

A STUDY OF THE METABOLISM OF ^{Certain}~~SOME~~ HETEROCYCLIC COMPOUNDS,
PARTICULARLY COUMARIN AND INDOLE.

By

MURRAY KAIGHEN

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Department of Biochemistry,
St. Mary's Hospital Medical School,
London, W.2.

Abstract of Thesis.

3-[^{14}C]Coumarin has been synthesised. Rabbits dosed with the labelled compound (50 mg./kg.) excreted nearly all the radioactivity in the urine within 48 hours; in rats (100 mg./kg.) the activity was almost equally distributed between urine and faeces. The metabolites in rabbits, measured by isotope dilution were: coumarin (0.5%), an acid-labile coumarin precursor (15.2%), 3-hydroxycoumarin (21.7%), 4-hydroxycoumarin (0.6%), 5-hydroxycoumarin (0.4%), 6-hydroxycoumarin (3.4%), 7-hydroxycoumarin (1.3%), 8-hydroxycoumarin (1.9%), *o*-hydroxyphenylacetic acid (20%), and *o*-hydroxyphenyllactic acid (3%). The hydroxycoumarins were mainly, and *o*-hydroxyphenylacetic acid partly, in conjugated forms. These metabolites accounted for nearly 95% of the excreted radioactivity, 70% of which was in the form of compounds containing the intact coumarin ring, and 30% as compounds in which the heterocyclic ring had been opened. Rats metabolise coumarin by ring opening to a greater extent than rabbits: only 3% of hydroxycoumarins were found as metabolites in the rat, whereas the amounts of hydroxyacids formed were similar to those in rabbits. No ^{14}C was found in the expired air of rabbits or rats.

3-Hydroxycoumarin is excreted by rabbits mainly as conjugates, but also as *o*-hydroxyphenylacetic and -lactic acids; in rats it is excreted mainly as the hydroxyacids. Coumarin is probably converted to the hydroxyacids by this route.

The extent to which the different positions of the coumarin molecule are hydroxylated in the animal is discussed in relation to the electron densities of the positions, and the stability of the lactone ring in vivo is also discussed.

About half of a dose of indole fed to rabbits (100 mg./kg.) is converted to indoxyl, of which roughly 60% is excreted as indoxyl sulphate and 40% as the glucuronide. Evidence was obtained for 5-hydroxylation of indole in vivo.

Preface.

The work described in this Thesis was carried out between January, 1957 and January, 1960 in the Department of Biochemistry, St. Mary's Hospital Medical School, under the supervision of Professor R.T. Williams. During part of this time, the work was aided by grants from the Medical Research Council and the American Instrument Co., Inc. For the rest of the time, funds were provided through the generosity of Professor Williams.

I am deeply indebted to Professor R.T. Williams, who made this work possible and guided it with great insight and judgment at every stage. I am grateful to Dr. D.V. Parke who freely gave help, advice and encouragement without regard to time or trouble, and to Dr. J.N. Smith for making available his knowledge and experience in many a discussion. Thanks are also due to the other members of the staff of the Biochemistry Department and to my colleagues for advice and discussion, and to Mr. Audas and his technical staff.

Vast numbers of compounds of synthetic and natural origin are today used as food additives, insecticides, crop sprays and drugs. The effects of these compounds on men or domestic animals and their metabolism are often completely unknown, and cases are known in which harmful effects have resulted from this ignorance. Coumarin has been widely used as a flavouring for foods and tobacco, and indole is normally formed in the body in appreciable amounts. Both of these compounds are toxic to animals and it seemed important therefore to try to elucidate their fate in the body as a contribution to understanding the cause of their toxicity.

M. Kaighen.

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M. Kaighen.

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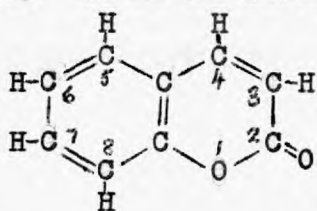
Chapter 1. Introduction.

The whole of this part of the thesis consists of a description of the metabolism in the animal of coumarin and two of its simple derivatives, 3-hydroxy- and 4-hydroxycoumarin. The only species used in these metabolic studies were rabbits and rats. The present investigation was undertaken as a continuation of the work of Mead et al. (1958a, b) with the aim of elucidating quantitatively the fate of coumarin in the mammal.

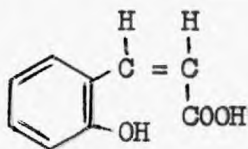
Outline of the Chemistry of Coumarin and its Simple Derivatives.

It is appropriate to commence with an account of the chemistry of the coumarin group of compounds, such as may help the subsequent discussion of the problem. The chemistry of coumarin has been extensively reviewed by Sethna and Shah (1945) and Wawzonek (1951). Most of the statements in what follows have been taken from these reviews; others will be separately acknowledged.

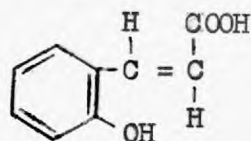
General. Coumarin is 2-oxo-1:2-benzopyran, the structure and numbering are given below. It is the lactone of o-hydroxy-cis-cinnamic



acid (coumarinic acid), the trans-form of which is o-coumaric acid.



o-hydroxy-cis-cinnamic acid



o-hydroxy-trans-cinnamic acid

The properties of coumarin are chiefly those of an unsaturated lactone, modified by the presence of the benzene ring.

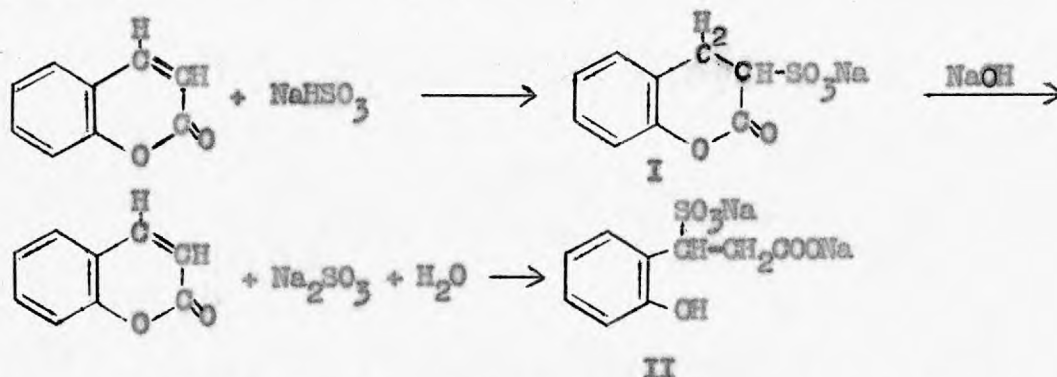
Properties of the 3,4-bond. The reactions of the 3,4-double bond of the heterocyclic ring are to be distinguished from those of the benzene ring, which are aromatic in nature, though not markedly so. The 3,4-double bond shows a marked tendency to add on reagents.

Coumarin can be reduced by a number of reagents under different conditions to form a variety of products. Catalytic reduction with Raney nickel at lower temperatures or with palladium and charcoal converts coumarin to dihydrocoumarin (melilotic anhydride), the 3,4-double bond only being affected. Further reduction to octahydrocoumarin may be brought about by Raney nickel at 200-250°C. By use of dilute solutions and sodium amalgam, or zinc and alkali, reduction of the 3,4-bond and ring opening occur with formation of o-hydroxyphenylpropionic acid (melilotic acid). A more complete reduction is achieved with copper chromite or sodium and alcohol, which yields γ -(o-hydroxyphenyl)propyl alcohol.

The 3,4-double bond adds bromine to form the 3,4-dibromide, which readily loses hydrogen bromide through the action of pyridine, leaving 3-bromocoumarin. This is an example of the saturation of the double bond under one set of conditions, and the regeneration of unsaturation under another. Mercuric salts will also add to the double bond, and here too the unsaturation may in certain cases be restored.

4-Cyanodihydrocoumarin is formed by addition of potassium cyanide. However, ammonia will not add to coumarin.

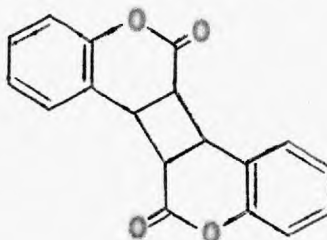
Sodium bisulphite adds very easily to coumarin to form sodium hydrocoumarin sulphonate (I) (Dodge, 1916). The sulphonate radical can be split off by addition of alkali with formation of neutral sulphite and coumarin, the latter combining with the sulphite to form the corresponding sulphonylhydrocoumaric acid (II):



The open chain sulphonate derivative can be converted fairly readily to o-coumaric acid by use of excess alkali. The same compound (II) can be lactonised using acetic anhydride, and the resulting hydrocoumarin-sulphonate (I) can be converted to coumarin with alkali. This affords a useful way of passing to the trans-series from coumarin, and back again. Dey and Row (1924) indicated that the sulphonic group of the hydro-coumaric compound (II) was in position 4.

The coumarin molecule has remarkable powers of self-combination. This is illustrated by the formation of an acid precursor of dihydrodicoumarin when concentrated solutions of the coumarin are reduced with sodium amalgam, zinc and alkali, or zinc and acetic acid. This dihydrodicoumarin is said to be joined through the 3,3'-positions. The tendency of coumarin to combine with itself through the 3,4-bond is also shown by the action of light on coumarin, which causes dimerisation. A

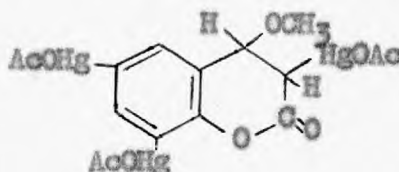
cyclobutane structure between the two coumarin moieties is postulated.



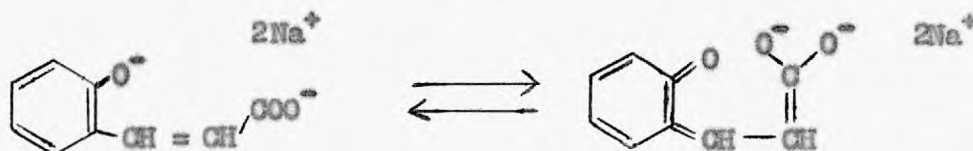
Substitution reactions. Coumarin is substituted mainly in the 6-position, and less readily in positions 8 and 3. Thus nitration gives mainly 6-nitrocoumarin with a little 8-nitrocoumarin. On further nitration, the former gives 3,6-dinitrocoumarin and the latter 6,8-dinitrocoumarin. The aromatic ring is more readily nitrated if it contains a hydroxyl group - thus it behaves like benzene.

Similarly sulphonation gives rise first to 6-coumarinsulphonic acid, then to 3,6-coumarin disulphonic acid. In the Elbs alkaline persulphate oxidation, position 6 is hydroxylated, and 6-aldehydocoumarin is formed by the Reimer and Tiemann reaction. Diazonium salts couple in position 6.

Halogenation of coumarin leads to an addition reaction at the 3,4-positions as described in the preceding section, but more vigorous treatment will give rise to 3,6- and 3,6,8- substituted derivatives. Coumarin reacts with mercuric acetate in methanol to give 3,6,8-tri-acetoxymercuri-4-methoxydihydrocoumarin, so that here too, true aromatic substitution takes place as well as addition to the double bond.



Opening of the lactone ring. The lactone ring of coumarin is opened by action of alkali with formation of a yellow salt of coumarinic acid. This has been assigned the o-quinonoid structure.



The conditions of ring opening vary for different coumarins. Coumarin itself is converted to the coumarinate at pH10. Coumarinic acid cannot exist in the free state and acidification of the coumarinate regenerates coumarin.

Prolonged action of alkali causes a cis - trans isomerisation with formation of the coumarate. At the same time a colour change takes place from yellow to a yellow-green fluorescence. The stable o-coumaric acid can be liberated from the salt by acidification. The cis-trans isomerisation can be effected more easily by the action of sodium ethoxide on coumarin; by boiling coumarin in dilute alkali with mercuric oxide and mercuric acetate (Sen and Chakravarti, 1930); or particularly by shaking in cold alkaline solution with freshly precipitated yellow mercuric oxide (Seshadri, 1954). Inversion can also be very conveniently achieved by addition of bisulphite followed by alkali treatment (p. 3). Seshadri (1934) attributes the catalytic action of the above agents as involving a preliminary addition to the 3,4-double bond, thereby facilitating rotation. The cis-trans inversion is caused by a repulsion between the negatively charged O and CO₂ groups.

The reverse change (trans to cis) can be brought about by heating o-coumaric acid with concentrated HCl (this laboratory) or by

keeping in glacial acetic acid saturated with HBr at 0° (Beilstein). Coumaric acid dissolved in benzene, methanol, or ethanol gives coumarin when exposed to u.v. light (Stoerner, 1909). The same change occurs almost quantitatively by boiling an aqueous or aqueous alcoholic solution of a coumaric acid with mercuric chloride (Seshadri, 1954; Rao, Sastri and Seshadri, 1939).

Coumarinic acids give coumarins on melting, whereas coumaric acids form styrenes with loss of CO₂. Fusion of coumarin with alkali leads to elimination of the pyrone ring with formation of salicylic acid.

Reactions of the carbonyl group. The carbonyl group of the pyrone ring is not a true keto group and will not yield an oxime or phenylhydrazone directly. However, treatment of coumarin with phosphorus pentasulphide gives thiocoumarin, which can be converted into the above derivatives. The oxime can also be obtained by action of hydroxylamine on iminocoumarin.

Some Simple Coumarin Derivatives. 3-Aminocoumarin is acidic, behaving as if it had the imino-structure. Hydrolysis of this compound gives 3-hydroxycoumarin, which exists as a keto-enol mixture. Thus, on the one hand, it is acidic, dissolving in sodium carbonate, and on the other hand, it forms a phenylhydrazone, and with *o*-phenylenediamine, a quinoxaline. Heating with sodium carbonate opens the lactone ring with formation of *o*-hydroxyphenylpyruvic acid. Thus the lactone structure is more labile than in coumarin.

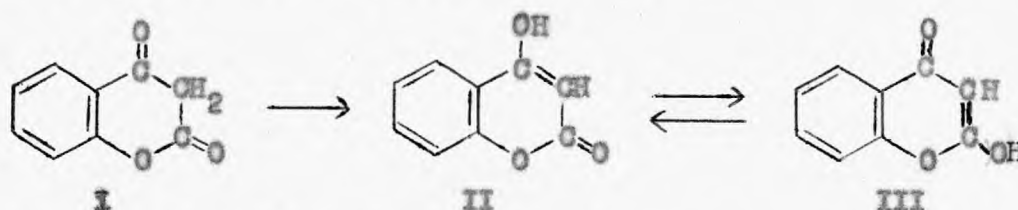
4-Hydroxycoumarin, or benzetetroneic acid, exists almost entirely in the enol form, as it has acid properties (pKa 5.8). The hydroxyl substituent in the 4-position activates the 3-position, since this point

is attacked in substitution reactions. 4-Hydroxycoumarin condenses easily with aldehydes to give 3,3'-alkylidene or arylidene bis-(4-hydroxycoumarins). The lactone ring of 4-hydroxycoumarin is opened by aqueous alkali, or by boiling with concentrated HCl, with subsequent decarboxylation and formation of o-hydroxyacetophenone. More vigorous alkali treatment gives salicylic acid. This behaviour contrasts with coumarin and 3-hydroxycoumarin, whose rings are opened by alkali but closed again by acid.

The hydroxycoumarins substituted in the benzene ring behave like normal phenols.

Coumarin-3-carboxylic acid is readily decarboxylated, as it is related to a substituted malonic acid. Coumarin-4-acetic acid is a vinylogue of malonic acid and behaves similarly, losing CO₂ at the melting point, and condensing with aromatic aldehydes in the Knoevenagel reaction.

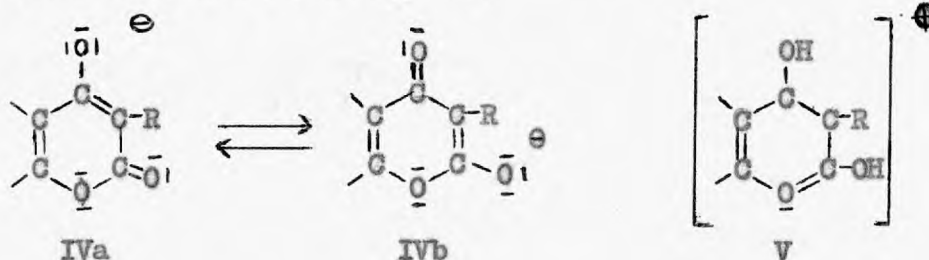
Tautomerism of 4-hydroxycoumarin. 4-Hydroxycoumarin contains a β -keto-lactone system which can undergo enolisation towards the keto carbonyl or towards the lactone carbonyl. Thus tautomerism between the 4-hydroxy-benz-2-pyrone (4-hydroxycoumarin) (II) and the 2-hydroxy-benz-4-pyrone (2-hydroxychromone) (III) is possible:



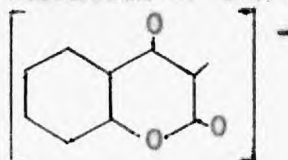
None of the keto form (I) is formed, but only enol, and the enolisation of the keto carbonyl is prevalent so that 4-hydroxycoumarin predominates in solution with a little 2-hydroxychromone. In the solid state the

compound is exclusively in the coumarin form (Arndt, 1949; Arndt et al., 1951). The hydroxyl attached to the lactone carbonyl is more acid than when attached to the keto carbonyl.

Arndt et al. (1951) came to the above conclusions by a study of the methylation of 4-hydroxycoumarin with diazomethane. Klosa (1953) also studied the reactions of 4-hydroxycoumarin and came to similar conclusions, pointing out that the chromone form may be favoured under special conditions of solvent and acidity. In alkali, II and III form the same synanion (IVa and b); in strong acid they both form the same syncation (V) (Arndt et al., 1951). Thus, in strong acid and alkali the difference between II and III disappears.



Chmielewska and Giecierska (1952) inferred from u.v. absorption data that 3-substituted derivatives of 4-hydroxycoumarin in 96% ethanol are completely ionised with formation of the following ion:

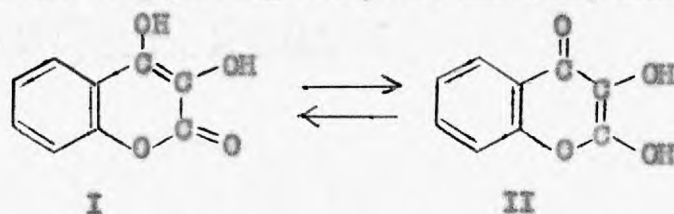


which presumably is the same as IVa and b above. They state that dicoumarol and Tromexan (pp.13-17) do not form ions of this type under similar conditions. Knobloch et al. (1952), on the basis of spectrographic and potentiometric measurements, suggested that the tautomeric equilibrium favours the 2-hydroxychromone form in the bis-hydroxy-

coumarin derivatives (cf. p. 63).

The electron shift involved in the 4-hydroxycoumarin-2-hydroxy-chromone tautomerism can explain the lability to alkali of 4-hydroxycoumarin glucuronide (Mead et al., 1958a) and of glycosides of 3-phenyl-4-hydroxycoumarin (Spero et al., 1949).

3,4-Coumarin diol: This compound (I) reacts with diazomethane in the chromone form (II) (Arndt et al., 1951). It is readily oxidised by iodine, silver nitrate, permanganate, and dichlorophenolindophenol,



and gives a blue or green colour with ferric chloride which soon fades (Arndt, 1949). This has been explained (Arndt et al., 1952) as being due to the formation of a coloured chelate cation from one Fe^{+++} ion and a monoanion of coumarindiol, reacting through its negative centre and the α -carbonyl. The ferric ion is reduced to ferrous with simultaneous oxidation of the chromone to the dicarbonyl compound, so that the colour fades.

Occurrence and Formation of Coumarin in Plants.

Coumarin and its hydroxy- and methoxy-derivatives occur widespread in plants, both free and combined as glycosides, especially in grasses, clovers, legumes, citrus fruits and orchids. Coumarin itself occurs particularly in the woodruff (*Asperula odorata*) and in white sweet clover (*Melilotus alba*). It was first isolated from tonka beans in 1870. Corcilius (1956) suggests that as many as 25 coumarins may be present in *Angelica archangelica*.

The distribution of the glycosides aesculin, fraxin and scopolin and the corresponding aglycones aesculetin, fraxetin and scopoletin in the horse chestnut was studied by Reppel (1956). Aesculetin and fraxetin may be converted into scopoletin; moreover, fraxetin and scopoletin appear to be precursors of lignin. The most widely distributed coumarin in higher plants is apparently scopoletin (6-methoxy-7-hydroxycoumarin) (Kala, 1956), which was sometimes found together with aesculin and aesculetin but not with coumarin itself.

Kosuge and Conn (1959) have found that $^{14}\text{CO}_2$ is incorporated into coumarin in white sweet clover and the coumarin is rapidly metabolised, being partly converted to melilotic acid (35% of radioactivity administered) and its glucoside (12%) with *o*-coumaryl glucoside (1%). Labelled *o*-coumaric acid was converted largely to its glucoside, with formation of a little melilotic acid and coumarin. Thus, *o*-coumaric acid could be the precursor of coumarin, the small amount of lactone appearing in the latter experiment being due to its rapid metabolism.

