Table 4.

The experiments with Fenton's reagent and coumarin were carried out as described by Boyland & Sims (1953). The ether extract was chromatographed in solvents *A*, *B* and *C*. 3-, 5- and 7-Hydroxycoumarins were readily detected together with traces of 6- and 8-hydroxycoumarins and *o*-coumaric acid. 4-Hydroxycoumarin and salicylic acid were not found.

DISCUSSION

Metabolism of coumarin. From a quantitative point of view the recovery of coumarin as urinary metabolites (see Table 2) amounts to no more than 25 % of the dose (0·1 g./kg.), and at present we have no information regarding the rest of the material. Qualitatively, however, it has been shown that in the rabbit coumarin is hydroxylated in the 3-, 7- and 8-positions, and the impression is gathered from the amounts of the metabolites isolated that 3-hydroxycoumarin is the major hydroxylated product. No evidence was obtained of ring fission, and *o*-coumaric acid and its β-oxidation product, salicylic acid, were not found as metabolites. The above three hydroxycoumarins are also formed in the ferret, guinea pig, mouse and rat, and in the first three of these animals 5-hydroxycoumarin was found as well. Coumarin was not hydroxylated in the 4- or 6- positions in any of the species examined (see Table 3). The absence of 4-hydroxycoumarin supports the view that coumarin probably does not suffer ring fission to *o*-coumaric acid, since 4-hydroxycoumarin is a metabolite of this acid (see below).

The charge densities of the carbon atoms of coumarin have been given by Isaac (1955) (see below) and the positions of highest-charge density are in the descending order, 3-, 6- and 8-. Position 4 is one of low-charge density.



Electrophilic substitution of coumarin may therefore occur at positions 3-, 6- and 8-, whereas nucleophilic substitution occurs at 4. Now Fenton's reagent is regarded as a source of hydroxyl-free radicals and with this reagent some hydroxylation of coumarin occurs at all positions except 4 (see Table 3). *In vivo*, hydroxylation occurs at all positions except 4 and 6. The ascorbic acid–iron system, however, did not yield the 3-, 4- or 8-hydroxycoumarins. Neither of the systems *in vitro* produced the same results as those obtained *in vivo*, except that none of them produced 4-hydroxycoumarin. The ascorbic acid–iron system, however, did hydroxylate aniline, nitrobenzene and cyanobenzene in the same positions as found *in vivo* (see Table 4). The main difference between the hydroxylation of coumarin *in vivo* and by the ascorbic acid–iron system *in vitro* was the absence of 3-hydroxycoumarin formation by the system *in vitro*. This is interesting because when coumarin is incubated with rabbit-liver microsomes, suitably fortified, the formation of 7- but not of 3-hydroxycoumarin is readily detected (unpublished data). It is thus possible that the 3-hydroxylation of coumarin occurs by a different mechanism from 5-, 7- and 8-hydroxylation. This might be expected since the 3:4 double bond of coumarin possesses true double character, whereas those in the benzo ring are aromatic in character.

The precursors of the hydroxycoumarins *in vivo* could be 1:2-dihydro-1:2-diols. Four of these could be postulated, namely, the 3:4-, 5:6-, 6:7- and 7:8-dihydrodiols. On dehydration, these compounds would be expected to yield mainly 3-, 6- and 8-hydroxycoumarins with smaller amounts of 5- and

7-hydroxycoumarins, but practically no 4-hydroxycoumarin since the 4-position of coumarin is of low-charge density. The formation of these diols in different amounts could in fact explain the formation *in vivo* of 3-, 5-, 7- and 8-hydroxycoumarins from coumarin.

Metabolism of o-coumaric acid. This acid (p*K* 4·6–4·7) is the *trans*-form of coumarinic acid (*o*-hydroxy-*cis*-cinnamic acid) which only exists as salts and normally occurs as the lactone, coumarin. We obtained no evidence that coumarin was converted *in vivo* into *o*-coumaric acid, but the latter on the other hand is converted into coumarin derivatives *in vivo*.

o-Coumaric acid, although it appears to be excreted unchanged to a considerable extent,

Table 2. *Conjugation of coumarin and o-coumaric acid*

Figures given are the averages for three animals (except where otherwise stated), the ranges being given in parentheses.

Compound fed	Dose (mg./kg.)	Percentage of dose excreted as: Glucuronide	Ethereal sulphate	Total conjugation	Umbelliferone excretion* (% of dose): As glucuronide	As ethereal sulphate	Total
Coumarin	100	18 (17–18)	7 (6–7)	25	5 (3–6)[5]	6 (5–8)[6]	11
o-Coumaric acid	200	14 (9–17)	1 (0–2)†	14	—	—	—

* Umbelliferone excretion was determined in a separate set of experiments and the number of animals used is indicated by the superior figures outside the parentheses.

† These figures are within the experimental error of the method and are not significant.