Coumarin could arise in plants from shikimic acid via a $\text{C}_6\text{-C}_3$ precursor (cf. Birch, 1957). Almost all the hydroxylated coumarin derivatives found in plants have a hydroxyl at position 7 (cf. Dean, 1952) and these could also arise from shikimic acid, or from hydroxylated aromatic derivatives by the scheme of Geissman and Hirciner (1952). It would seem unnecessary to postulate a hydroxylation of the coumarin nucleus in the plant.

Physiological Effects of Coumarins.

The plant growth effects and pharmacology of coumarins have been reviewed by Dean (1952), Reppel (1954), and Mead (1957). In the

following sections, selected topics only are discussed.

Growth effects of coumarins on plants. The most constant effects of coumarin are its inhibition of the germination of seeds and of the growth of roots. Some of its derivatives have similar effects, for example, coumarin, 3-methylcoumarin and 7-hydroxycoumarin inhibited germination of chisory seeds 30-35%, 4-hydroxy- and 6,7-dihydroxycoumarins having less effect (Graillot, 1951).

The effects of coumarin on the germination and growth of plants have been related to its effects on biochemical activity. Poljakoff-Mayber (1953) showed that coumarin inhibits the activity of the proteolytic enzymes in imbibing lettuce seeds in vitro, but not in more developed seedlings; it also inhibits the lipases (cf. also Poljakoff-Mayber and Mayer, 1955). The formation of amylase in germinating wheat is prevented by coumarin.

Coumarin blocks mitosis in young roots of onion (*Allium cepa*) and lily (*Lilium longiflorum*), growth also being inhibited (Cornman, 1947). Steinegger and Lupi (1955) reported similar effects in the onion for umbelliferone and aesculetin, but not aesculin.

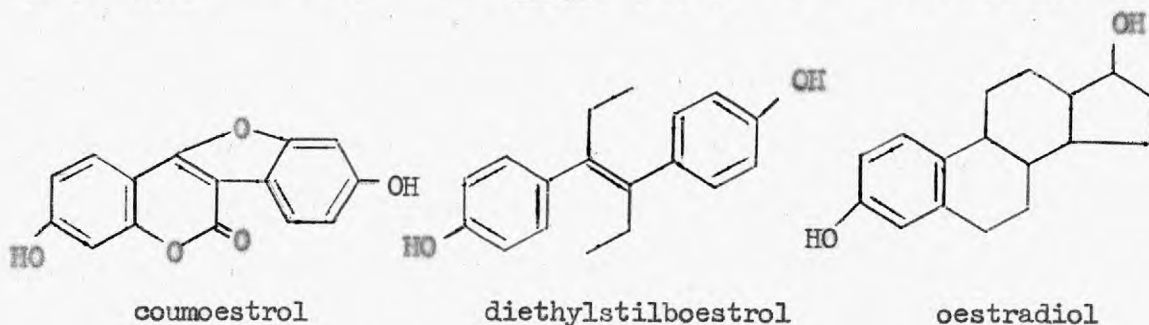
Pharmacological action of coumarins. Coumarin has relatively slight pharmacological action in man; it is somewhat toxic, a dose of 4 g. producing symptoms of illness and weakness, and inhibiting the sympathetic nervous system. It is also toxic for dogs, sheep, horses and cattle, the lethal dose for a sheep being 5 g. and for horses and cattle, about 40 g.

Some values for toxicities of coumarin have been cited but the data are not comprehensive. Dieke et al. (1947) report the acute

toxicity of coumarin for domestic rats to be more than 500 mg./kg. (L.D.50), administered orally, as a solution in propylene glycol. Hazleton et al. (1956) quote figures for the oral L.D.50 in rats as 290 mg./kg. given as a 10% solution in propylene glycol and 520 mg./kg. given as a 6% solution in corn oil. Zwickey and Nelson (1956) state that weanling rats fed 150 mg. coumarin per kg. per day in their diet for 27 weeks showed only slight liver changes, while 60 mg./kg./day produced no effect. In dogs, however, 100 mg./kg./day was fatal in two weeks; on 50 mg./kg./day, the dogs survived 1-9 months. At these levels, and even in some cases at 25 mg./kg./day, there were anorexia, weight loss and weakness, with damage to the liver.

Coumarin has a narcotic action in rabbits, frogs and earthworms, and is a sedative and hypnotic for mice.

The oestrogenic activity of some coumarin derivatives is of interest. 3-(p-hydroxyphenyl)-4-propyl-7-hydroxycoumarin was the most active oestrogen of a number tested in ovariectomised rats by Gley and Mentzer (1945), being about 0.02 as active as oestradiol. A naturally occurring plant oestrogen, coumestrol, has been isolated from ladino clover (Bickoff et al., 1957) and characterised as 6',7-dihydroxybenzofuro-[3',2':3,4]coumarin by Bickoff et al. (1958). It has been pointed out



that coumestrol has a stilbene-like structure analogous to diethylstilboestrol (compare also oestradiol) (cf. Bickoff et al., 1960).

Pharmacology and metabolism of the coumarin anticoagulants. The coumarins which are of the greatest practical importance pharmacologically are certain derivatives of 4-hydroxycoumarin used as anticoagulants and rodenticides. There is a very large literature on these compounds dealing with pharmacological effects and synthesis of new derivatives.

Dicoumarol, the agent in spoiled sweet clover hay causing the haemorrhagic disease in cattle, was first isolated by Link and his co-workers, and identified by them as 3,3'-methylene bis(4-hydroxycoumarin) (Campbell and Link, 1941; Stahmann, Huebner and Link, 1941). This compound prolongs the coagulation and prothrombin times of animals (cf. Roche e Silva, 1943; Lester, 1944; Butt et al., 1941). The action is not harmful to man and can be controlled. It is now widely used clinically as an anticoagulant as it has the advantages of effectiveness when administered orally, cheapness and prolonged action. On the other hand, response varies greatly among individuals and the action is delayed 1 or 2 days after administration.

Toxicities of dicoumarol in various species were determined by Rose et al. (1942). The acute intravenous lethal dose for mice, rats and guinea pigs was 50-70 mg./kg., but was several times greater by the oral route. Rats and mice fed 0.005% dicoumarol in their diet, and rabbits and dogs receiving 1-2 mg./kg. and 5-50 mg./kg. respectively daily by mouth all died in less than 4 weeks. Haemorrhage into the tissues and sometimes necrosis of the liver were the changes observed on autopsy. Roche e Silva (1943) gave a figure of 10-120 mg. per day for two days for

the lethal dose by injection into rabbits.

Ethyl bis(4-hydroxy-3-coumarinyl)acetate (ethyl biscoumatate; Tromexan; Pelentan) was found to have certain advantages over dicoumarol as an anticoagulant, as it acts more quickly with fewer side effects (Hausner, et al., 1951), but leads to a more transient hypoprothrombinaemia, and has only 1/5 of the effect of dicoumarol in humans (Burke and Wright, 1951; Barker et al., 1952; Hausner et al., 1951).

Both Tromexan and dicoumarol are strongly antagonised by vitamin K, which is structurally related to these drugs (Barker et al., 1952; Link, 1943).

The pharmacological importance of the 4-hydroxycoumarin anticoagulants has provided a stimulus for metabolic studies. Thus, Quick (1945) found that some dogs were more resistant to the anticoagulant effects of dicoumarol than others and concluded that this was due to a highly developed detoxication mechanism. Evidence obtained by intravenous injection of ^{14}C -dicoumarol into mice indicated that the drug disappeared rapidly from the blood and was taken up by the liver, being partly fixed there unchanged and partly excreted into the intestine in the bile. The radioactivity then appears to be reabsorbed from the intestine and excreted in the urine, with some appearing in the faeces. In the rabbit, the pathway is similar but no activity remains in the faeces (Jaques and Spinks, 1950; Lee et al., 1950). These workers found that administration of vitamin K caused a more rapid displacement of dicoumarol from the liver, and suggested that the time unchanged dicoumarol remains fixed in the liver is related to the suppression of prothrombin formation. However, it should be noted that vitamin K does not exert its effect by altering

the rate of metabolism of the drug (Weiner et al., 1950).

In man, dicoumarol is slowly and erratically absorbed from the alimentary tract, and slowly metabolised and excreted, although the rate of transformation varies widely for different subjects. The slow transformation can be related to a low free plasma level due to fixation of the drug on plasma and tissue proteins. The time lag in response to dicoumarol is partly due to the slow absorption, but also to its mode of action, which is to stop production of prothrombin by the liver: the prothrombin already present in plasma then has to be used up before the prothrombin time is raised (Quick, 1945; Lupton, 1947).

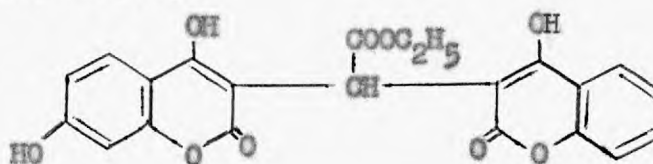
The metabolic products of dicoumarol were not identified in man, dog, mouse, or rabbit, but included little or no unchanged dicoumarol, although Hausner et al. (1951) claim on paper chromatographic evidence that some unchanged ^{14}C -dicoumarol was present in urine of rats after oral administration, and report that seven possible metabolites were formed. Labelled CO_2 was not expired by mice following intravenous injection of ^{14}C -dicoumarol (Jaques and Spinks, 1950), or by rats after oral administration (Hausner et al., 1951).

Although dicoumarol can be degraded chemically to salicylic acid, and salicylates administered to rats and dogs may induce hypoprothrombinaemia (Field, 1953), salicylic acid was not found as a urinary metabolite of dicoumarol in rats (Lester, 1944), nor in man or dog (Weiner et al., 1950). No glucuronide was formed in man or dog, and formation of an ethereal sulphate is unlikely as the pK_a of dicoumarol is low (5.7) (cf. R.T. Williams, p. 630).

Use of ^{14}C -Tromexan in rats showed that absorption after oral administration was rapid, and that there was a high initial liver concentration, a more rapid drop in body retention, and a high urinary excretion rate in the first few hours, as compared with ^{14}C -dicoumarol (Hausner et al., 1951). A small amount of $^{14}\text{CO}_2$ was formed which was assumed to arise from the carboxyl group. Three possible metabolites, which were not identified, were reported, and some unchanged Tromexan.

Tromexan is also absorbed and metabolised more quickly than dicoumarol in man (Brodie et al., 1952), so that after discontinuation of the drug, the effects disappeared more rapidly, but as with dicoumarol the rate of transformation varied widely among individuals. Tromexan is bound on plasma and tissue proteins to a considerable extent, but less so than dicoumarol, and this may be a factor in its more rapid metabolism.

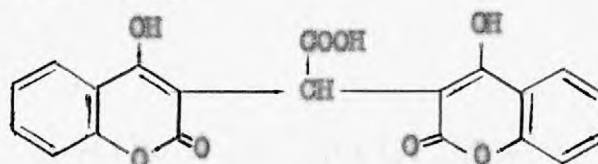
In man, Tromexan is hydroxylated in one of the benzene rings, meta to the lactone bridge, to the extent of 5-15% of an oral dose (Burns et al., 1953 a,b). No glucuronides were formed from Tromexan or hydroxytromexan, and indeed conjugation is unlikely, as both compounds have pKa values of 3.1. The drug is metabolised in the liver, the metabolite is excreted into the intestine via the bile, then is reabsorbed and eliminated in the urine. The hydroxylated metabolite is present in the body for longer than the parent drug, which suggests that it could be the agent causing the therapeutic effect. However, hydroxytromexan had no anticoagulant activity.



Hydroxytromexan

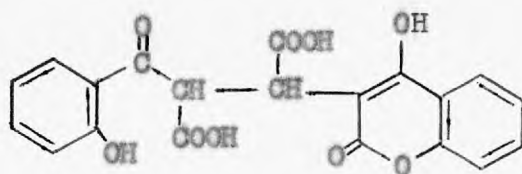
Metabolism of Tromexan was complete in man, but other metabolites were not identified. Tromexan acid was not formed.

In the rabbit, the rate of metabolism is similar to that in man, but in the dog it is very much slower (Burns *et al.*, 1953a). Tromexan is partly excreted unchanged (15%) by the rabbit, and there is no hydroxylation of the rings. The main metabolite (20%) is Tromexan acid, formed by de-esterification.

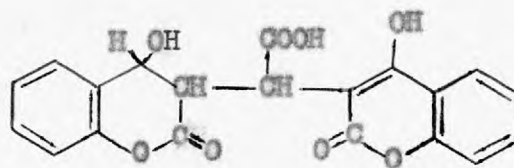


Tromexan acid

Pulver and von Kaulla (1948) reported that two Tromexan acids were isolated from the urine of rabbits fed Tromexan, the one occurring in greater amount having a lactone ring opened, and being devoid of toxicity and anticoagulant effect, the other, found only in small amounts, being partially reduced. The structures below have not been confirmed (cf. R.T. Williams, p. 634).



A



B

A number of coumarin derivatives tested by Lehmann (1943) were stated to have no advantage over 3,3'-methylene-bis(4-hydroxycoumarin) as anticoagulants, but Fantl (1943) reported that 3,3'-ethylidene-bis(4-hydroxycoumarin) (one of the compounds tested by Lehmann) gave a shorter

and less severe hypoprothrombinaemia than dicoumarol, promising greater safety in control. Certain other coumarins lacking the 4-hydroxyl group also show some activity, such as 7-methoxy-4-methylcoumarin and some dihydroxycoumarins (Bose et al., 1945).

Some of the 4-hydroxycoumarin anticoagulants also find a use as rodenticides. This application is based on the fact that these compounds induce fatal tissue haemorrhages in rats and mice when given in frequent small doses, but cause no untoward effects in man and domestic animals at these concentrations. The principal compounds used are dicoumarol, and warfarin (3-(α -acetylbenzyl)-4-hydroxycoumarin) (cf. Pollitzer, 1950, for review).

Anthelmintic activity. Numerous coumarin derivatives have been tested in particular against earthworms and roundworms. 3-Phenylumbelliferone was the most toxic to earthworms of a group of coumarins tested by Khorana and Motiwala (1950), and 3-methyl- and 7-methylcoumarins were found to be particularly toxic to Ascaris suis (Nakabayashi et al., 1953). It is of interest that coumarin and santonin, an established anthelmintic, both have a lactone ring.

Effects on bacteria. Many coumarins are antibacterials. Compounds including coumarin, 4-hydroxycoumarin, dicoumarol and 4-methylaesculetin were shown by Brodersen and Kjaer (1946) to have a bacteriostatic effect on many different species of bacteria, mostly pathogenic, dicoumarol being the most active. Some soil micro-organisms, on the other hand, are apparently able to utilise coumarin as sole carbon source (Robbins, 1946). Bellis (1958) isolated two species of Penicillium from soil which could use either coumarin or o-coumaric acid as sole source of carbon. The

evidence suggested that o-coumaric acid was converted by the organisms to 4-hydroxycoumarin and 3,3'-methylene bis(4-hydroxycoumarin), and if sucrose was present in the medium as well, a little umbelliferone was formed from coumarin.

Metabolism of Coumarin and its Simple Derivatives in Animals.

Prior to the work of Mead et al. little work had been done on the metabolic conversion of coumarin itself, or its simple hydroxylated derivatives, in the animal, except that Vasilin et al. (1938) demonstrated that coumarin was not converted to benzoic acid by the sheep.

Mead et al. (1955a, 1958b) showed that coumarin was hydroxylated in the animal. 3-, 7- and 8-hydroxycoumarins were found to be metabolites of coumarin in rabbits, rats, mice, guinea pigs, and ferrets by paper chromatographic techniques, and 5-hydroxycoumarin was also found in the last three species. 3-Hydroxy- and 7-hydroxycoumarin were the major metabolites, and these were isolated from the urine of coumarin-fed rabbits, the 3-isomer as its glucuronide and ethereal sulphate (Mead et al., 1958b). 3-Hydroxycoumarin was thought to be the chief metabolite from the amount isolated, and 7-hydroxycoumarin was shown by a fluorimetric method (Mead et al., 1954, 1955b) to be formed to the extent of 10% of the dose. 4- and 6-Hydroxycoumarins were not found as metabolites, nor were o-coumaric acid and salicylic acids.

While the present work was in progress, Furuya (1958) confirmed the above results in rabbits by detection of 3-, 7-, and 8- but not 4-, 5-, or 6-hydroxycoumarins. Booth et al. (1959) also have isolated 3- and 7-hydroxycoumarins from the urine of rabbits receiving coumarin, and a new metabolite, o-hydroxyphenylacetic acid. o-Hydroxyphenyllactic acid was

tentatively identified as a urinary metabolite, and they claim that melilotic acid, melilotoyl glycine, o-coumaric acid and o-hydroxyphenyl-hydroacrylic acid are formed, the last being regarded as the precursor of o-coumaric acid. They were unable to detect 3- or 7-hydroxycoumarins in the urine of coumarin-fed rats, but found o-hydroxyphenylacetic acid as a major metabolite, and reported the detection of all the other metabolites as for rabbits.

Hydroxycoumarins. The glucuronide of 3-hydroxycoumarin was isolated by Flatow (1940) from the urine of a man receiving the free phenol; that of 4-hydroxycoumarin by Roseman et al. (1954) (dogs); and Sieberg (1924) reported that 7-hydroxycoumarin, and 6,7- and 7,8-dihydroxycoumarins were excreted in unknown conjugated forms after injection into rabbits. Mead et al. (1955b, 1958a) studied the fate of the monohydroxycoumarins in the rabbit. They are metabolised mainly by conjugation, all six forming a glucuronide, and all except 4-hydroxycoumarin forming an ethereal sulphate. The glucuronides are formed in greater amount than the ethereal sulphates. All the glucuronides and some of the ethereal sulphates were isolated from the urine of rabbits fed the appropriate hydroxycoumarin. 4-Methyl-7-hydroxycoumarin is metabolised in a similar way (Mead et al., 1955b).

Aesculin (6-glucoside-7-hydroxycoumarin) is excreted by rats partly as the aglycone, aesculetin, and partly as unchanged aesculin. O-Methylation of aesculetin occurs in the rat, as it is converted into scopoletin (6-methoxy-7-hydroxycoumarin) and probably also into 6-hydroxy-7-methoxycoumarin (Yang et al., 1958). When scopoletin is fed to rats, it is partly demethylated to aesculetin, and both aglycones are excreted as conjugates with glucuronic acid, sulphate and glycine, although these

have not all been distinguished (Braymer et al., 1960). Rats can also demethylate herniarin (Fujita and Furuya, 1958).

7-Hydroxycoumarin is excreted as the glucoside by some insects, instead of the glucuronide (Smith, 1955); the ethereal sulphate is formed as in vertebrates.

o-Coumaric acid and melilotic acid. o-Coumaric acid is partly eliminated unchanged by rabbits, and partly conjugated with glucuronic acid at the phenolic hydroxyl group and with glycine at the carboxyl group (Mead et al., 1958b). 4-Hydroxy- and 7-hydroxycoumarins are also metabolites of o-coumaric acid in the rabbit, which proves that an in vivo trans-cis isomerisation and a cyclisation take place.

Further, it was shown by Mead et al. (1958b) that melilotic acid was converted by rabbits to o-coumaric acid and its metabolites, but that o-coumaric acid was not reduced to melilotic acid. On the other hand, Booth et al. (1959) report that o-coumaric acid fed to rats and rabbits is partly converted to melilotic acid and its glycine conjugate. They found a small amount of 4-hydroxycoumarin, postulated as arising from o-hydroxyphenylhydracrylic acid, but no 7-hydroxycoumarin. o-Hydroxyphenylacetic acid was also reported.

Other coumarin derivatives. Ethyl coumarin-3-carboxylate injected into a mouse, or applied on excised liver or kidney was partly hydrolysed to coumarin-3-carboxylic acid (Ichibagase, 1955). Ethyl 7-nitrocoumarin-3-carboxylate was chiefly hydrolysed and reduced to 7-aminocoumarin-3-carboxylic acid, part of which was acetylated to 7-acetamidocoumarin-3-carboxylic acid.

Chapter 2. Materials and Methods.

Reference Compounds. The following compounds were purchased, and purified further where indicated: Coumarin (B.D.H. Ltd.), m.p. 69-70° from n-hexane, *o*-coumaric acid monohydrate (B.D.H. Ltd.), m.p. 208°, 7-hydroxycoumarin (B.D.H. Ltd.), m.p. 230-1° after several recrystallisations from water with charcoal, *o*-hydroxyphenylacetic acid (Light and Co.), m.p. 145-6°, and *o*-hydroxyacetophenone (Light and Co.), colourless liquid, chromatographically pure. 4-Hydroxycoumarin, m.p. 216°, was donated by Aspro Ltd.

The following hydroxycoumarins were synthesised: 3-hydroxy- m.p. 153° from water (Linch, 1912), 6-hydroxy- m.p. 252° from a large volume of water (Bargellini and Monti, 1915), and 8-hydroxycoumarin, m.p. 159-60° from water (Cingolani, 1954).

5-Hydroxycoumarin was synthesised according to Adams and Bockstahler (1952), except that the final decarboxylation stage in the synthesis was found to be unreliable and was modified. Thus, 5-hydroxycoumarin-3-carboxylic acid (2 g.) and sodium metabisulphite (5 g.) were warmed with 25 ml. water until all the solid had dissolved. Warming was continued until effervescence had ceased, then the solution was gently evaporated over a flame and the pasty orange-red residue was dried in vacuo over P_2O_5 . The dry solid was heated in a bath of molten solder metal for $4\frac{1}{2}$ hours, initially at 140°, then for the last hour at 180°. The cooled mixture was extracted several times with ether and the combined extracts were evaporated. The residue, after recrystallisation from water, yielded 1.2 g. 5-hydroxycoumarin (76%) as white woolly needles, m.p. 228°.

6,7-Dihydroxycoumarin (aesculetin) was obtained from its glucoside, aesculin (Light and Co.), by acid hydrolysis (0.5 N HCl for 0.5 hour), m.p. 269-70° from water.

Dihydrocoumarin (6.3 g.) (Light and Co.) refluxed with 50 ml. of 10% KOH for 1 hour, the acidified hydrolysate extracted with ether, and the residue from the evaporated extract recrystallised from carbon tetrachloride yielded 5.9 g. of o-hydroxyphenylpropionic acid (melilotic acid) as white needles, m.p. 87°. Melilotic acid readily recyclises to dihydrocoumarin if left exposed to light and air; a brownish liquid is formed, consisting of a mixture of the acid and its lactone.

o-Hydroxyphenyllactic acid was synthesised by reduction of an alkaline solution of 3-hydroxycoumarin with sodium amalgam, cf. Plöchl and Wolfrum (1885). These authors do not describe the experimental procedure for the reduction. The following procedure was found to be best; variations of this gave a mixture of products which could not be separated:

3-Hydroxycoumarin (5 g.) was dissolved in 25 ml. of 2 N Na_2CO_3 solution and diluted with water to about 150 ml. About 1 ml. of 40% NaOH solution was added, and the yellow solution was boiled for 15 minutes under reflux to convert the 3-hydroxycoumarin to o-hydroxyphenylpyruvic acid. Sodium amalgam, from 2.15 g. sodium and 180 g. mercury, was added in small portions to the solution cooled at 0°, with continuous mechanical stirring, during 0.5 hour. The mixture (greenish-yellow aqueous layer and mercury layer) was transferred to a 250 ml. centrifuge bottle, stoppered with a rubber bung and shaken for 15 minutes. The colour of the solution was almost discharged by this procedure. The supernatant solution was decanted, the mercury was washed with water, and solution and washings

acidified with concentrated HCl. The solution was extracted with 4 x 50 ml. ether, the extract dried over anhydrous Na_2SO_4 , and the solvent removed. The residue was a dark reddish oil, weighing 5.7 g. Paper chromatography in benzene - acetic acid - water (1:1:2, by vol.) showed a spot at R_f 0.09, giving a dark blue colour with Gibbs's reagent. Only traces of impurities were found. To the crude o-hydroxyphenyllactic acid (5.7 g.) were added 20 ml. of water, then calcium hydroxide was added gradually, with warming and stirring, until the pH remained at 8. The mixture was heated to boiling, filtered, and cooled. After removal of a sticky material by filtration, the solution on standing and cooling at 0° deposited white crystals, which were filtered off. This material, dried in vacuo over CaCl_2 , yielded 3.4 g. of white microcrystals, which were found to be chromatographically pure. This calcium salt was a hemihydrate; the calcium o-hydroxyphenyllactate of Flöchl and Wolfrum (loc. cit.) contained $6\text{H}_2\text{O}$.

Calc^{d.} for $(\text{C}_9\text{H}_9\text{O}_4)_2 \text{Ca} \cdot 0.5\text{H}_2\text{O} \cdot \text{H}_2\text{O}$, 2.2.

Found: H_2O , 2.1 (loss at 110°).

o-Hydroxyphenylpyruvic acid, the lactone of which is 3-hydroxycoumarin, was prepared as follows: 3-Hydroxycoumarin (1 g.) was dissolved in 50 ml. of 2N Na_2CO_3 and boiled under reflux for 0.5 hour. The solution, cooled to 0° , was acidified to pH 2 by cautious addition of ice-cold concentrated HCl, and extracted immediately with 3 20 ml. portions of ether. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent was removed to leave a yellowish syrup (0.32 g.). Paper chromatography of this material in 3 solvent systems (see Table 2.1) showed a main spot which gave characteristic colour reactions (Table 2.2) and was assumed to

be o-hydroxyphenylpyruvic acid, and a weak spot corresponding to 3-hydroxycoumarin. The syrup could also be distinguished by its reactions in aqueous solution (Table 2.3). Thus with aqueous 1% FeCl_3 it gave a violet-red colour, and with Gibbs's reagent a green colour. 3-Hydroxycoumarin gives a green colour and o-hydroxyphenylacetic acid no colour with ferric chloride. The syrup gradually crystallised in 2 or 3 days to a mixture consisting of 3-hydroxycoumarin and the decarboxylation product, o-hydroxyphenylacetic acid. The decarboxylation can be observed on paper chromatograms run in ammoniacal solvents (C, Table 2.1) by spraying with Gibbs's reagent; the o-hydroxyphenylacetic acid then appears as a crescent-shaped area running at the front of the yellow-green o-hydroxyphenylpyruvic acid spot. According to Erlenmeyer and Stadlin (1904) the free pyruvic acid cannot be obtained as it reverts to 3-hydroxycoumarin.

o-Hydroxyphenylhydracrylic acid: attempts were made to prepare this compound by sodium amalgam reduction of an aqueous-ethanolic solution of 4-hydroxycoumarin. However, a mixture of products was obtained, from which almost the whole of the starting material was re-isolated. The remaining product consisted of about 0.5 ml. of a yellow aqueous solution. Paper chromatography showed the presence of three unidentified spots, which were acidic, giving a yellow colour on a green background when sprayed with a 0.1% aqueous solution of bromothymol blue, and a blue colour with Gibbs's reagent. R_f values in benzene - acetic acid - water (1:1:2, by vol.) were 0.05, 0.40, and 0.92, respectively. This preparation was used for paper chromatography. Attempts to isolate a calcium salt of o-hydroxyphenylhydracrylic acid from the mixture were unsuccessful.

α -Benzamino-o-hydroxycinnamic acid (salicylalhippuric acid) was synthesised by the method of Rebuffat (1889) in 25% yield, white needles

from ethanol-water, m.p. 197-197.5° (lit. 195°). o-Tyrosine was obtained by reduction of the above compound to N-benzoyl-o-tyrosine with sodium amalgam, and removing the benzoyl grouping by boiling with HCl (Blum, 1908). o-Tyrosine formed small white needles, m.p. 249-50° (decomp.).

2,4-Dihydroxycinnamic acid was prepared in low yield (10%) as a yellowish powder, m.p. 240° (decomp.) by treatment of 7-hydroxycoumarin with alkali (Fosen, 1881).

o-Hydroxy-trans-cinnamoyl glycine (o-coumaryl glycine) was synthesised according to Mead et al. (1958b), forming white prisms from methanol, m.p. 226-7° (decomp.).

The glucuronides of 3-, 4-, and 7-hydroxycoumarins were biosynthesised as described by Mead et al. (1955, 1958a). 3-Hydroxycoumarin glucuronide, m.p. 212-213° (decomp.) from water; 4-hydroxycoumarin glucuronide monohydrate, m.p. 184-5° from water; 7-hydroxycoumarin glucuronide monohydrate, m.p. 149-50° from ethanol. The isolation of these compounds from the urine of rabbits receiving coumarin will be mentioned later.

The benzylamine salts of the o-hydroxyphenyl acids were prepared by adding the theoretical amount of benzylamine to the hydroxy acid dissolved in a small volume of ethyl acetate. The benzylamine salt separated rapidly after stirring for a few seconds, it was filtered off quickly, washed with ethyl acetate and recrystallised from the same solvent. These salts have the advantage of being quickly and easily made and purified, and of having sharp, characteristic melting points. The benzylamine salt of o-hydroxyphenyllactic acid tended to decompose if

there was any delay in purification.

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

Calc^{d.} 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 acid formed white woolly needles from ethyl acetate, m.p. 146-7°.

Calc^{d.} 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°.

Calc^{d.} for $C_{16}H_{19}NO_4$: C, 66.4; H, 6.6.

Found: C, 66.2; H, 6.6.

Other derivatives used are described under isotope dilution methods.

[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 hours, the solution was neutralised with 6.2 ml. of approximately 2*N* HCl and 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 hours. To the dry mixture of malonic acid and sodium chloride, salicylaldehyde (0.85 ml.) and aniline hydrochloride (0.1 g.) were added. After thorough mixing, the material was warmed on a water bath at 50-60° for 6 hours and then kept 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 hours. The hard cake which had then formed was triturated with 5 ml. water, drained, and washed successively with 2 ml. of absolute ethanol and 2 ml. of ether. The yield of coumarin-3-carboxylic acid was 0.94 g. 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 CO₂ had ceased. The solution was evaporated in vacuo at 60-70° and the pasty mass of sodium hydrocoumarinsulphonate was dried in vacuo over P₂O₅ (yield 1.3 g.). The dry salt was then heated under reflux at 190° for 6 hours. After cooling, the residue was extracted repeatedly with ether and the extracts on evaporation yielded 0.6 g. of ¹⁴C-coumarin, m.p. 61° (recovery of ¹⁴C was 387.6 µc. or 77%). After recrystallisation 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 hours, a further 23 mg. of coumarin, m.p. 63-65°, was obtained.

Animals. Chinchilla doe rabbits (about 3 kg.) and female albino rats (3 months old; 150-200 g.) maintained on a constant diet, were used. Rabbits received 80 g. of diet 41 (Associated Flour Millers Ltd.) and 150 ml. of water per day; rats received 10 g. of diet 41 and 20 ml. of water per day. Compounds were finely ground and administered orally to the animals as a suspension in water containing a little bile salt. A stomach tube was used for rabbits, a syringe and wide-bore needle for rats. The latter were lightly anaesthetised with ether before dosing. The animals were kept in metabolism cages for collection of urine and faeces except for experiments in which expired air was collected. Urine was collected when

it was passed which was usually every 24 hours for rabbits, although sometimes two collections per day were possible, and twice daily for rats. Faeces were usually collected at the end of the experiment (2-5 days); for one rat, daily collections were taken. The rat faeces were carefully rinsed with water, and the washings were added to the urine.

Collection of expired air. An animal was housed in a perspex tank through which air could be drawn and having an arrangement for collecting urine periodically without breaking the air current (Parke and Williams, 1950, 1953; Elliot, Parke and Williams, 1959). For measurement of coumarin in expired air, the absorption train consisted of two Dreschel bottles containing anhydrous magnesium perchlorate followed by two tubes containing 50 ml. of ethanol immersed in an acetone-solid CO_2 mixture. In experiments for collection of $^{14}\text{CO}_2$, these were followed by two Dreschel bottles containing 5N NaOH.

Determination of coumarin in expired air. Coumarin in the 6-hourly ethanolic samples from the absorption train was estimated spectrophotometrically at 275 m μ . against a blank through which laboratory air had been drawn for the same period. Where small differences between sample and blank were obtained, the entire spectra between 210 and 300 m μ . were compared. Recovery of coumarin was checked by using thin films of coumarin on clock glasses. No coumarin was removed from the chamber in two experiments by aeration for 12 and 30 hours respectively.

Measurement of radioactivity. Measurements were carried out on solid or liquid samples of "infinite thickness" in an end-window Geiger-Muller counter. Where the amount of material available was insufficient to make an "infinitely thick" layer, a correction was applied (Calvin et al.,

1949). Solid samples were counted on aluminium planchettes, 1.8 sq. cm., and liquid samples on inverted planchettes, 5.0 sq. cm. The specific activities were determined by comparison with stable reference polymers, having activity 0.1 $\mu\text{C./g.}$ The different counters used had background counts of 8-12 and 17-21 counts per minute.

The total radioactivity in urine was measured by counting the liquid urine, and occasionally by counting the residue obtained by evaporation of the urine under infra-red lamps. Results from the two methods did not differ significantly except for low-activity urine, when the latter was more accurate. Radioactivity in faeces was measured by homogenising with water and counting wet or after drying. Tissues were homogenised in a Waring blender or in a small glass homogeniser, with or without the addition of water, according to the consistency of the tissue, and counted wet. The percentage radioactivity was calculated on the basis of the wet weight of the tissue. CO_2 in expired air was assayed for radioactivity by trapping in NaOH and counting as BaCO_3 (Calvin et al., 1949).

Counting Accuracy. Samples were counted for periods of 10 minutes, with a 10 minute background count between each, until sufficient total counts had been taken to give a standard error of less than 2% for urine or faeces fractions, or metabolites containing more than 3% of the radioactivity in the dose. The standard error was often greater for less important fractions: thus, it was 3-4% for metabolites containing 1-3% of the activity, and usually less than 5% for metabolites containing less than 1% of the activity. Standard errors for some tissues containing only small amounts of radioactivity were occasionally as high as 20%.

When the amount of material on the planchette was less than 20 mg. per sq. cm., the number of counts obtained was corrected from self-absorption curves.

Qualitative Tests.

A number of preliminary tests were carried out on the urine of animals fed coumarin derivatives, and these tests were also applied, where appropriate, to pure compounds, and to various extracts from biological material.

Reducing substances were tested for with Benedict's qualitative sugar reagent: 2 ml. of reagent were boiled for $\frac{1}{2}$ minute, a few drops of urine were added and the mixture was heated at 100° for 2 minutes. A green coloration indicated slight reduction, a brown or yellow precipitate, strong reduction.

The pH of urine was tested approximately with B.D.H. wide range universal indicator paper, and a closer approximation was sometimes obtained with the appropriate narrow range paper.

Glucuronides: a few mg. of naphthoresorcinol were dissolved in 0.5 ml. concentrated HCl, an equal volume of urine or a few mg. of glucuronide gum or pure compound were added, and the mixture was boiled for one minute, cooled, and extracted with an equal volume of ether. A purple colour in the ether layer denoted the presence of glucuronide. Normal urines gave a rather faint purple, those containing much glucuronide, or solutions of pure glucuronide, gave a deep purple.

Ethereal sulphate: 0.5 ml. of a 1% solution of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in 2N HCl was added to 0.5 ml. of test solution. Any precipitate formed (including inorganic sulphate) was filtered off, and the clear solution was boiled for

30 seconds and cooled. A precipitate or cloudiness indicated the presence of ethereal sulphate.

Gibbs's test: to 0.5 ml. test solution, an equal volume of 0.1% 2,6-dichloroquinone chloroimide in ethanol was added, followed by 0.5 ml. of saturated aqueous NaHCO_3 . This test was not usually performed with urine, because normal urines give a blue colour with this reagent.

Brentamine test: 0.5 ml. of a 0.01% aqueous solution of a Brentamine salt (usually Brentamine fast blue B salt) was added to 0.5 ml. of test solution, followed by 0.5 ml. of saturated aqueous NaHCO_3 . This was only occasionally used for urine as such (but cf. Chapter 4).

Ferric chloride: a few drops of a 1% aqueous neutral solution of ferric chloride were added to 0.5 ml. of test solution. Distinctive colours are often obtained with phenolic compounds. This was used as a routine for urine.

Brady's reagent: carbonyl groups were tested for by addition of a few drops of a 2% solution of 2,4-dinitrophenylhydrazine in $6N \text{ H}_2\text{SO}_4$ (Brady's reagent) to 0.5 ml. of urine. A yellow or orange precipitate, separating in the cold or on warming, indicates a keto or aldehyde grouping.

Ninhydrin: 0.5 ml. of a 0.1% ethanolic solution of ninhydrin added to 0.5 ml. of test solution, and warmed if necessary, was used as a test for α -amino acids.

Fluorescence: urine often showed fluorescence characteristic of certain compounds when illuminated by ultra-violet light (principal wavelength 270 m μ . or 360 m μ .). Addition of concentrated ammonia, glacial acetic acid, or HCl sometimes modified the fluorescence response.

Paper Chromatography. (cf. Mead et al., 1958a; Swain, 1953).

Descending chromatography on Whatman No. 4 paper was used (occasionally Whatman No. 1 or other paper as indicated). Rf values for the following solvent systems are given in Table 2.1.

- A. Benzene - glacial acetic acid - water (1:1:2).
- B. n-Butanol - glacial acetic acid - water (4:1:5 or 4:1:2; the upper layer of the former is equivalent to the latter mixture).
- C. n-Propanol - ammonia solution (sp. gr. 0.88) (7:3).
- D. Ethyl methyl ketone saturated with 2N ammonia solution.
- E. n-Butanol - ammonia solution (sp. gr. 0.88) - water (8:1:11).
- F. Benzene-n-butanol-2N ammonia (1:2:3).
- G. Benzene-n-propanol-ammonia solution (sp. gr. 0.88) (3:7:3).

All proportions are by volume.

Of these solvent systems, A, B, C, D, and E were found to give the best results. F and G were satisfactory for many coumarin derivatives and were occasionally tried to achieve better separation between particular groups of compounds. Several other solvents were of little or no value, on account of poor separation, tailing, streaking, compounds running at the solvent front, etc.

n-Butanol - benzene - water (5:2:2) gave a spot at Rf 0.68 for o-hydroxyphenylpyruvic acid, with some tailing, and was sometimes used for this labile compound as it is a neutral solvent system.

Urine, or compounds dissolved in an appropriate solvent were applied to the paper with fine glass capillary tubes. About 50 μ l. of urine, 50-100 μ g. of a solid extract, or 20 μ g. of a pure compound were applied to the paper, to form a spot about 0.75 cm. in diameter. The chromatogram

was developed in the solvent mixture without prior equilibration of the paper with the vapour phase, and without special precautions regarding temperature. Under these conditions, Rf values varied from time to time, from one batch of solvent mixture to another, and from one tank to another. Also, Rf values for pure compounds were often slightly different from those for the metabolites in biological material. Accordingly, reference compounds were always run alongside urine or extracts. Rf values quoted in Table 2.1 are averages of several of the best runs, and give an indication of the relative positions of the spots. Runs were of 12-13" from the origin.

The spots were detected on paper by the following procedures:

Fluorescence. Papers were exposed to u.v. light (wavelength 270 mμ. or 360 mμ.). Many compounds fluoresce or quench the background fluorescence. Exposure of the paper to ammonia fumes sometimes modifies the fluorescence.

Photocatalytic fluorescence. The paper was sprayed with N NaOH and exposed to u.v. light (360 mμ.). Coumarin develops a blue-green fluorescence within one minute of exposure. This reaction is given by coumarins with no free hydroxyl groups and no substituent in position 4 (Feigl et al., 1955; Mead et al., 1958a).

Other spray reagents. Gibbs's reagent (0.1% 2,6-dichloroquinone-chloroimide in ethanol) followed by saturated aqueous NaHCO_3 .

Brentamine fast blue B salt (0.01% in water) followed by saturated aqueous NaHCO_3 .

Ferric chloride, 1% in water.

Ninhydrin, 0.1% in ethanol.

Bromothymol blue, 0.1% in water, for acidic compounds.

m-Dinitrobenzene, 2% in ethanol, followed by N NaOH. This reagent gives a red colour with o-hydroxyacetophenone.

Colours given on paper by the compounds used in this study are quoted in Table 2.2. These are not always exactly the same as colour reactions given in a test tube (cf. Table 2.3.).

Autoradiography. This was carried out by placing chromatograms of radioactive urine or extracts in contact with X-ray film and developing for 2 weeks.

Isotope Dilution Methods.

The general procedure for carrying out isotope dilutions was as follows. An aliquot of urine was taken and a weighed amount of inactive carrier was added. The volume of urine and the amount of carrier were chosen so that when the metabolite was re-isolated it would have an appreciable count, even if present in small amount. When it was desired to estimate the amount of a metabolite occurring free in urine, the carrier was re-isolated after allowing the mixture to stand for 0.5 hour to ensure homogeneity of inactive carrier and active metabolite. In the estimation of the total amount of the compound present in urine, including conjugates, the mixture was boiled under reflux with an equal volume of concentrated HCl for 3 hours, when it was desired to hydrolyse glucuronides, and with 1/20 volume of concentrated HCl for 20 minutes to hydrolyse ethereal sulphates only.

Coumarin (a) (Total). Coumarin (0.4-0.5 g.) was added to urine

Table 2.1.

 R_F values of coumarin and its possible metabolites (cf. p. 33).