Table 3. *Metabolites of coumarin in various species compared with the products of the action of ascorbic acid and of Fenton's reagent on coumarin*

Doses of coumarin: ferrets, 0·1 g./kg. orally with meat; guinea pigs, 0·14 g./kg. orally in gelatin capsules; mice, 0·4 g./kg. orally in arachis oil (this dose was fatal in 12–24 hr.); rabbits, 0·17 g./kg. orally in water; rats, 0·2 g./kg. orally in arachis oil or in gelatin capsules, and subcutaneously in arachis oil.

+, Indicates that the compound was readily detected by paper chromatography; −, the compound was not found; tr., the compound was detected in traces.

Metabolites sought	Metabolites found in the urine of: Ferret	Guinea pig	Mouse	Rabbit	Rat	Products of the action of: Ascorbic acid	Fenton's reagent
3-Hydroxycoumarin	+	+	+	+	+	−	+
4-Hydroxycoumarin	−	−	−	−	−	−	−
5-Hydroxycoumarin	tr.	+	+	−	−	+	+
6-Hydroxycoumarin	−	−	−	−	−	tr.	tr.
7-Hydroxycoumarin	+	+	+	+	+	+	+
8-Hydroxycoumarin	+	+	+	+	+	−	tr.
6:7-Dihydroxycoumarin	−	−	−	−	−	.	.
o-Coumaric acid	−	−	−	−	−	tr.	tr.
Melilotic acid	−	−	−	−	−	.	.
Salicylic acid	.	.	.	−	.	−	−

Table 4. *Hydroxylation of some monosubstituted benzenes by ascorbic acid–iron compared with that found* in vivo

+, Indicates that the phenol was formed; −, indicates that it was not; where negative results were obtained a check was made on the stability of the authentic phenol in the presence of the reagents. A blank space indicates that authentic phenol was not available for comparison.

Position of hydroxylation	Aniline: Ascorbic acid–Fe	*in vivo**	Nitrobenzene: Ascorbic acid–Fe	*in vivo*†	Cyanobenzene: Ascorbic acid–Fe	*in vivo*‡	Benzoic acid.§ Ascorbic acid–Fe
ortho-	+	+	−	tr.	+	+	+
meta-	−	−	+	+	+	+	+
para-	+	+	+	+	+	+	+
2:3-	.	.	−	−	.	.	−
2:5-	.	.	+	+	.	.	+
3:4-	.	.	+	+	+	+	−

* Smith & Williams (1949); Parke & Williams (1956).

† Parke (1956).

‡ Smith & Williams (1950); Bray, Hybs & Thorpe (1951).

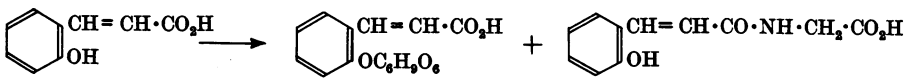
§ Benzoic acid is not hydroxylated *in vivo*.

undergoes two types of reactions *in vivo*, namely, direct conjugations at both the carboxyl and hydroxyl groups, and cyclization. Conjugation with glucuronic acid at the phenolic hydroxyl group occurs to a small extent and this was proved by the isolation of the non-reducing *o*-glucosiduronono-*trans*-cinnamic acid. There was no evidence that it formed an ethereal sulphate and in this respect it behaved like salicylic acid, of which it is a vinylogue. Conjugation at the carboxyl group was proved by the detection of *o*-hydroxy-*trans*-cinnamoylglycine in the urine. Whether or not it formed a reducing ester glucuronide (i.e. *o*-hydroxycinnamoyl glucuronide) was not proved. The urine was slightly reducing but this could have been due entirely to the reducing 4-glucosiduronocoumarin which was isolated from the urine. The conjugation reactions of *o*-coumaric acid appear to be those shown in scheme 1.

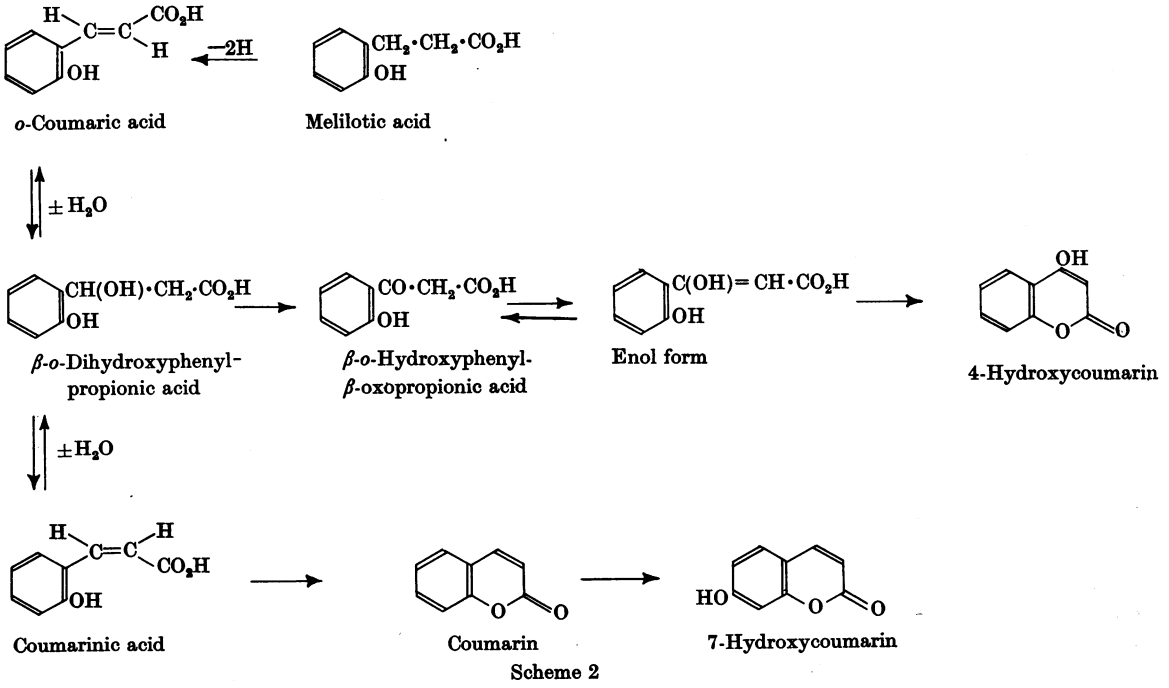
The more interesting aspect of *o*-coumaric acid metabolism is its conversion into the coumarin derivatives, 4- and 7-hydroxycoumarins, which involves a *trans–cis*-isomerization and a cyclization *in vivo*. The formation of the coumarin derivatives could be explained by assuming that *o*-coumaric acid undergoes the normal processes of β-oxidation (although it is not finally oxidized to salicylic acid) and that some of the intermediates of β-oxidation cyclize according to scheme 2.