<u>Compound</u>	<u>R_F values in solvent</u>						
	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>G</u>
Coumarin	0.88	0.92	0.96	0.96	0.93	0.93	0.91
3-Hydroxycoumarin	0.72	0.91	0.86	0.44	0.53	0.30	0.61
4-Hydroxycoumarin	0.15	0.91	0.72	0.18	0.46	0.24	0.59
5-Hydroxycoumarin	0.12	0.87	0.81	0.64	-	0.30	0.62
6-Hydroxycoumarin	0.05	0.85	0.78	0.85	-	0.55	0.67
7-Hydroxycoumarin	0.15	0.86	0.60	0.51	-	0.25	0.60
8-Hydroxycoumarin	0.62	0.88	0.66	0.60	-	0.26	0.56
6,7-Dihydroxycoumarin	0.00	0.77	0.27	-	-	-	-
Potassium 3-hydroxycoumarin sulphate	0.00	0.50	0.77	-	-	-	-
Potassium 7-hydroxycoumarin sulphate	0.00	0.48	0.73	-	-	-	-
3-Hydroxycoumarin glucuronide	0.00	0.58	0.11	0.03	-	-	-
4-Hydroxycoumarin glucuronide	0.00	0.59	0.29	0.04	-	-	-
7-Hydroxycoumarin glucuronide	0.00	0.52	0.25	0.02	-	-	-
<u>o</u> -Coumaric acid	0.20	0.90	0.36	0.07	-	0.09	0.34
Salicylic acid	0.79	0.92	0.77	0.38	0.51	-	-
<u>o</u> -Hydroxyphenylpropionic acid	0.43	0.92	0.67	0.27	0.46	0.29	0.60
<u>o</u> -Hydroxyphenylacetic acid	0.28	0.89	0.87	0.35	0.47	0.27	0.63
<u>o</u> -Hydroxyphenyllactic acid	0.09	0.88	0.76	0.18	0.32	0.12	0.42
<u>o</u> -Hydroxyphenylpyruvic acid	0.08	0.80	0.60	-	-	-	-
<u>o</u> -Hydroxyphenylhydracrylic acid	0.05 (0.40) (0.92)	0.81	0.73	-	-	-	-
2,4-Dihydroxycinnamic acid	0.00	0.81	0.31	0.02	0.05	-	-
<u>o</u> -Tyrosine	0.00	0.26	0.15	0.00	0.00	0.00	0.09
<u>o</u> -Hydroxyacetophenone	0.83	0.96	0.96	-	-	-	-
Phenol	0.87	0.93	0.95	0.97	0.94	-	0.94

Table 2.2

Colour reactions on paper of coumarin and its possible metabolites (cf. pp. 34-35).

Compound	Fluorescence			Gibbs	Brentamine (+ NaHCO ₃)	Ferric Chloride
	270 mμ.	360 mμ.	360 mμ. (NH ₃ Fumes)			
Coumarin	Q	-	-	-	-	-
3-Hydroxycoumarin	-	-	-	Red → Green	Blue (→ blue Fading to pink-yellow)	-
4-Hydroxycoumarin	Purple	Very weak purple	Very weak purple	Red-purple	Pink (→ red)	-
5-Hydroxycoumarin	Pale blue	-	Yellow	Green → Blue → purple	-	-
6-Hydroxycoumarin	Purple-blue	Purple-blue	Purple-blue	Slowly developing very weak blue	-	-
7-Hydroxycoumarin	Blue	Blue	Intense blue	Very weak red-violet, darkening	-	-
8-Hydroxycoumarin	Q	-	Q	Green → blue → mauve	-	-
6,7-Dihydroxy- coumarin	-	-	Blue	-	-	-
<u>o</u> -Coumaric acid	Green	Blue	Bright green	Blue	-	-
Salicylic acid	Purple	-	-	Blue (slowly developing)	-	Purple
<u>o</u> -Hydroxyphenyl- propionic acid	Q	-	-	Blue	Weak yellow (→ yellow → pink)	-
<u>o</u> -Hydroxyphenyl- acetic acid	Q	-	-	Blue	Weak orange (→ weak orange purple > 50 μg.)	-
<u>o</u> -Hydroxyphenyl- lactic acid	Q	-	-	Blue	(Yellow → orange → pink)	Pale yellow → pink or purple
<u>o</u> -Hydroxyphenyl- pyruvic acid	-	Weak purple	Bright green rapidly → yellow	Yellow- green	Weak red (→ pale yellow)	Green slowly → blue
<u>o</u> -Hydroxyphenyl- hydracrylic acid	-	-	-	Blue	-	-
2,4-Dihydroxy- cinnamic acid	-	Purple-blue	Intense blue	Crimson slowly → purple	Violet (→ purple-red)	-
<u>o</u> -Tyrosine	-	-	-	Red-purple	-	-
Phenol	-	-	-	Blue (slow)	Yellow (→ orange → pink)	-
<u>o</u> -Hydroxyacetophenone	Q	-	-	Very weak blue (100 μg.)	-	-

Q means quenching of the background fluorescence.

Table 2.3

Colour reactions in a test tube of some possible metabolites of coumarin.

Compound	Fluorescence			Alcohol Sol ⁿ .	Gibbs	Brentamine (+ NaHCO ₃)	Ferric Chloride
	pH 2	360 mμ. pH 8	pH 13				
3-Hydroxycoumarin	-	-	-	-	Red	Purple-red (→ purple-blue)	Green
6,7-Dihydroxy-coumarin	-	Blue-green	-	-	-	-	Green
<u>o</u> -Hydroxyphenyl-propionic acid	-	-	-	-	Blue	Weak yellow (→ red-orange)	-
<u>o</u> -Hydroxyphenyl-acetic acid	-	-	-	-	Blue	(Yellow to orange)	-
<u>o</u> -Hydroxyphenyl-lactic acid	-	-	-	-	Blue	Weak yellow (→ red)	Faint greenish
<u>o</u> -Hydroxyphenyl-pyruvic acid	Weak blue	Bright green	Green	Green-blue	Intense green rapidly → deep blue	-	Violet-red
2,4-Dihydroxy-cinnamic acid	-	-	-	Weak green-blue	Crimson	(Intense purple-red)	-
<u>o</u> -Tyrosine	-	-	-		Red-purple	(Orange to salmon-pink fading to yellow)	-
Phenol	-	-	-	-	Blue	Bright yellow (→ orange-red fading)	Blue
<u>o</u> -Hydroxyacetophenone	-	-	-	-	Blue	-	-

(1-5 ml., containing 1/100 to 1/40 of the total urinary radioactivity), diluted with water (10-20 ml.) and an equal volume of concentrated HCl was added. The mixture was boiled under reflux for 3 hours (usually with a little ethanol to help to dissolve all the coumarin). The solution was cooled to 0°, the coumarin which separated was filtered off, and the filtrate was extracted with ether (3 x 20 ml.). The combined extracts, dissolved in ether, were dried over anhydrous NaSO_4 and evaporated. The residue was recrystallised from n-hexane and from light petroleum (b.p. 100-120°) until the activity was constant (m.p. 69-70°).

(b) (Free). Coumarin (0.3-0.5 g.) was added to urine (1-5 ml.; 1/100 to 1/20 of the radioactivity) and diluted with water (10-20 ml.). The mixture was warmed under reflux, with addition of a little ethanol (3-5 ml.) until all the solid had dissolved. After standing 0.5 hour at room temperature, the solution was cooled to 0°; coumarin separated out and was filtered off and recrystallised as above.

3-, 5-, 6-, 7-, and 8-Hydroxycoumarins (a) Total. Hydroxycoumarin (0.1-0.5 g.) was added to urine (1-5 ml.; 1/100 to 1/20 of the radioactivity), diluted with water if necessary and hydrolysed as for total coumarin. 3-, 7- and 8-hydroxycoumarins were extracted from the cooled solution by shaking with several portions of ether or by continuous extraction with ether. 5-Hydroxycoumarin was re-isolated by continuous extraction with chloroform for 24 hours. The extracts were dried (anhydrous NaSO_4) and the solvents distilled off. 6-Hydroxycoumarin separated from the hydrolysate simply on cooling to 0°, and was filtered off.

3-Hydroxycoumarin was recrystallised from water (charcoal) and occasionally also from benzene and ethanol, and counted as such (m.p. 152-3°)

until the activity was constant. It was then converted into 2-oxo-3-phenylhydrazonochroman (m.p. 173-4°) by treatment with N NaOH and phenylhydrazine hydrochloride (Erlenmeyer and Stadlin, 1904), and this was recrystallised from aqueous ethanol to constant activity. 5- (m.p. 228°), 6- (m.p. 252°), 7- (m.p. 231°), and 8- (m.p. 159-60°) hydroxycoumarins were recrystallised from water, with charcoal for the first two or three times, and counted as such and after conversion into the corresponding acetoxycoumarins (5-, m.p. 85°; 6-, m.p. 145-6°; 7-, m.p. 140-1°; and 8-, m.p. 133°, after recrystallisation from water).

(b) Mild hydrolysis. Hydroxycoumarin (0.2-0.3 g.) was added to urine (5 ml.; 1/50 activity), an aqueous volume of 0.6 N HCl was added and the mixture was boiled under reflux for 20 minutes, with gradual addition of 0.3 N HCl as necessary until the compound was dissolved. On cooling the solution to 0°, the hydroxycoumarin crystallised out, and was filtered off and recrystallised to constant activity as above. The same derivatives were prepared.

(c) Free. Hydroxycoumarin was added to urine as before and the mixture was diluted with water and warmed to dissolve the carrier. 3-, 6- and 7-hydroxycoumarins separated on cooling, 8-hydroxycoumarin was extracted with ether. The compounds were recrystallised and counted as usual.

4-Hydroxycoumarin. Prolonged heating with acids converts 4-hydroxycoumarin and its conjugates into o-hydroxyacetophenone. 4-Hydroxycoumarin (0.3-0.5 g.) was added to urine (2-5 ml.; 1/50 to 1/20 activity) which was diluted and made 5N with respect to HCl as usual. The mixture was boiled under reflux for 6 hours to complete the conversion of 4-hydroxycoumarin to the ketone, and the latter was then isolated from the cooled solution by

continuous ether extraction for 8 hours. After removal of the solvent, the residue was taken up again in a little ether, washed with 5% aqueous NaHCO_3 , then with water, and dried over anhydrous Na_2SO_4 . The solvent was removed and the oil obtained was dried in vacuo to constant weight. The oil was then treated with the theoretical amount of 2,4-dinitrophenylhydrazine in concentrated HCl (1-2 ml.) at 100° for 1 hour. The 2,4-dinitrophenylhydrazone of *p*-hydroxyacetophenone was obtained as long orange needles, m.p. 216° from ethanol (Roseman et al., 1954), and was recrystallised to constant activity.

6,7-Dihydroxycoumarin (aesculetin). This compound was decomposed by heating with acid, so the following procedure was adopted. Urine (5 ml.) was mixed with the gastric juice of *Helix pomatia* (1 ml.) which contains β -glucuronidase, and acetate buffer (5 ml., pH 4.6), and incubated for 1 hour at 37° . Aesculetin (0.4 g.) was added to the mixture, which was diluted with water (100 ml.), boiled to dissolve the compound, treated with charcoal and filtered. Aesculetin (m.p. 269°) separated on cooling and was recrystallised from water. It was also converted into 6,7-diacetoxycoumarin (m.p. 133°) (Liebermann and Knietzsch, 1880) which was recrystallised from ethanol until the activity fell to zero. The aesculetin was added after incubation to avoid inhibition of the β -glucuronidase by a large excess of the product of hydrolysis.

3-, 4-, and 7-Hydroxycoumarin glucuronides. 3-Hydroxycoumarin glucuronide (0.1 g.) was added to urine (2.5 ml.; 1/100 activity) and warmed slightly to dissolve the solid; for the 4- derivative (0.05 g.), the urine (2.5 ml.) was diluted with water and boiled to dissolve the solid. The 7- derivative (0.2 g.) was dissolved in a few drops of 2N aqueous

ammonia and the urine (2.5 ml.) was added. The solution (acidified with ice-cold 2N HCl for the 7- isomer) was kept at 0° overnight. 3- and 4-hydroxycoumarin glucuronides crystallised out and were recrystallised from water to constant activity (m.p.s 208° and 184°, respectively). The 7-hydroxycoumarin glucuronide which separated was dissolved again in 2N ammonia (0.8 ml.) and acidified with 2N HCl. On cooling, the glucuronide slowly separated in small colourless needles (m.p. 147°) and was recrystallised from ethanol to constant activity.

o-Coumaric acid. o-Coumaric acid (0.2-0.5 g.) was added to urine (1-5 ml.; 1/100 to 1/20 activity), dissolved by warming and adding a little acetone, and re-isolated after standing for 0.5 hour by evaporating the acetone, diluting with water and cooling to 0°. The o-coumaric acid (m.p. 208°) was recrystallised from acetone-water to constant activity and was then converted into acetyl-o-coumaric acid (m.p. 158°), which was recrystallised from benzene until the activity fell to zero. Total o-coumaric acid could not be estimated because on heating with acid it is converted into coumarin.

o-Coumarylglycine. o-Coumarylglycine (0.1-0.2 g.) was added to urine (5 ml.), diluted slightly with water and boiled to dissolve the carrier. On cooling, o-coumarylglycine separated and was recrystallised from water and acetone-water until the activity disappeared.

o-Hydroxyphenylpropionic acid (melilotic) (a) Total. The acid (0.3-0.5 g.) was added to urine (5 ml.), which was diluted with water, made 5N with respect to HCl and hydrolysed for 3 hours. The cooled solution was saturated with NaCl and extracted with ether (5 x 20 ml.). The dried extract (anhydrous Na₂SO₄) was evaporated to a dark-coloured oil which did

not crystallise (dihydrocoumarin). The oil was triturated with calcium hydroxide and a few ml. of water with warming to form the calcium salt. The mixture was cooled and filtered. The filtrate was evaporated to 1 ml., filtered and cooled to 0°. Calcium melilotate separated and was dissolved in 2N HCl (2 ml.) and the free acid extracted into ether. The extract was dried (anhydrous Na_2SO_4) and yielded melilotic acid (m.p. 87°) which was recrystallised from carbon tetrachloride-petroleum ether (b. 100-120°) and from carbon tetrachloride to constant activity. It was further purified as the benzylamine salt (m.p. 146-7° from ethyl acetate) when the activity fell to zero.

(b) Free. The procedure was exactly as above but without acid hydrolysis.

o-Hydroxyphenylacetic acid. The acid (0.2-0.4 g.) was added to urine (1-5 ml.) and the procedures for total or free melilotic acid were followed. The ether extract of the hydrolysate or of the non-hydrolysed urine yielded o-hydroxyphenylacetic acid, m.p. 145-6° after recrystallisation from ether-petroleum ether (b.p. 60-80°) and from chloroform to constant activity. It was also converted into its benzylamine salt, m.p. 165°, which was recrystallised from ethyl acetate to constant activity.

o-Hydroxyphenyllactic acid. Calcium o-hydroxyphenyllactate (0.4 g.) was added to urine (1-5 ml.) which was hydrolysed as for total melilotic acid and extracted with ether (5 x 10 ml.). The residual oil from the dried extract was converted to the calcium salt which was recrystallised from water, reconverted to the free acid and thence to the benzylamine salt. The later (m.p. 130-131°) was recrystallised from ethyl acetate to constant activity.

o-Tyrosine. DL-o-Tyrosine (0.2 g.) was added to urine (5 ml.)

which was hydrolysed in the usual way, the solution was evaporated to about 1 ml., cooled to 0°, and o-tyrosinehydrochloride was precipitated by gradual addition of ethanol (5 ml.). The latter was recrystallised from ethanol and treated with benzoyl chloride and alkali. N-Benzoyl-o-tyrosine was isolated as small white needles and prisms, m.p. 187°, and was recrystallised from ethanol until the activity disappeared.

For free o-tyrosine, the urine containing the carrier was either brought to pH2 with concentrated HCl and after 0.5 hour the carrier was re-isolated as for total o-tyrosine, or was precipitated directly by addition of ethanol (20 ml.), filtered, and washed with ethanol and ether.

Chapter 3. The Metabolism of 3-Hydroxycoumarin.

The fate of 3-hydroxycoumarin in man and the rabbit has been investigated by Flatow (1910) and Mead et al. (1958a). It was necessary in this work to pursue the investigation in order to ascertain if the hydroxy-aromatic acids formed from coumarin (Chapter 5) were formed via 3-hydroxycoumarin.

Experimental.

The fate of 3-hydroxycoumarin in the rabbit.

Biosynthesis of 3-hydroxycoumarin glucuronide. Three rabbits received collectively 6 g. of 3-hydroxycoumarin. The 24 hour urine (total volume 410 ml.) had a pH of 6-7, gave a strong naphthoresorcinol reaction and a strongly positive test for ethereal sulphate. Paper chromatography of the urine in solvent systems A, B and C (Table 2.1) indicated the presence of free 3-hydroxycoumarin, its ethereal sulphate, and its glucuronide. After acid hydrolysis (5 ml. of urine refluxed for 3 hours with an equal volume of concentrated HCl) only 3-hydroxycoumarin could be detected in the hydrolysate. Paper chromatographic examination of ether extracts of the urine before and after acid hydrolysis showed that 3-hydroxycoumarin was present in increased amount after acid hydrolysis.

The bulk of the urine, worked up by the lead acetate procedure as described by Mead et al. (1958a) yielded a total of 1.6 g. of 3-hydroxycoumarin glucuronide hemihydrate, m.p. 212-213° (decomp.) from water.

Identification of o-hydroxyaromatic acids. A rabbit (3.4 kg.) was fed 0.84 g. of 3-hydroxycoumarin orally. The 24 hour urine (volume 60 ml.) had a pH of 6.5 and gave a strongly positive naphthoresorcinol reaction

and ethereal sulphate test. With aqueous ferric chloride, the urine gave a brown-buff precipitate, which on standing developed a weak green colour. No precipitate was formed with Brady's reagent, and Benedict's and Fehling's solutions were not reduced.

When irradiated by ultra-violet light (360 m μ .) at pH 6.5 the urine had a greenish blue fluorescence. At pH 10 (2N NH₄OH), the fluorescence was light green; at pH 4 (glacial acetic acid), it was blue; at pH 2 (concentrated HCl), the fluorescence was almost destroyed, being only a very faint greenish colour. Thus, as the pH is decreased the fluorescence changes from green to blue.

Urine samples collected during the following 30 hours gave similar qualitative reactions.

The untreated urine (50 μ l.) was applied to paper chromatograms and developed in solvent systems A, B, C, D and E (Table 2.1). 3-Hydroxycoumarin and its ethereal sulphate and glucuronide were detected with solvents A, B, C and D. o-Hydroxyphenylpyruvic acid was detected with A and B; solvents C and D, being ammoniacal, usually decomposed the labile acid, but the reference compound, when applied in high concentrations (more than 100 γ) could sometimes be detected in C. o-Hydroxyphenylacetic acid was detected with all five solvent systems, and a smaller amount of o-hydroxyphenyllactic acid with A, B, C and D.

Various other spots were detected which could not be identified. The principal ones are described in Table 3.1.

Table 3.1.

R _F values in solvent			Gibbs	Fluorescence 270 mμ.
A	B	C		
0	0	0	-	yellow to yellow-green
-	0.53	0.03	Red-purple or purple	blue

The pooled 60 hour urine (150 ml.) was acidified to pH 2 with concentrated HCl, saturated with NaCl and extracted continuously with ether for 12 hours. A bulky, flocculent white precipitate separated from the aqueous phase when it was mixed with ether. This was filtered off and dried (0.68 g.). Paper chromatography showed it to be mainly 3-hydroxycoumarin glucuronide. After recrystallisation from water, this material had m.p. and mixed m.p. with authentic 3-hydroxycoumarin glucuronide, 208°.

The ether extract, after removal of the solvent, yielded a dark, acid-reacting gum (0.58 g.). This dissolved in water to a strongly acid solution (pH 2-3). The naphthoresorcinol test was strongly positive (due to traces of glucuronide carried over in emulsion during the extraction). The ethereal sulphate test was negative. With Gibbs's reagent the extract gave a dark blue colour, with aqueous ferric chloride a weak red-brown colour. It gave no precipitate with Brady's reagent.

Paper chromatography of the gum (50 μg.) in solvents A, B and C showed the presence of 3-hydroxycoumarin, o-hydroxyphenylacetic acid and o-hydroxyphenyllactic acid. No trace of o-hydroxyphenylpyruvic acid was detected.

The gum was extracted several times with hot benzene, and after evaporation of the solvent, the residue was dissolved in saturated aqueous NaHCO₃ (5 ml.) and extracted with ether (2 x 5 ml.). The aqueous phase

was then acidified with 2N HCl and extracted again with ether.

The residue from the first ether extract was a whitish solid which did not melt below 250° and gave negative naphthoresorcinol and ethereal sulphate tests. Paper chromatography revealed only one spot, corresponding to 3-hydroxycoumarin. Acidification, extraction with ether, removal of the solvent and recrystallisation from water gave 3-hydroxycoumarin (10 mg.), m.p. and mixed m.p. 152°.

The residue from the second ether extract was a gum (22.6 mg.) which was shown by paper chromatography in three solvents to consist almost entirely of a mixture of o-hydroxyphenylacetic acid and o-hydroxyphenyl-lactic acid. There were also two minor spots having the characteristics shown in Table 3.2.

Table 3.2.

R_f (solvent A)	Gibbs	Fluorescence
		<u>360 mμ.</u>
0.00	blue	-
0.02	-	bright blue, green in NH_3 fumes

The gum became partly crystalline on keeping at 0°. An attempt was made to separate it into its constituents as follows. The gum (22 mg.) was dissolved in a small amount of ether and applied in a band to a paper chromatogram (Whatman No. 3 paper) previously washed for 24 hours with solvent A and dried. After development of the chromatogram in A (12 inch run) the zones were located by spraying a narrow strip cut from the side of the paper with Gibbs's reagent. The areas corresponding to o-hydroxyphenylacetic and -lactic acids were cut out and eluted chromatographically with ethanol. Only very small traces of non-crystalline material were obtained which could not be crystallised, but chromatography in A, B and C

indicated that the two fractions contained only o-hydroxyphenylacetic acid and o-hydroxyphenyllactic acid, respectively.

Conjugated o-hydroxyaromatic acids. After ether extraction of the urine in the experiment described above, it was made 5N with respect to HCl, hydrolysed by heating for 3 hours under reflux, and extracted continuously with ether for 48 hours. The extract was dried (anhydrous Na_2SO_4) and the solvent evaporated to yield brownish residues (290 mg.). This partly crystalline material chromatographed in solvents A, B, C and D revealed the presence of 3-hydroxycoumarin, o-hydroxyphenylacetic acid, and o-hydroxyphenyllactic acid, but no o-hydroxyphenylpyruvic acid. The intensity of the spots suggested that 3-hydroxycoumarin and o-hydroxyphenylacetic acid were major metabolites and o-hydroxyphenyllactic acid was a relatively minor, though significant, metabolite.

The extract was fractionated as described in the preceding section and afforded 190 mg. of 3-hydroxycoumarin, m.p. and mixed m.p. 152°. The second fraction was a sticky brownish solid (93.5 mg.). Paper chromatography showed mainly o-hydroxyphenylacetic and -lactic acids, the former comprising the main bulk of the material. An attempt to isolate these acids by the technique described above was unsuccessful. However, the two eluates obtained contained only o-hydroxyphenylacetic and o-hydroxyphenyllactic acids, respectively, as before.

The fate of 3-hydroxycoumarin in the rat.

Five rats (each weighing 200 g.) were given 0.2 g. each of 3-hydroxycoumarin by mouth. The pooled 24 hour urine had a pH of 6 and gave a positive naphthoresorcinol reaction but not so strongly positive as for rabbit urine. No distinctive colour was obtained with aqueous ferric

chloride, and Brady's reagent gave no precipitate. Benedict's reagent was reduced.

Paper chromatography of the urine in solvents A, B and C showed the presence of o-hydroxyphenylacetic acid (main metabolite), o-hydroxyphenyl-lactic acid (strong spot), and 3-hydroxycoumarin (only just detectable). In addition, a number of unidentified spots was observed, all of which were not necessarily detectable in all solvent systems. The most prominent and consistently appearing are recorded in Table 3.3.

Table 3.3.

R _F values in solvent			Gibbs's reagent
A	B	C	
0.00	0.05	0.21	red-purple to red-brown
-	0.22	0.16	yellow
-	-	0.12	red-orange to yellow-brown

The metabolites were still detectable in the urine in similar relative amounts 72 hours after dosing. The urine collected from five rats over three days was pooled (150 ml.), hydrolysed, and ether extracted as described for rabbit urine. The residue after removal of the ether consisted mainly of the two hydroxy acids with only traces of 3-hydroxycoumarin. An attempt to isolate and purify the acids was not successful.

Discussion.

3-Hydroxycoumarin appears to be fairly completely absorbed and excreted in about 2.5 days by the rabbit, judging by the amounts of metabolites isolated from the urine (recovery of the dose; Table 1, Appendix 1). In the case of the rat, metabolites were still being excreted after 3 days.

The findings of Mead et al. (1958a) that the major portion of administered 3-hydroxycoumarin was excreted by the rabbit in conjugated forms, the glucuronide (57%) being quantitatively more important than the ethereal sulphate (14%), were confirmed in this work qualitatively (chromatography) and semi-quantitatively (isolation). It was also found that a small amount of 3-hydroxycoumarin was excreted in the free form. In the rat, the picture is rather different. Glucuronide and ethereal sulphate are not present in large amounts in rat 3-hydroxycoumarin - urine, and the conjugates of 3-hydroxycoumarin were not detected at all by paper chromatography.

The small extent of glucuronide formation in the rat is connected with the very small amounts of 3-hydroxycoumarin found in the urine. The main difference between the metabolism of 3-hydroxycoumarin in rabbits and in rats lies in the fact that rats cleave the lactone ring to a relatively much greater extent than rabbits. Thus, while significant amounts of o-hydroxyphenylacetic and o-hydroxyphenyllactic acids are excreted by the rabbit, most of the administered material is left with the lactone ring intact. In rats, on the other hand, most of the hydroxycoumarin is converted into the o-hydroxyphenyl- acids.

These hydroxy- acids are partially conjugated in the animal (rabbit and rat) because after removal of the free acids from the urine, acid hydrolysis of the residual solution liberates a further amount of them. The hydroxy-acids could be conjugated with glucuronic acid to form ester or ether glucuronides (which is unlikely, because of the weak naphthoresorcinol reaction of rat urine), or more likely, may be conjugated with glycine to form compounds related to phenaceturic acid. These conjugates are present in fairly large amounts in the rat, and this would account for the reducing

power of rat 3-hydroxycoumarin urine. No experiments to isolate and identify the conjugates were undertaken, so the point was not proved.

That the lactone ring of 3-hydroxycoumarin is easily opened in vitro under mild alkaline conditions has been mentioned (p.6). Chromatographic evidence has been obtained that the opening of the lactone ring in vivo proceeds via o-hydroxyphenylpyruvic acid. The acid was detected in untreated urine of rabbits fed 3-hydroxycoumarin, although the pH of the urine was as low as 6.5. This precludes the possibility that o-hydroxyphenylpyruvic acid was formed as an artifact by alkaline conditions in urine. Although the labile acid is difficult to detect in alkaline solvent systems, it sometimes appeared using solvents A and B (Table 2.1). Since some of the hydroxyphenylpyruvic acid survived cyclisation in these acid solvents it may be supposed that more of it was originally present in the urine than was detected.

The ease of oxidative decarboxylation of o-hydroxyphenylpyruvic acid to o-hydroxyphenylacetic acid in vitro suggests the route by which the latter is formed in the body. It is unlikely to have been formed from its precursor in urine at pH 6.5. The formation of a conjugate of o-hydroxyphenylacetic acid is conclusive evidence of its biological origin.

The formation of o-hydroxyphenyllactic acid, albeit to a minor extent compared with o-hydroxyphenylacetic acid, indicates an alternative route of o-hydroxyphenylpyruvic acid.

Chapter 4. The Metabolism of 4-Hydroxycoumarin in the Rabbit. A Method for the Quantitative Estimation of 4-Hydroxycoumarin in Rabbit Urine.

4-Hydroxycoumarin was found to be excreted mainly unchanged by the dog (Roseman et al., 1954). It seemed desirable to investigate what other metabolites might be formed, for the following reasons:

If 4-hydroxycoumarin were formed from coumarin in the rabbit, its metabolites would also be coumarin metabolites. The metabolism of 4-hydroxycoumarin is also itself of great interest as it is the parent compound of a number of important anticoagulant drugs. Finally, it is of theoretical interest to compare the metabolism of 3-hydroxy- and 4-hydroxycoumarins, these being the hydroxycoumarins with the substituent in the pyrone ring.

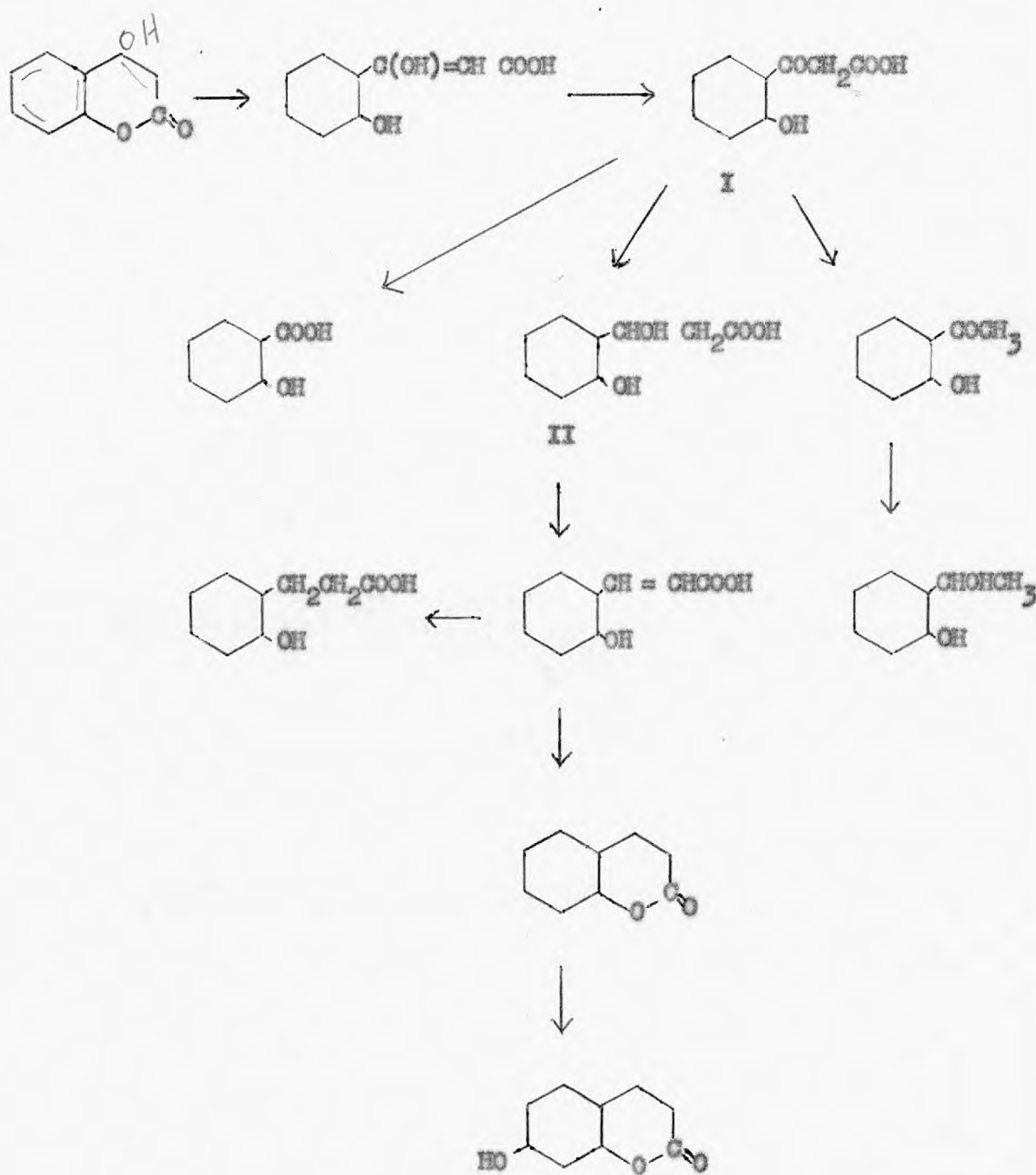
The most likely route to be considered for the breakdown of 4-hydroxycoumarin in the body is opening of the lactone ring, with modification of the products by oxidation, reduction, decarboxylation, or removal of the elements of water. Scheme 4.1, p. 54 represents the possibilities.

Experimental.

In a preliminary experiment, a rabbit (3 kg.) was fed 1.5 g. of 4-hydroxycoumarin orally. The 24 hour urine (volume 90 ml.) had a pH of 9, gave a strong naphthoresorcinol reaction and a weak ethereal sulphate test. Benedict's reagent was reduced somewhat after two minutes boiling. No characteristic colour was observed with aqueous ferric chloride and no precipitate was obtained with Brady's reagent.

The urine had a weak greenish-blue fluorescence when neutral or when acidified (pH 4 and 2) and gave a green fluorescence at pH 13.

Scheme 4.1.



A portion of the urine was acidified with concentrated HCl (pH 2) and extracted with ether. The extract gave a weakly positive naphthoresorcinol reaction and did not reduce Benedict's reagent or give a precipitate with Brady's reagent.

Portions of the urine were hydrolysed with acid: (a) urine was made 0.3N with respect to HCl and boiled under reflux for 15 minutes; (b) urine was made 5N to HCl and boiled for 4 hours. Ether extracts were made of the hydrolysates. The various fractions were chromatographed on paper in solvents A, B, C and D (Table 2.1). The original urine and the mildly hydrolysed urine contained 4-hydroxycoumarin and its glucuronide. The ether extracts showed the presence of 4-hydroxycoumarin only. The strongly hydrolysed urine showed no indication of either 4-hydroxycoumarin or its glucuronide, but o-hydroxyacetophenone was detected in the ether extract.

The intensity of the spots on paper chromatograms suggested that the 4-hydroxycoumarin excreted unchanged represented a major part of the dose. In order to isolate this compound and to estimate it quantitatively, 4 rabbits (3 kg. each) were fed 1.5 g. of 4-hydroxycoumarin each. Qualitative tests for the 24 hour urines were similar to the preceding experiment.

From two of the urines, colourless crystals separated on standing. The precipitate (3.75 g.) was collected after centrifuging. This material appeared to be only partially soluble in methanol, ethanol and acetone. It was soluble both in saturated aqueous NaHCO_3 and in 2N HCl. A red-purple colour was obtained with Gibbs's reagent, a weak red with Brentamine fast blue B salt, and yellow with Brentamine fast red 3GL.

The naphthoresorcinol reaction was positive. These tests suggested a mixture of 4-hydroxycoumarin and its glucuronide. Paper chromatography in three solvents showed only a small amount of 4-hydroxycoumarin. The material was extracted with acetone (100 ml.) and the solvent evaporated, yielding 4-hydroxycoumarin (20 mg.).

The residue was in the form of feathery rosettes and did not melt below 250°. Heated on a spatula, it charred and left a white ash, which was insoluble in cold water but soluble in 2N HCl. The flame test (brick red) suggested the presence of calcium. The salt was dissolved in the minimum amount of hot water and acidified with 3 drops of concentrated HCl. On cooling, a colourless microcrystalline precipitate separated, m.p. 184-5°, mixed m.p. with authentic 4-hydroxycoumarin glucuronide 184-5°. Chromatography showed only one spot, corresponding to the glucuronide, and hydrolysis with 5N HCl for a few minutes, followed by chromatography, showed only one spot, identical with 4-hydroxycoumarin. A total of 0.25 g. of the pure glucuronide was obtained from the glucuronide salt (0.5 g.). The latter was not further investigated.

The other two urines deposited a precipitate, mainly phosphate, which was separated and cautiously acidified with concentrated HCl. The filtered solution, on cooling, deposited a brownish solid, which on recrystallisation from hot water (charcoal) gave 4-hydroxycoumarin glucuronide (55 mg.). The total yield of 4-hydroxycoumarin glucuronide monohydrate from the 4 rabbits was 0.3 g.

Portions of each urine (10 ml.) were retained for quantitative work. The remainder was pooled (800 ml.), brought to pH 2 with concentrated HCl, and extracted continuously with ether for 30 hours. Crude 4-hydroxy-

coumarin (1 g.) separated from the ether extract. Recrystallised from water (charcoal), this gave 0.75 g. of 4-hydroxycoumarin, m.p. 211-213°, showing one spot on paper chromatograms. Further recrystallisation gave m.p. and mixed m.p. 215-216°.

The rest of the ether extract yielded on evaporation a further amount (1 g.) of crude brown material, consisting mainly of 4-hydroxycoumarin. This was used for paper chromatography.

A rabbit was fed 1 g. of 4-hydroxycoumarin orally and the 24 hour urine was collected. 10 ml. of this was kept for qualitative tests and chromatography, the remainder (120 ml.) was brought to pH 3 with concentrated HCl and extracted continuously with ether for 6 hours. The ether extract was dried over anhydrous Na_2SO_4 . The residual urine plus washings (150 ml.) was divided into two parts. One fraction (50 ml.) was mixed with an equal volume of acetate buffer (pH 4.6), 50 mg. of a partially purified β -glucuronidase preparation (Wight and Co.) were added, and the mixture was incubated at 37° for 18 hours. The mixture was saturated with NaCl and extracted continuously with ether for 6 hours, and the extract was dried (anhydrous Na_2SO_4). The second fraction (100 ml.) was made 5N with respect to HCl and boiled under reflux for 4 hours. The hydrolysate was extracted continuously with ether for 8 hours, and the extract was dried.

The pooled 24 hour urine (800 ml.) from three rabbits which had received a total of 1.8 g. of 4-hydroxycoumarin was made N with respect to NaOH and warmed for 1.5 hours on a water bath at 80°. The solution was acidified to pH 3 with HCl and continuously extracted with ether for 30 hours. After drying the extract and removal of the solvent, a crude

brown product (0.6 g.) was obtained.

All the fractions described above were examined by paper chromatography in solvent systems A, B, C and D. The unchanged urine contained 4-hydroxycoumarin as the main constituent; there was also a strong spot for the glucuronide. The ether extracts of acidified urine, enzyme-hydrolysed urine, and alkali-hydrolysed urine consisted almost entirely of 4-hydroxycoumarin. o-Hydroxyacetophenone was not detected in any of the above fractions, but it was found in the ether extract of strongly acid-hydrolysed urine, where it appeared to be the only constituent, apart from a little 4-hydroxycoumarin.

Chromatography of the original urine in solvent A, showed a spot giving a blue Gibbs colour, which appeared to correspond to the main component of the o-hydroxyphenylhydracrylic acid mixture, having R_f 0.05 (Table 2.1). However, this could not be confirmed in other solvent systems, nor with any of the extracts.

No trace was detected in the urine or any of the extracts of salicylic acid, o-coumaric acid, 7-hydroxycoumarin, melilotic acid, or coumarin. A number of minor unidentified spots were observed, giving colours with Gibbs's reagent, particularly in the ether extract of acid-hydrolysed urine. The chromatographic behaviour in solvent A of the spots detected in the latter extract is described in Table 2, Appendix 1.

Quantitative estimation of 4-hydroxycoumarin.

Attempts were made to apply the method of Robinson et al. (1953) for the estimation of 8-hydroxyquinoline to the estimation of 4-hydroxycoumarin. Problems of pigment precipitation were encountered and led to the adoption of Brentamine fast red 3GL salt (BFR) and distilled water as

solvent instead of phosphate buffers. 4-Hydroxycoumarin gave a yellow colour with BFR; using a 0.1% aqueous solution of BFR and a substrate concentration range 5-30 $\mu\text{g./ml.}$, precipitation of the pigment occurred at the higher concentrations. The colour was soluble in ethanol, but did not develop in ethanol solution. Therefore, the colour was developed in aqueous solution and ethanol was then added to clear the solution. The colour developed immediately at room temperature. λ_{max} was 440 $\text{m}\mu$.

Method of Assay. The standard curve was prepared as follows: 3 ml. of an aqueous solution of 4-hydroxycoumarin (5-30 $\mu\text{g./ml.}$) were treated with 1 ml. of 0.1% aqueous BFR and mixed (test tubes with ground glass stoppers were used). Ethanol (2 ml.) was added immediately, the solution was mixed by shaking, and the colour was measured without delay in the Unicam S.P. 500 spectrophotometer at 440 $\text{m}\mu$. in 1 cm. cells. The blank consisted of 3 ml. of water, 1 ml. BFR reagent and 2 ml. of ethanol. The standard graph obtained was a straight line and was the mean of 18 separate determinations; individual variations in the upper part of the curve amounting to $\pm 10\%$. Thus, even with pure solutions of 4-hydroxycoumarin, reproducibility was not good. As far as was possible, the lower part of the curve was used in estimations.

Recoveries were made by dissolving 4-hydroxycoumarin in normal rabbit urine (2-4 mg./ml.) and diluting for assay as required. Estimations were made at substrate concentrations of 20 $\mu\text{g./ml.}$ Normal urine with no added substrate was diluted by the same amount and the colour developed compared with that of urine containing added substrate. The reading for normal urine was usually appreciable, and was, moreover, variable.

Recoveries (typical average values from a number of experiments) are shown in Table 4.1.

Thus, recoveries are consistently too high (at least 10%) even after subtraction of values due to the colour developed in normal urine.

Table 4.1.

Recoveries of 4-hydroxycoumarin from urine.

Concentration of 4-hydroxycoumarin. ($\mu\text{g./ml.}$)	Found ($\mu\text{g./ml.}$)	Corrected for normal urine values ($\mu\text{g./ml.}$)	Recovery %
20.0	26.4	22.0	110
20.0	28.4	24.0	121
20.0	26.4	22.0	110
20.0	26.4	21.6	108

Estimation of 4-hydroxycoumarin and its glucuronide in rabbit urine.

Glucuronides were estimated by the method of Hanson *et al.* (1944).

4-Hydroxycoumarin and glucuronide estimations were carried out on the urine of 3 rabbits before and after feeding 4-hydroxycoumarin (0.2 g./kg.).

4-Hydroxycoumarin was also estimated in the urine of 4 rabbits used in the experiment cited on p.55 (0.5 g./kg.).

A strong, bright yellow colour was given by the urine with BFR reagent on the first experimental day (i.e. the first 24 hours after the dose) but only a feeble yellowish on subsequent days.

The urine was made up to 200 ml. with water on each day; then for 4-hydroxycoumarin estimations, 1 ml. of this solution was made up to 100 ml; for glucuronide estimations, 1 ml. was made up to 25 ml. on normal days and to 100 ml. on experimental days. 4-Hydroxycoumarin was estimated by treating 3 ml. of diluted urine with 1 ml. of BFR reagent and 2 ml. of ethanol as described above. All determinations were carried out in

triplicate.