In scheme 2 it is assumed that coumaric acid (and melilotic acid) undergoes β-oxidation as far as the keto acid stage, the enol form of the keto acid then cyclizing to 4-hydroxycoumarin. For the formation of 7-hydroxycoumarin, the intermediate formation of coumarin is tentatively postulated. Coumarin could be formed via coumarinic acid from β-*o*-dihydroxyphenylpropionic acid. If coumarin were formed, then 3- as well as 7-hydroxycoumarin could be expected, but only the 7-derivative was detected.

The occurrence in the urine of phenylketonurics of *ortho*-hydroxylated compounds derived from phenylalanine (see Boscott & Bickel, 1954) suggests that, in view of our findings with *o*-coumaric acid, it is not impossible that coumarin derivatives may be trace metabolites of phenylalanine and should be searched for.



Scheme 1



Scheme 2

SUMMARY

1. Coumarin when fed to rabbits is hydroxylated to 3-, 7- and 8-hydroxycoumarins, which are excreted in conjugation. This also occurs in the ferret, guinea pig, mouse and rat, the first three of which also excrete 5-hydroxycoumarin. However, not more than 25 % of the coumarin fed (dose 0·1 g./kg.) can be accounted for as conjugated glucuronic acid and ethereal sulphates excreted in the urine.

2. The ethereal sulphate and glucuronide of 3-hydroxycoumarin were isolated from the urine of rabbits fed on coumarin. 7-Hydroxycoumarin was also isolated after acid hydrolysis of the urine. 5- and 8-Hydroxycoumarins were not isolated but were detected in the hydrolysed urine by paper chromatography. The hydroxylation of coumarin has also been carried out with Fenton's reagent and an ascorbic acid–iron system. The results, which were not exactly the same as those found *in vivo*, have been discussed.

3. *o*-Coumaric acid appears to be excreted unchanged to a large extent by rabbits. It is, however, directly conjugated to a small extent since its ether glucuronide, *o*-glucosidurono-*trans*-cinnamic acid, was isolated and its glycine conjugate, *o*-hydroxy-*trans*-cinnamoylglycine, was detected by paper chromatography in the urine.

4. *o*-Coumaric acid is also cyclized *in vivo*, since small amounts of the glucuronide of 4-hydroxycoumarin were isolated from the urine and 7-hydroxycoumarin was detected chromatographically. Melilotic acid, the corresponding saturated acid (dihydrocoumaric acid), also yields 4- and 7-hydroxycoumarin in the rabbit.

5. The mechanism of the cyclization of *o*-coumaric acid has been discussed and it is suggested that the coumarin derivatives formed arise from β-oxidation products of *o*-coumaric acid.

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The Preparation and Properties of Lithium Hydroxypyruvate and Hydroxypyruvic Acid

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Interest in the possible biological importance of hydroxypyruvate has been revived by the recent demonstrations that it acts as an effective substrate for the L-lactic dehydrogenases of muscle (Meister, 1952) and yeast (Dickens & Williamson, 1956*a*), and for the specific D-glyceric dehydrogenase (hydroxypyruvate reductase) present in certain higher plants (Stafford, Magaldi & Vennesland, 1954), being thus reduced respectively to L- or D-glyceric acid. Hydroxypyruvate is also a substrate for transketolase (Horecker, Smyrniotis & Klenow, 1953; Haba, Leder & Racker, 1955) and for carboxylases of yeast (Dickens & Williamson, 1956*b*) and of muscle (Dickens & Williamson, unpublished work). A transaminase present in animal tissues is able to transfer the amino group of L-alanine to