The percentages of administered 4-hydroxycoumarin excreted free and as glucuronide are presented in Tables 3 and 4, Appendix 1. The values given were obtained by subtracting the average normal values for the two days preceding the dose from the values on experimental days. The unchanged 4-hydroxycoumarin was all excreted on the first experimental day; on the second and third experimental days, no increase above normal was obtained. The average values are summarised below, with the ranges in parentheses.

		<u>Percentage of dose.</u>
Free 4-hydroxycoumarin	(7 rabbits)	40 (27-51)
Glucuronide	(3 rabbits)	30 (29-32)
Total	(3 rabbits)	66 (57-83)

Discussion.

Of an orally administered dose of 4-hydroxycoumarin, only 66% could be accounted for as urinary metabolites, and this consisted of the unchanged compound, which was rapidly excreted partly as free 4-hydroxycoumarin and partly as its glucuronide. The remainder of the dose may have been present in the faeces, but only the urine was examined in these experiments.

The extent of conjugation of 4-hydroxycoumarin is relatively less than that of 3-hydroxycoumarin and this may be related to the acidity (pK_a 5.8) of 4-hydroxycoumarin.

There are no available data for comparison of excretion of 4-hydroxycoumarin in rabbit and rat. The pattern of excretion in the dog (Roseman *et al.*, 1954) is similar to that in the rabbit. These workers accounted for 75% of the intravenously injected sodium salt, 50% as the free

hydroxycoumarin and 25% as the glucuronide.

The available evidence indicates that the lactone ring of 4-hydroxycoumarin, in contrast to that of 3-hydroxycoumarin, remains intact in the animal. The data presented in the present work shows that o-hydroxyacetophenone is not a metabolite of 4-hydroxycoumarin in the rabbit, the ketone detected after acid hydrolysis of the urine being an artifact. Any conjugated o-hydroxyacetophenone should have been revealed by the enzymic or mild alkaline hydrolysis of the urine, which procedures do not break down 4-hydroxycoumarin. Although reference compounds for some of the expected intermediate products shown in Scheme 4.1 were not available, the absence of the stable metabolites which might be formed from these is reasonably good evidence against opening of the ring, as o-hydroxybenzoylacetic acid (I) and o-hydroxyphenylhydracrylic acid (II) are unstable and should be transformed. The latter is known to lose a molecule of water fairly easily by acid treatment (Booth et al., 1959) to form o-coumaric acid, and likely routes for the former are degradation to salicylic acid, or decarboxylation to o-hydroxyacetophenone. o-Coumaric acid, coumarin, 7-hydroxycoumarin and salicylic acid are easily detected in small amounts. A weak point in the argument is that o-hydroxyacetophenone may be converted in considerable amount to o-hydroxyphenylmethylcarbinol, and this compound was not available.

Roseman et al. (1944) reported that 4-hydroxycoumarin has 1/20 of the anticoagulant activity of dicoumarol, and the question arises whether it possesses this activity per se or whether it is partly converted to dicoumarol or to a similar compound. Also, the 4-hydroxycoumarin may be converted to a derivative of 2-hydroxychromone in vivo (cf. p. 7).

Knobloch et al. (1952) attribute the anticoagulant effect of the bis(4-hydroxycoumarins) to the chromone form of the fully dissociated anions at pH 7-8 (pp. 8-9).

Chapter 5. The Metabolism of [^{14}C] Coumarin.

This chapter deals mainly with experiments conducted with rabbits and rats using [^{14}C] coumarin.

A preliminary experiment was carried out with inactive coumarin to check the possibility that a dose of coumarin might be partially eliminated unchanged via the lungs. The expired air of a rabbit (3 kg.) which had received 0.25 g. of coumarin contained no detectable amount of coumarin during the 24 hours after dosing.

[^{14}C] Coumarin Experiments.

Rabbits. Three rabbits received [^{14}C] coumarin (40-50 mg./kg.). Daily urine volumes varied usually from about 50-200 ml. When two urine specimens could be obtained in the 24 hours, the specimen excreted during the night had a larger volume (100-200 ml.) than the daytime sample (0-40 ml.). The urine was slightly alkaline (pH 7-9). The urine as such, or after adding 2N ammonia showed a strong bright blue fluorescence when illuminated by ultraviolet light at 270 or 360 m μ ., which persisted for 6 days (rabbit 1) or for the duration of the experiment (3-4 days) (rabbits 2 and 3). The acidified urine fluoresced a much weaker blue-green. The first 24 hour urine gave a strong positive naphthoresorcinol reaction; later urines gave weaker reactions, not more intense than normal urine. The urine did not reduce Benedict's reagent even on prolonged boiling for 2-5 minutes, and gave no precipitate with Brady's reagent. With aqueous ferric chloride no distinctive colouration was observed, but sometimes dubious greenish-brown colours or precipitates were obtained.

Paper chromatography of the urine as such in solvent systems A, B, C and D (Table 2.1) revealed the presence of 3-hydroxy- and 7-hydroxy-

coumarins, a little 8-hydroxycoumarin, o-hydroxyphenylacetic acid, and a weak spot for o-hydroxyphenyllactic acid. An ether extract of the acidified urine showed the same metabolites, and an ether extract of the urine after acid hydrolysis (5 ml. of urine and 5 ml. of concentrated HCl refluxed for 0.5 hour) contained the hydroxycoumarins in increased amounts, the hydroxy-acids, and, in addition, coumarin. A little 6-hydroxycoumarin was detected in some extracts by its weak u.v. fluorescence; it did not show up on chromatograms sprayed with Gibbs's reagent, due no doubt to inadequate separation from the hydroxy-acids, which give strong colours with this reagent. No trace was discovered on the chromatograms of 4-hydroxy- or 5-hydroxycoumarins, 6,7-dihydroxycoumarin, o-hydroxyphenylpyruvic acid, o-coumaric acid, melilotic acid, salicylic acid, or phenol.

The pooled faeces (3-4 days) contained only a very small percentage of the radioactivity fed (0.2% to 0.65%). The dried faeces from one rabbit were extracted with boiling acetone and the extract was concentrated and chromatographed on paper. No trace of any metabolites was detected, and the faeces were not investigated further.

Rats. The urine from three rats which had been fed [^{14}C] coumarin (100 mg./kg.) had a daily urine volume of 2-25 ml. (usually below 10 ml.) and a pH of 7-8. Before and after addition of 2N ammonia, the urine showed a similar, but weaker, fluorescence to that of rabbit urine, while the acidified urine fluoresced blue-green. This effect persisted in the urine samples for at least three days. The first and second 24 hour urines gave a fairly strong positive naphthoresorcinol reaction. The urine reduced Benedict's reagent quite strongly in 30 seconds. No distinctive colours were seen with ferric chloride solution and no precipitate was obtained with Brady's reagent.

The untreated urine when chromatographed as above showed a weak spot corresponding to o-hydroxyphenyllactic acid, and a strong spot for o-hydroxyphenylacetic acid. 3-Hydroxycoumarin was barely detectable and 7-hydroxycoumarin was sometimes only detectable by its u.v. fluorescence after exposure to ammonia vapour. An ether extract of acid-hydrolysed urine contained the same compounds and coumarin. A trace of 6-hydroxycoumarin could sometimes be detected in extracts after a higher dose of inactive coumarin by its fluorescence in u.v. light. A strong spot appeared on chromatograms of untreated urine which had similar R_f values and gave similar colour reactions to o-tyrosine (but was not identical with the latter compound, see p. 73). No indication was obtained of the presence of 4-hydroxy-, 5-hydroxy-, or 8-hydroxycoumarins, 6,7-dihydroxycoumarin, o-hydroxyphenylpyruvic acid, o-coumaric acid, melilotic acid, salicylic acid, 2,4-dihydroxycinnamic acid or phenol.

Faeces. Rat faeces contained considerable amounts of radioactivity (Table 5.1). The pooled faeces (4 days) from each of two rats were dried over anhydrous CaCl_2 and extracted several times with boiling acetone. The combined extracts were filtered, taken almost to dryness and made up to 25 ml. with ethanol, forming a green solution (Extract 1). The residues were extracted twice with warm $2N$ Na_2CO_3 solution to remove any conjugates. The filtered solution was made up to 100 ml. or 200 ml. with water (Extract 2). No further activity was extractable from the residue by mechanical shaking with Na_2CO_3 for 6 hours.

A portion of the freshly extracted wet faeces residue (20 g.) from one rat was boiled with $5N$ HCl (100 ml.) under reflux for 3 hours, and the hydrolysate was extracted continuously with ether for 8 hours. The solid residues were extracted with several portions of acetone, and the acetone

and ether extracts were combined and concentrated. The concentrate was made up to 100 ml. with acetone and counted (Table 8, Appendix 1). There was no radioactivity in the acid hydrolysate after it had been ether-extracted. A solid residue (1.3 g.) remaining after the above extraction was a black powder, appearing to be elemental carbon (Table 8). A portion of the faeces residue from another rat was similarly treated after keeping for several weeks, during which time a large part of its radioactivity disappeared. During acid hydrolysis of this fraction, any evolved CO_2 was trapped in 5N NaOH and counted as BaCO_3 .

The above extracts (1 and 2) were chromatographed on paper in solvent systems A, B, C, and E (Table 2.1). Traces of free coumarin were detected in both extracts and a little *o*-hydroxyphenylacetic acid in Extract 1 only. Some other prominent spots were seen on the chromatograms but none were identified. There was no trace of any of the monohydroxy-coumarins, *o*-coumaric acid, melilotic acid, 2,4-dihydroxycinnamic acid or phenol.

Low Dose Experiments. Three rabbits and three rats received [^{14}C] coumarin (690 $\mu\text{C.}/\text{g.}$) orally at a dose level of 1 mg./kg.; about 2 $\mu\text{C.}$ of activity for rabbits and about 0.1-0.2 $\mu\text{C.}$ for rats. In the case of rats, an aqueous suspension was considered unsuitable for administration of such a small dose, so the compound was dissolved in the minimum amount of ethanol, made up to a definite volume with water, and a measured volume was administered. Total faeces of each rabbit for 3 days were homogenised in an equal weight of water (sp. gr. of water, 1.0), and the 2 day faeces of each rat (2-4 g.) were homogenised in 10 ml. of water and counted wet.

Results and Discussion.

In a preliminary experiment to determine the general pattern of

excretion of radioactivity, a dose (40 mg./kg.) of [^{14}C] coumarin containing a low activity (1.3 $\mu\text{c.}$) was fed to one rabbit. Two other rabbits later received doses (50 mg./kg.) containing 50-60 $\mu\text{c.}$ of activity. For rats, a higher dose level (100 mg./kg.) was used so that sufficient radioactivity (10-15 $\mu\text{c.}$) could be administered to the smaller animals to enable isotope dilutions to be carried out on the urine.

The excretion of radioactivity by rabbits and rats after dosing is shown in Table 5.1. Almost all of the administered radioactivity was recovered, amounting to 83-101% for rabbits and 86-100% for rats, but there is a considerable difference in the route of elimination between the two species. Rabbits excrete practically all the radioactivity in the urine, with very little in the faeces, whereas while rat urine contains more than half of the excreted radioactivity (47-61%), a considerable amount (32-39%) is eliminated in the faeces.

The rates of excretion can be seen from Tables 5,6 and 7, Appendix 1, and Figures 1, 2a, 2b, 3a and 3b. In the first rabbit experiment, some 92% of the radioactivity was excreted in the urine in the 4 days following dosing, but the urine was still weakly active after 12 days. Most of the activity (50%) appeared on the fourth day, accompanied by a correspondingly large urine volume (268 ml.). This animal was kept without food until the fourth day. Thus, the sudden increase in excretion was clearly due to retention of urine. In later experiments (rabbits 2 and 3), excretion was virtually complete in 48 hours (90.2, 80.4%), most of the activity (78.4, 79.1%) appearing within the first 24 hours. For rats, the excretion of radioactivity was slightly slower (Figure 3), but again, most of the urinary activity (43-54% of the dose) had appeared within 48 hours (Table 6,

Appendix 1). For one rat, separate daily faeces samples were counted (Table 7, Appendix 1); the excretion of activity was uneven, but all had appeared within 4 days after dosing.

The distribution of radioactivity in various tissues of rabbits and rats killed 3-4 days after dosing is presented in Table 9, Appendix 1. The percentage of the total activity remaining in the tissues is very small. One rat was homogenised whole and counted. The liver of another rat contained more activity than the livers of the rabbits, but the contents of the gastro-intestinal tract of a rabbit contained a little activity, while the tract of a rat contained none.

The distribution of the radioactivity in the low-dose experiments (Table 5.2) is similar to that for the larger doses (Table 5.1) except that a higher percentage of the activity fed to rabbits was found in the faeces; in one rabbit this was considerable (23%). Also, the recovery was not complete for rabbits. However, both these discrepancies are probably due to a lower counting accuracy, as small amounts of activity were present in large amounts of excreta. In the rat experiments, higher counts were found in smaller amounts of excreta.

Metabolism of coumarin in rabbits. In the rabbit, almost all of the radioactivity found in the urine was accounted for as metabolites by isotope dilution. The amounts of each metabolite excreted are quoted in Table 5.3. Three main kinds of metabolites can be distinguished: hydroxycoumarins, hydroxyaromatic acids, and acid-labile precursor or precursors of coumarin.

The most important group of compounds quantitatively is the monohydroxycoumarins. Together these compounds represent some 40% of the dose

when estimated after acid hydrolysis of the urine. Isotope dilution for the free compounds gave a total value of only about 4%. Thus, the hydroxycoumarins are excreted mainly in conjugated forms. The conjugates have been shown (Mead et al., 1958a) to be the glucuronides and ethereal sulphates. Isotope dilution for free glucuronides (those of 3-, 4-, and 7-hydroxycoumarin only) and for hydroxycoumarins after mild acid hydrolysis, which splits the ethereal sulphates, suggests that the amount of ethereal sulphate formed under these conditions is approximately equal to, or even slightly greater than glucuronide (Table 5.4).

All six of the monohydroxycoumarins are found to be metabolites of coumarin, the main one being 3-hydroxycoumarin (20%). 7-Hydroxycoumarin is also a major metabolite (12%) but the other isomers are minor metabolites - 6-hydroxycoumarin (3%) and 8-hydroxycoumarin (2%) - or occur only in traces - 5-hydroxycoumarin (0.4%) and 4-hydroxycoumarin (0.6%). The experimental accuracy does not permit any distinction to be made between the 4- and 5- isomers. 6,7-Dihydroxycoumarin was not detected in the urine by paper chromatography and isotope dilution confirmed its absence. No definite statement can be made about the other dihydroxy-isomers.

Two aromatic hydroxy-acids were found by paper chromatography and isotope dilution, the main one being o-hydroxyphenylacetic acid, which amounted to about 20% of the dose. The other was o-hydroxyphenyllactic acid (3% of the dose). The o-hydroxyphenylacetic acid occurred partly conjugated (about 1/4 to 1/3) and partly free (Table 5.3). The nature of the conjugation was not investigated, however. Another possible hydroxy-acid, o-hydroxyphenylpropionic acid, was not detected by paper chromatography nor by isotope dilution for the free and total acid.

Only about 0.5% of free coumarin was found, but after strong acid hydrolysis of the urine about 15% of the radioactive dose was accounted for as coumarin. Thus an acid-labile precursor or precursors of coumarin was present in coumarin-urine from rabbits.

Free o-coumaric acid and its conjugate, o-coumaryl glycine were not found by isotope dilution or paper chromatography.

Some isotope dilutions were carried out on the first and second 24 hour urines for Rabbit 2, and it can be seen from Table 5.5 that the greater part of each metabolite was excreted in the first 24 hours after dosing.

Metabolism of coumarin in rats. The relative proportions of metabolites excreted by the rat differ significantly from those excreted by the rabbit, and only about half of the urinary radioactivity (19-29% of the dose) was accounted for as metabolites (Table 5.3).

Hydroxylation of the coumarin nucleus occurred also in rats, most of the monohydroxycoumarins being found in the urine by isotope dilution. However, the amounts of hydroxycoumarins in rat urine were very much less than in rabbit urine amounting together to not more than 3-4% of the dose (or 6-8% of the urinary radioactivity). In rats, as in rabbits, 3-hydroxycoumarin was quantitatively the most important hydroxycoumarin (about 2% of the dose), while 6-hydroxy-, 7-hydroxy- and 8-hydroxycoumarins were found in approximately equal amounts (0.3-0.5%). No distinction can be drawn between the last three isomers from these results. Isotope dilution was not carried out for 5-hydroxycoumarin, due to paucity of the reference compound, and 4-hydroxycoumarin (0.5%) was found in only one rat. These figures all represent total amounts, after acid hydrolysis of the urine.

o-Hydroxyphenylacetic and o-hydroxyphenyllactic acids were found in rat urine in amounts quite similar to those found in rabbit urine. The former acid accounted for the same proportion of the dose as in rabbits (20%); the amount of o-hydroxyphenyllactic acid was rather less in rats (0.6-0.9%) than in rabbits (about 3%). The o-hydroxyphenylacetic acid was partly conjugated and partly excreted free, as in rabbits, but the extent of conjugation appeared to be variable. o-Hydroxyphenylpropionic acid was not found.

An acid-labile precursor of coumarin was found in rats, but in smaller and more variable amounts than in rabbits (3-7.5%). The small amounts of unchanged coumarin found were similar to those for rabbits.

No free o-coumaric acid was found by paper chromatography and isotope dilution. No o-tyrosine was found by isotope dilution and no 2,4-dihydroxycinnamic acid by paper chromatography. A number of unidentified spots were observed on paper chromatograms, however, some of which are described in Table 10, Appendix 1.

The relative importance of the metabolites in rabbits and in rats is compared in Table 5.6, in which the amounts of metabolites excreted as percentages of the dose fed, and as percentages of the radioactivity in urine, are presented.

Rat faeces. Attempts to identify the metabolites in rat faeces, which represented an important part of the dose (30-40%), met with little success. The distribution of activity among various fractions prepared from faeces of two rats is shown in Table 8, Appendix 1. The amount of activity extractable into acetone is small (1% or less of the dose). Some of this was found by paper chromatography to be due to traces of unchanged coumarin

and small amounts of o-hydroxyphenylacetic acid, but an unidentified compound was also present (Table 11, Appendix 1) which could have been another hydroxyaromatic acid.

The amount of activity extractable into Na_2CO_3 was variable (20.5%, 6.7%) and could have been due to conjugated metabolites. Paper chromatography showed a trace of unchanged coumarin, together with an unidentified major metabolite which closely resembled o-tyrosine in its chromatographic behaviour. Isotope dilution for o-tyrosine in rat urine, which contained a similar compound, yielded negative results, however, and was not carried out with the faeces extract. The above spot usually showed up in two parts, the central part giving a green, and the outer part a purple Gibbs colour. Thus, two inseparable compounds could be present, or one compound which can change into another form (cf. o-hydroxyphenylpyruvic acid, which gives a green-purple Gibbs spot). Another unidentified metabolite, running very close to the above compound on paper chromatograms, or surrounded by it, was observed as a green to greenish-brown Gibbs spot. There was also another compound present in smaller amount which gave a blue Gibbs colour and appeared to be the same as that observed in Extract 1 (see Table 11, Appendix 1).

The activity remaining in rat faeces after extraction (17%, 26%) was not due to a free phenolic compound or an acid or conjugate, as it was not extractable into acetone or Na_2CO_3 . In the course of several weeks, most of this activity disappeared, and it was also lost from the fresh material on strong acid hydrolysis. Thus, a considerable amount of a fairly volatile substance, or precursors thereof, was present (11-23% of the dose), which was insoluble in polar solvents and probably neutral. No

$^{14}\text{CO}_2$ was evolved by acid hydrolysis of the residue after spontaneous loss of activity. The ether extracts of the acid hydrolysates and the aqueous phases contained very small or negligible amounts of activity. The stable part of the activity was located in a solid residue of elemental carbon which could have been derived from carbohydrate. Thus, this portion of the radioactivity (5.8%; 2.1%) could have been incorporated into carbohydrate of the intestinal flora of the rat.

Table 5.1.

Excretion of Radioactivity by Rabbits and Rats after Dosing with [^{14}C] Coumarin.

<u>Experiment No.</u>	<u>Rabbits.</u>			<u>Rats.</u>		
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>
Weight (Kg.)	3.25	3.0	2.9	0.14	0.2	0.4
Dose of Coumarin (mg.)	125.0	150.0	147.0	14.45	21.0	39.85
Dose of Coumarin (mg./Kg.)	40	50	50	100	100	100
Dose of ^{14}C ($\mu\text{C}/\text{animal}$)	1.345	58.42	47.65	9.977	14.49	10.22
Duration of experiment (days)	12	4	3	4	5	6.75
<u>Percentage of radioactivity recovered in:</u>						
Urine	101.3	92.2	81.1	47.0	58.9	60.5
Faeces	-	0.2	0.8	38.7	33.4	32.4
Washing from tank, etc.	-	-	-	-	4.5	-
Tissues	-	0.1	1.2	0.3	2.9	-
<u>Total radioactivity recovered</u>	<u>101.3</u>	<u>92.3</u>	<u>83.1</u>	<u>86.0</u>	<u>99.7</u>	<u>93.0</u>

Table 5.2.

Excretion of Radioactivity by Rabbits and Rats after a small oral dose of [^{14}C] Coumarin.

<u>Experiment No.</u>	<u>Rabbits.</u>			<u>Rats.</u>		
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>
Weight (Kg.)	2.8	2.5	3.0	0.175	0.15	0.165
Dose of Coumarin (mg.)	2.83	2.5	3.07	0.175	0.150	0.165
Dose of Coumarin (mg./Kg.)	1.0	1.0	1.0	1.0	1.0	1.0
Dose of ^{14}C ($\mu\text{c.}/\text{animal}$)	1.95	1.73	2.07	0.12	0.10	0.11
<u>Duration of Experiment (days)</u>	3	3	3	2	2	2
<u>Percentage of radioactivity recovered in:</u>						
Urine	56.2	52.2	44.5	55.0	54.9	41.4
Faeces	4.7	4.6	22.6	51.8	44.7	56.1
<u>Total radioactivity Recovered:</u>	<u>60.9</u>	<u>56.8</u>	<u>67.1</u>	<u>106.8</u>	<u>99.6</u>	<u>97.5</u>

Table 5.3.

The Metabolites of [^{14}C]coumarin in rabbits and rats.

Experiment No.	Rabbits			Rats		
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>
Dose of coumarin (mg./kg.)	40	50	50	100	100	100
Dose of ^{14}C ($\mu\text{C.}/\text{animal}$)	1.3	58.4	47.6	10.0	14.5	10.2
Duration of experiment (days) ¹	4	2	1.7	3.7	5	6.75
<u>Compounds examined:</u>	<u>Percentage of dose</u>					
Coumarin after acid hydrolysis	12.3	14.8 (0.7)	16.7 (0.4)	3.1 (1.6)	7.4 (0.6)	-
3-Hydroxycoumarin	18.1	19.9	23.4 (2.8)	1.8	1.7	-
4-Hydroxycoumarin	-	0.3	0.9	0	0.5	-
5-Hydroxycoumarin	-	0.3	0.5	-	-	-
6-Hydroxycoumarin	-	4.7	2.0 (0.2)	0.3	0.3	-
7-Hydroxycoumarin	10.1	16.0	10.0 (0.7)	0.26	0.45	-
8-Hydroxycoumarin	-	2.5	1.3 (0.3)	0.3	0.5	-
6,7-Dihydroxycoumarin	-	0	0	-	-	-
Total coumarins	-	58.5	54.8	5.8	10.9	-
<u>o</u> -Tyrosine	-	-	-	0	0	0
<u>o</u> -Coumaric acid (free)	-	0	0	0	0	-
<u>o</u> -Coumaryl glycine	-	0	0	-	-	-
<u>o</u> -Hydroxyphenylpropionic acid	-	0	0	0(0)	0(0)	-
<u>o</u> -Hydroxyphenyllactic acid	-	3.5	2.6	0.6	0.9	-
<u>o</u> -Hydroxyphenylacetic acid	-	22.1	18.1 (12.9)	12.5 (11.2)	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	80.2	47.0	58.9	60.5

1. Time during which urine was collected for isotope dilution (see Tables 5 and 6, Appendix 1).

2. Figures in parentheses are for the free metabolite; all other figures are for metabolites after acid hydrolysis.

Table 5.4.

Free and conjugated metabolites of [^{14}C] coumarin in a rabbit.

Experiment No.3. Dose of coumarin 50mg./kg. Dose of ^{14}C 47.6 μC .

Percentage of dose.

<u>Compound</u>	<u>Total (A)</u>	<u>Free</u>	<u>Glucuronide</u>	<u>Glucuronide (B)</u>	<u>A - B</u>	<u>mild hydrolysis.</u>
Coumarin	16.7	0.4	-	-	-	-
<u>o</u> -Coumaric acid	-	0	-	-	-	-
<u>o</u> -Coumatyl glycine	-	0	-	-	-	-
3-Hydroxycoumarin	23.4	2.8	9.0	11.8	11.6	11.8
4-Hydroxycoumarin	0.9	-	0.12	-	-	-
5-Hydroxycoumarin	0.5	-	-	-	-	-
6-Hydroxycoumarin	2.0	0.2	-	-	-	0.8
7-Hydroxycoumarin	10.0	0.7	3.1	3.8	6.2	5.3
8-Hydroxycoumarin	1.3	0.3	-	-	-	0.8
<u>o</u> -Hydroxyphenyl- acetic acid	18.1	12.9	-	-	-	-
<u>o</u> -Hydroxyphenyl-lac- tic acid	2.6	-	-	-	-	-

Table 5.5.

Metabolites of [^{14}C] coumarin excreted by a rabbit on successive days.

Experiment 2. Dose of coumarin 50 mg./kg. Dose of ^{14}C 58.4 μc .

Percentage of dose in urine from:

<u>Compound</u>	<u>1st day</u>	<u>2nd day</u>	<u>Total</u>
Coumarin	14.2(0.6)	0.6 (0.1)	14.8 (0.7)
3-Hydroxycoumarin	18.2	1.7	19.9
4-Hydroxycoumarin	0.3	0.04	0.3
5-Hydroxycoumarin	0.3	-	0.3
6-Hydroxycoumarin	4.2	0.5	4.7
7-Hydroxycoumarin	14.4	1.6	16.0
8-Hydroxycoumarin	2.3	0.2	2.5

Figures in parentheses are for the free metabolite.

Table 5.6.

Percentages of radioactivity fed and of urinary radioactivity excreted as metabolites by rabbits and rats.

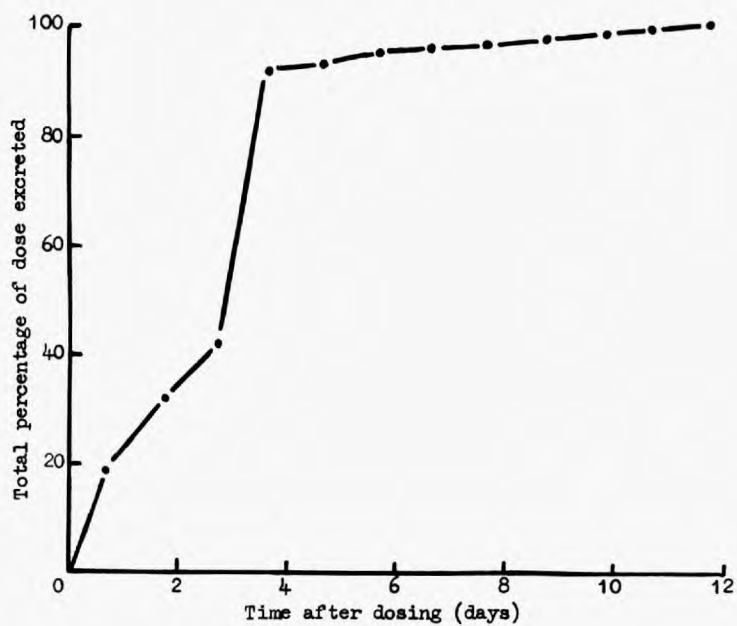
Experiment No.	Rabbits		Rats	
	2	3	4	5
Dose of coumarin (mg./kg.)	50	50	100	100
Dose of ^{14}C ($\mu\text{C.}/\text{animal}$)	58.4	47.6	10.0	14.5
Duration of experiment (days)	2	1.7	3.7	5

Metabolites excreted as percentage of:

Compound	dose	urinary activity	dose	urinary activity	dose	urinary activity	dose	urinary activity
Coumarin	14.8	16.4	16.7	20.8	3.1	6.6	7.4	12.6
3-Hydroxycoumarin	19.9	22.1	23.4	29.2	1.8	3.8	1.7	2.9
4-Hydroxycoumarin ⁸	0.3	0.3	0.9	1.1	0	0	0.5	0.85
5-Hydroxycoumarin	0.3	0.3	0.5	0.6	-	-	-	-
6-Hydroxycoumarin	4.7	5.2	2.0	2.5	0.3	0.6	0.3	0.5
7-Hydroxycoumarin	16.0	17.7	10.0	12.5	0.26	0.55	0.45	0.76
8-Hydroxycoumarin	2.5	2.8	1.3	1.6	0.3	0.6	0.5	0.85
Total coumarins	58.5	64.8	54.8	68.3	5.8	12.2	10.9	18.5
<i>o</i> -Hydroxyphenyl-acetic acid	22.1	24.5	18.1	22.6	12.5	26.6	27.2	46.2
<i>o</i> -Hydroxyphenyl-lactic acid	3.5	3.9	2.6	3.2	0.6	1.3	0.9	1.5
Total acids	25.6	28.4	20.7	25.8	13.1	27.9	28.1	47.7
Sum of urinary metabolites	84.1	93.2	75.5	94.1	18.9	40.1	39.0	66.2
Radioactivity of urine	90.2		80.2		47.0		58.9	

Excretion of radioactivity in the urine of
a rabbit receiving ^{14}C -coumarin orally
(dose, 40 mg./kg.).

Figure 1.
Experiment 1.



Excretion of radioactivity in the urine of
rabbits receiving ^{14}C -coumarin orally
(dose, 50 mg./kg.).

Figure 2a.

Experiment 2.

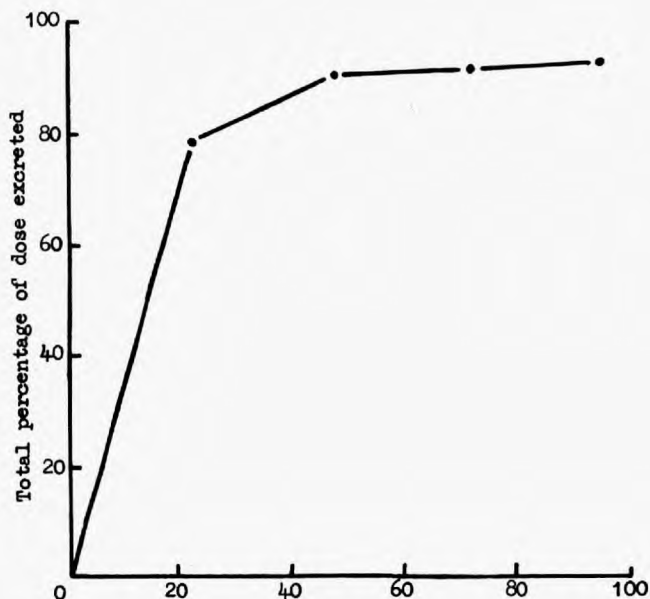
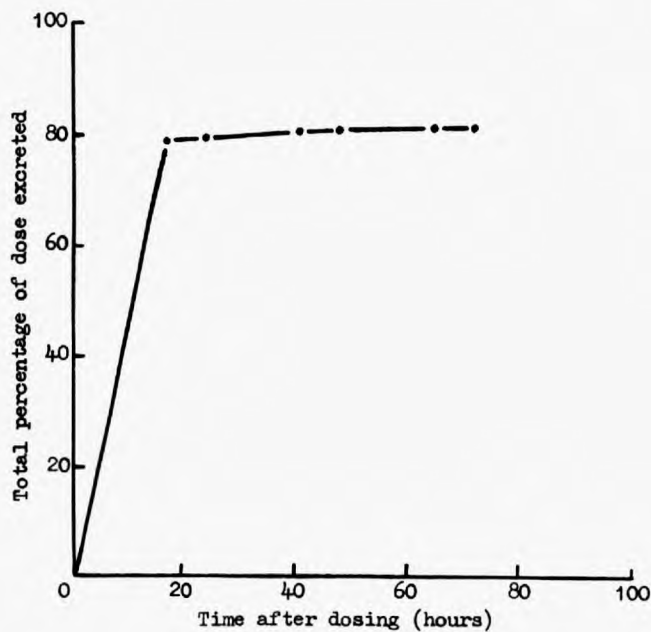


Figure 2b.

Experiment 3.



Excretion of radioactivity in the urine of
rats receiving ^{14}C -coumarin orally
(dose, 100 mg./kg.).

Figure 3a.

Experiment 4.

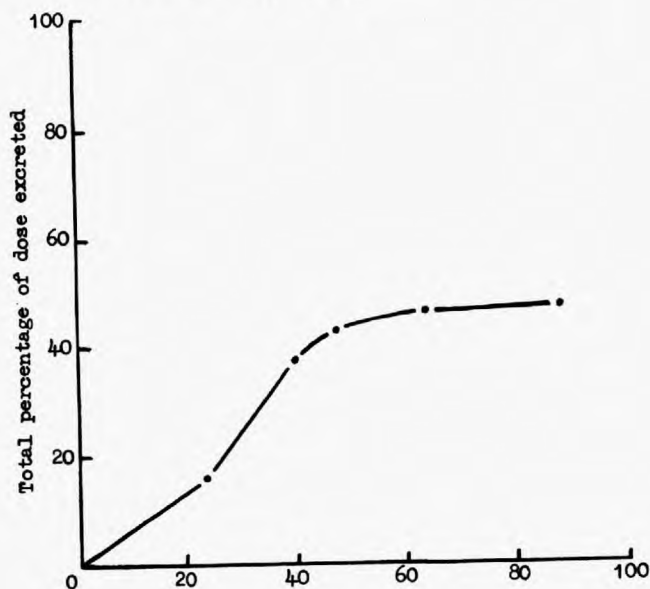
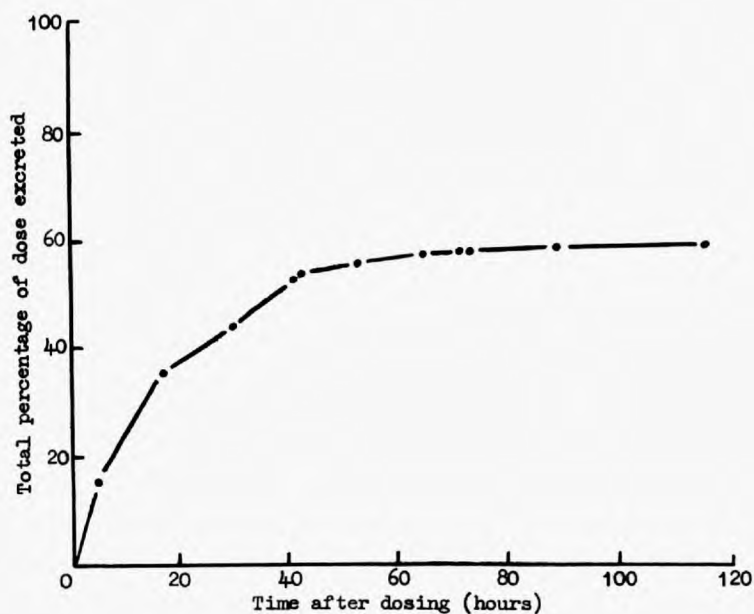


Figure 3b.

Experiment 5.



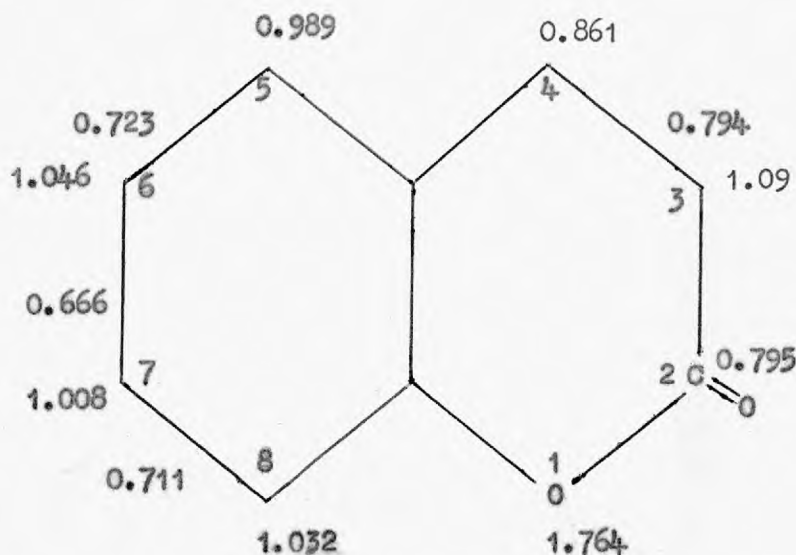
Chapter 6. Discussion.

The demonstration in the present work that coumarin is metabolised by hydroxylation at all the possible positions of the molecule is of great theoretical importance. A body of evidence has accumulated over a long period of time which shows that large numbers of drugs and other foreign compounds are metabolised in the animal by hydroxylation. The introduction of hydroxyl groups into the molecule does not result in formation of all the theoretically possible hydroxy-derivatives but only certain favoured isomers, and increasing evidence has made possible, in the case of benzene derivatives, the formulation of tentative rules by which the orientation of hydroxylation may in certain cases be predicted (Smith, 1950).

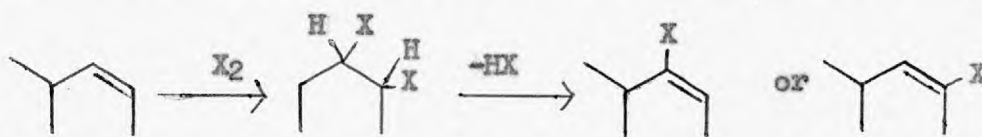
Not so much evidence is available for the heterocyclic compounds, but certain trends can be seen. Thus, Mead et al. (1958b) demonstrated 3-hydroxy-, 7-hydroxy-, and a trace of 8-hydroxy-coumarin as metabolites of coumarin in the rabbit. It now appears that the first two isomers are major metabolites, and that the other hydroxy-isomers are formed, but in amounts not detectable by the methods previously used.

The reactivities of the various positions of the coumarin nucleus may be related to the electron densities at these positions, the carbon atoms having the highest electron densities being those normally attacked by electrophilic reagents in vitro.

Samuel (1955) has determined the electron densities of all the atoms in the coumarin nucleus, and also the bond orders of the carbon-carbon links. The molecular diagram for coumarin is shown overleaf.



The positions placed in order of decreasing electron density are: 3 6 8 7 5 4. Thus, position 3 has the highest electron density of the carbon atoms and would be expected to have the greatest reactivity towards electrophilic reagents. This is true only to a limited extent, as although the 3 position is the most reactive with reagents that can add on to and saturate the 3,4 double bond, with most electrophilic reagents the 6-position is that which is most readily substituted, and the 8-position is the next most reactive (Chapter 1, p. 4). These last two carbon atoms have the highest electron densities in the benzene ring and are typically aromatic. The characteristic readiness of the 3,4 double bond to add on reagents, say X_2 , frequently with subsequent elimination of HX to restore the unsaturation (p. 2) is not a typically aromatic substitution, nor is it



an ethylenic addition. The 3,4 bond can be compared with the phenanthrene double bond or the 1,2 double bond of naphthalene, but is more reactive than either (cf. Badger, 1949). Its bond order (0.794) is the highest of the coumarin molecule.

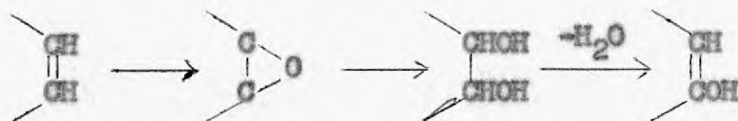
It has been suggested that the biological reactivities of the different positions of aromatic and heterocyclic compounds also are a function of their electron densities. This is largely supported by the present work, which indicates that position 3 of coumarin is by far the most reactive in vivo, and the biological activities of the other positions are also in agreement with this, with the sole exception of position 7.

In the rabbit, about 20% of a dose of coumarin is excreted as 3-hydroxycoumarin, and about 23% as o-hydroxy-acids, which have been shown to be derived from 3-hydroxycoumarin. That is to say, nearly 45% of the dose has been metabolised by means of reaction at position 3. Similarly, in rats about 22% of the coumarin is degraded via position 3, which represents about 75% of the urinary radioactivity which was accounted for in these animals. This overwhelming importance of position 3 is surprising even although this atom has the highest electron density, but it has to be borne in mind that if the hydroxylation proceeded via the dihydrodiol (p. 87) the addition is the type of reaction which is favoured by the properties of this grouping.

6-Hydroxy- and 8-hydroxycoumarins would be expected to be next most abundant after 3-hydroxycoumarin. However, 7-hydroxycoumarin occurs to the extent of 13% of the dose in the rabbit, whereas the average figures for the 6- and 8-isomers are 3.4% and 1.9%, respectively. This anomalous behaviour has not been explained, but some suggestions are made later. The small amount of hydroxylation at positions 4 and 5 is in agreement with the low electron densities at these positions. Further metabolism of 4-hydroxycoumarin by ring opening or by other means would not account for any but an insignificant percentage of a dose of coumarin, as most of the

4-isomer fed to rabbits was eliminated unchanged.

Mechanisms of hydroxylation. The hydroxylation of the coumarin molecule in vivo could take place by direct substitution by hydroxyl groups (by an ionic or free radical mechanism), or by the intermediate formation of a 1,2-dihydro-1,2-diol. Dihydrodiols are known to be formed in the animal body, a number of them having been isolated as metabolites of polycyclic hydrocarbons (e.g. Young, 1947). The currently favoured theory regarding the mechanism of their formation involves the direct addition of oxygen to the double bond to form an epoxide (Boylan, 1950), which could then undergo hydration to the dihydrodiol. These diols lose water on treatment with acid and yield the monophenol.

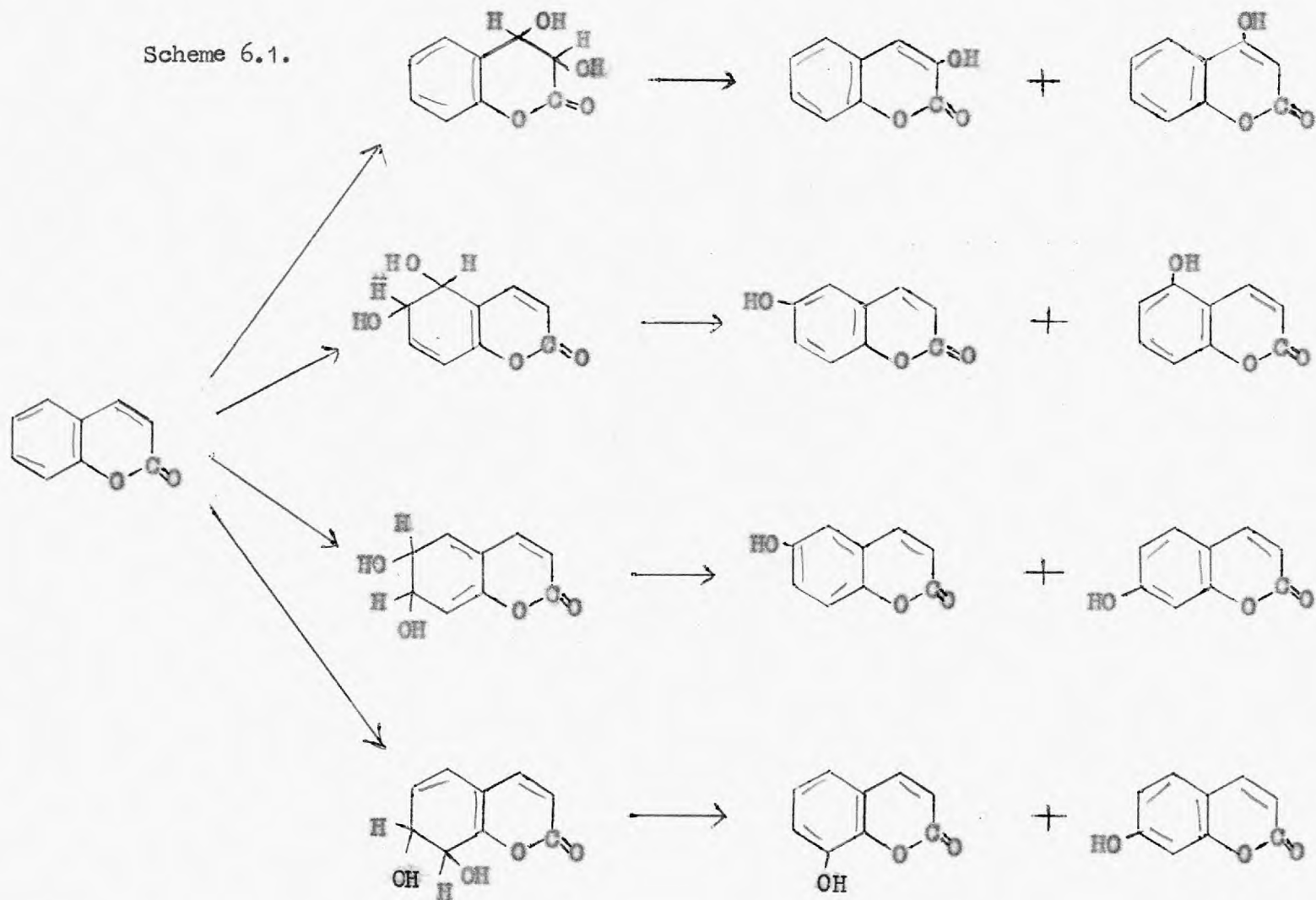


The hydroxyl group left attached to the ring is located on the carbon atom which has the highest electron density in the parent compound (Badger, 1949; cf. Smith, 1950).

Coumarin could give rise to four possible dihydrodiols, the 3,4-dihydro-3,4-dihydroxy-, 5,6-dihydro-5,6-dihydroxy-, 6,7-dihydro-6,7-dihydroxy-, and 7,8-dihydro-7,8-dihydroxy-derivatives. Two different monohydroxycoumarins could be formed from each of these by removal of the elements of water. The possibilities are shown in Scheme 6.1.

If the hydroxyl groups remained on the carbon atoms having the highest electron density, 3-, 6-, and 8-hydroxycoumarins should be formed. The present findings indicate, however, that all 6 hydroxycoumarins are formed. This can be explained by assuming that a mixture of both possible

Scheme 6.1.



hydroxycoumarins is formed from each dihydrodiol, with one isomer being produced in greater amount than the other. Thus, I should give rise almost entirely to 3-hydroxycoumarin, with very little of the 4-derivative. 6-Hydroxycoumarin should be the main product from both II and III, and IV would give rise mainly to the 8-isomer. 7-Hydroxycoumarin would be the minor product from both III and IV, but this would not account for the relatively large amount of this compound which was found.

Free radicals. Another mechanism of hydroxylation of the coumarin nucleus which could be postulated is direct substitution by free radicals. One in vitro source of free hydroxyl radicals is Fenton's reagent (cf. Boyland and Sims, 1953), and this has been shown (Mead et al., 1958b) to hydroxylate coumarin in all except the 4 position. Boyland and Sims (1953) detected 7-hydroxycoumarin as a product of this reaction.

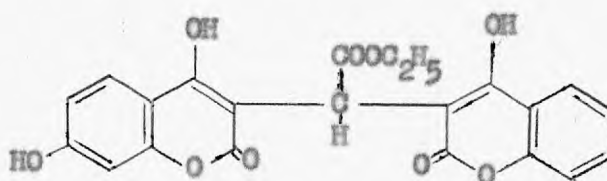
Thus, the pattern of hydroxylation seems qualitatively similar here to that found in vivo; however, no quantitative results are available. It should also be pointed out that Boyland and Sims (1953) did not find complete similarity between the metabolites of naphthalene in rabbits and rats and the hydroxylation products of naphthalene using Fenton's reagent or aqueous hydrogen peroxide irradiated with u.v. light.

The model hydroxylating system of Udenfriend et al. (1954), containing ascorbic acid, ferrous ions, and ethylenediamine tetra-acetate, has been shown by Mead et al. (1958b) to hydroxylate coumarin in a somewhat different way from that found in vivo. The main products are 7-hydroxy- and 5-hydroxycoumarins, with traces of the 6-isomer, whereas in vivo position 3 is chiefly substituted. It was suggested (Udenfriend et al., 1954) that this in vitro system might function by generation of

hydrogen peroxide, and that this reaction too could involve free radicals.

The evidence available seems to point to different mechanisms for the hydroxylation of position 3 and of the aromatic ring carbons. Thus, perhydroxylation could take place at the 3,4 double bond and free radical hydroxylation could occur in the benzene ring. This may account for the unexpected proportions of isomers hydroxylated in the benzene ring, as free radical reactions do not follow the usual rules of substitution.

Furthermore, the mechanism of formation of 7-hydroxycoumarin may be different from that of the other hydroxycoumarins substituted in the benzene ring. This gives rise to the suggestion that the biological site for the formation of 7-hydroxycoumarin could be different from that for the other hydroxy-isomers. It is of interest to note that ethyl biscoumacetate is metabolised in man by hydroxylation at position 7 of one of the coumarin rings (Burns et al., 1953a, b), although the other positions in the benzene ring are free.



The compounds chosen as substrates by Udenfriend et al. were shown by them to be oxidised in the same way by the model hydroxylating system as in the intact animal. Now these are foreign compounds which have also been shown (Mitoma et al., 1956) to be oxidised by the oxygen - and TPNH - dependent enzyme system of liver microsomes. It may be inferred from this that since 7-hydroxycoumarin is formed from coumarin by the ascorbic acid-iron system in vitro, it may be formed in vivo by the liver microsomes,

3-hydroxylation being effected by a different enzyme system.

However, consideration should also be given to the fact that the oxygen used for hydroxylation of acetanilide by liver microsomes is derived from oxygen, not from water, and this makes it less likely that free radicals are involved in the reaction (Udenfriend et al., 1956; cf. Parke and Williams, 1959). This leads to the final possibility that instead of electrophilic substitution (involving attack by a cationic reagent), or a reaction with free radicals, the active agent in 7-hydroxylation of coumarin could be an anion (i.e. nucleophilic substitution), which would attack position 7 in preference to positions 6 or 8. This too presupposes a separate site for 7-hydroxylation, and it would have to be supposed that position 5 was not available, for example, through binding to a tissue constituent. It could be postulated that a molecule of oxygen could give rise to two ions, O^- and O^+ . It would be of great interest to discover whether ^{18}O from molecular oxygen or from water were incorporated into the hydroxyl of 7-hydroxycoumarin.

The importance of 7-hydroxylation of coumarin in the rabbit probably cannot be compared with the widespread occurrence of 7-hydroxycoumarin derivatives in plants, as the latter are probably synthesised from phenols, not obtained by hydroxylation of the coumarin nucleus (cf. Chapter 1, p. 10).

Dihydroxycoumarins. With regard to the possible occurrence of dihydroxycoumarins as metabolites, the isomers most likely to be found could be predicted on the basis of the electron densities of the various positions of the coumarin molecule. Two pairs of adjacent carbons could be perhydroxylated to form three possible bis(dihydro-dihydroxy-)coumarins, each of which could give rise on dehydration to four possible dihydroxy-

coumarins, the relative amounts of these being predicted by reference to the charge on the carbon atoms (p. 85). The relative likelihood in which these isomers might be expected to be formed, if they were formed at all, is: 3,6->6,8->3,8->3,7->3,5->6,7->5,8->4,8-; 5,7-; 4,7-; 4,6-; and 4,5-dihydroxycoumarins. The o-substituted compounds 3,4-, 5,6-, and 7,8-dihydroxycoumarins do not seem feasible at all according to this scheme, but the possibility may also be considered of the formation of dihydroxycoumarins from dihydrodiols by removal of hydrogen. There is evidence that in monocyclic aromatic compounds, the 1,2-dihydroxy derivative is the principal product of the dihydrodiol (Parke and Williams, 1959) and if this held for coumarins the most likely derivatives would be 3,4-, 6,7-, 7,8-, and 5,6-dihydroxycoumarins.

The only dihydroxycoumarin looked for was 6,7-dihydroxycoumarin (because it was available) and it was not found, which does not favour the latter theory. However, according to the former theory, five other isomers seem more likely to occur than the 6,7-derivative, and the most likely, the 3,6-isomer, is known. 3,6-Dihydroxycoumarin does not fluoresce in u.v. light as does aesculetin, but gives a green colour with ferric chloride (Neubauer and Flatow, 1907). No metabolite was detected in urine of animals dosed with coumarin which gave a green spot with ferric chloride on paper chromatograms, but it should be pointed out that 3-hydroxycoumarin gives a green colour in a test tube with this reagent, but was not detected in coumarin-urine by ferric chloride sprayed on chromatograms. To establish whether or not 3,6-dihydroxycoumarin is formed as a metabolite it would be necessary to synthesise it.

Small, or perhaps in the rat, relatively large, amounts of dihydroxycoumarins may be formed from derivatives already containing one hydroxyl

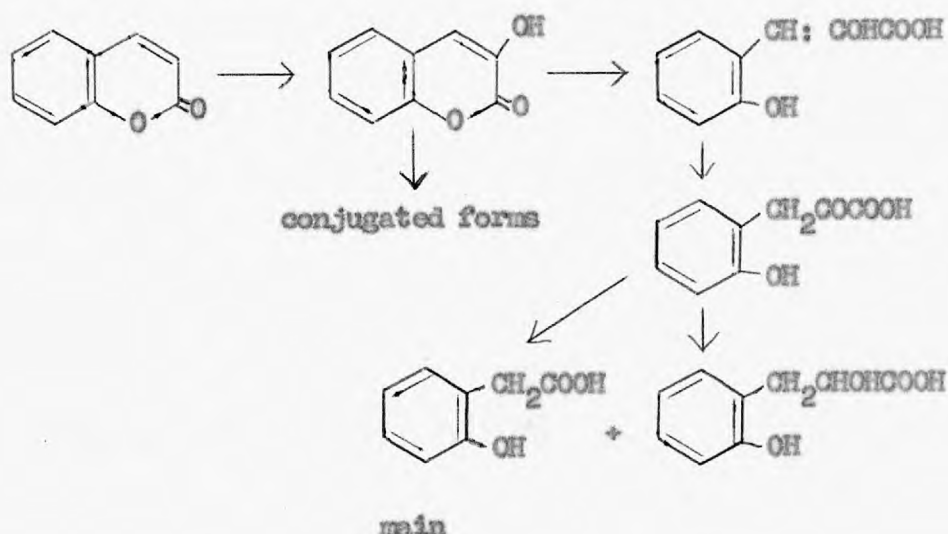
group in the benzene ring, i.e. the mechanism would not involve simultaneous perhydroxylation at 4 positions. Thus, Mead et al. (1958a) found small amounts of 6,7-dihydroxycoumarin as a metabolite of 6-hydroxycoumarin in the rabbit.

To sum up, the evidence seems to indicate that perhydroxylation is not the mode of hydroxylation of the benzene ring of coumarin, but that this mechanism is probably operative for positions 3 and 4.

Opening of the lactone ring. The discovery that the heterocyclic ring of coumarin is opened in vivo is an important step in the elucidation of the fate of this compound, and may help in understanding its pharmacological activity. It was previously believed (Mead et al., 1958b) that since o-coumaric acid, salicylic acid and melilotic acid were not formed from coumarin in vivo the heterocyclic ring remained intact. Booth et al. (1959), while the present work was in progress, reported the detection of o-hydroxyphenylacetic acid and o-hydroxyphenyllactic acid as metabolites of coumarin in rabbits and rats, and claimed that o-coumaric acid and melilotic acid were also found. Work with isotopically labelled coumarin has established conclusively that o-hydroxyphenylacetic acid is a major metabolite in rabbits and rats and that hydroxyphenyllactic acid is also formed in small amounts but confirmed the findings of Mead et al. (1958b) that the other acids were not metabolites.

At an early stage in this work it was thought that 3-hydroxycoumarin might be converted by the organism to o-hydroxyphenylpyruvic acid, and the finding of the other two hydroxy-acids (above) as coumarin metabolites suggested that the pathway of their formation was via 3-hydroxycoumarin. This was established by administering the latter compound to rabbits and to

rats, and finding all three of the above acids as metabolites. This indicated the importance of hydroxylation of position 3 in the organism. The main route of metabolism of coumarin in rabbits is shown below.



Flatow (1910) reported that 3-hydroxycoumarin was not converted to o-hydroxyphenylacetic acid by man but isolated this acid from the urine of a rabbit dosed with "o-hydroxyphenylpyruvic acid". The latter was prepared by heating 3-hydroxycoumarin in aqueous NaOH and then saturating with CO₂, a procedure which would give rise to substantial amounts of o-hydroxyphenylacetic acid in any case.

The present results are of theoretical importance, indicating that the opening of the heterocyclic ring in vivo is subject to certain conditions. Coumarin is converted to o-coumaric acid in vitro only under alkaline conditions (pH 10) (p. 5), and o-coumaric acid is not formed as an artifact even in the alkaline urine (pH 8-9) of the rabbit. However, saturation of the 3,4 double bond renders the lactone ring much more labile, and any dihydrocoumarin formed in vivo would exist largely as melilotic acid in the urine. Since melilotic acid was not found, direct reduction at the 3,4 positions does not occur in the coumarin ring as such. Again, if position

3 is substituted by a hydroxyl group, the lactone structure is more readily split in vitro than the parent molecule. That this is the case in the body also is shown by the formation of o-hydroxyphenylpyruvic acid as a metabolite of 3-hydroxycoumarin. The alkaline conditions in urine are not sufficient to open the ring of 3-hydroxycoumarin, nor is o-hydroxyphenylacetic acid produced as an artifact in urine (cf. p. 52).

Reduction at the 3-position does occur, after the lactone ring of 3-hydroxycoumarin has been opened, but this is not a reduction of the 3,4 bond of coumarin, but of the carbonyl group of the side-chain of the acid to form a secondary alcoholic group.

The route to the hydroxy-acids outlined above helps to explain the non-formation of salicylic acid from coumarin. This would not be expected to arise from an acid with a two carbon side-chain, and the lactic acid is resistant to oxidation.

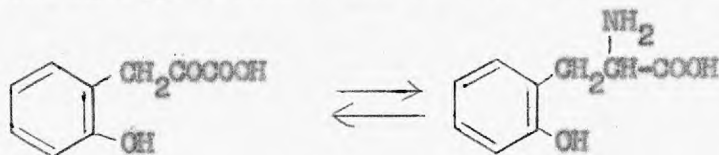
The possibility exists that substitution by hydroxyl in the benzene ring of coumarin may affect the stability of the lactone ring in vivo to a certain extent. If 7-hydroxycoumarin underwent opening of the heterocyclic ring in vivo, 2,4-dihydroxycinnamic acid would be the product. However, this acid was not found as a metabolite in rats or rabbits. The formation of 2,5- and 2,3-dihydroxycinnamic acids (via 6- and 8-hydroxycoumarin, respectively) was not investigated.

Since it appears from the present evidence that the coumarin lactone ring is not opened in the body unless position 3 is hydroxylated, it is suggested that dihydroxylation, in position 3 and in one other position, may be followed by ring opening. Thus, for example, 3,6-dihydroxycoumarin could give rise to 2,5-dihydroxyphenylacetic acid.

The importance of hydroxyaromatic acids as coumarin metabolites.

The formation of o-hydroxyphenyl acids from coumarin in rabbits and rats is of great interest, as o-hydroxyphenylacetic acid, normally occurring in very small amounts in human urine, is excreted in greatly increased amounts in the metabolic disorder, phenylketonuria (Boscott and Bickel, 1953; Armstrong et al., 1955). It is probably derived from o-tyrosine, which is formed in greater amount from phenylalanine following blockage of the normal route to p-tyrosine (Jervis, 1947; Udenfriend and Bessman, 1953; Armstrong and Shaw, 1955). It has been demonstrated that o-tyrosine gives rise to o-hydroxyphenylacetic acid in man (Blum, 1908) and in the rabbit (Flatow, 1910). Blaschko (1949) showed that o-tyrosine was a substrate for amino acid oxidases, and suggested therefore that it could be degraded to o-hydroxyphenylacetic acid via o-hydroxyphenylpyruvic acid.

It was considered a possibility that o-hydroxyphenylpyruvic acid might be converted into o-tyrosine, and if this were the case, the latter

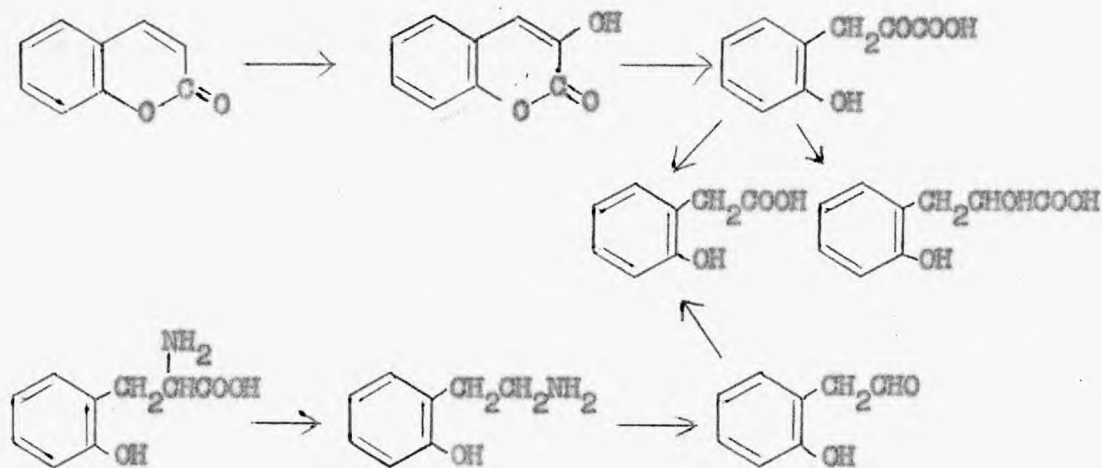


could be a metabolite of coumarin. Some evidence that a reversal of the usual direction of transamination may occur was furnished by Umezawa (1959), who found p-methylphenylalanine as well as p-methylphenylacetic acid in the urine of rabbits injected with p-methylphenylpyruvic acid. However, the compound having similar chromatographic properties to o-tyrosine which was observed in urine and faeces of rats fed coumarin was found by isotope dilution not to be o-tyrosine.

DL-o-Tyrosine fed to rats was found to be partly eliminated unchanged in the urine and partly converted to o-hydroxyphenylacetic acid. No o-hydroxyphenyllactic acid was detected in the urine and this is in agreement with the observations of Armstrong and Shaw (1955) that this acid was not excreted by normal or phenylketonuric subjects fed o-tyrosine.

Blaschko (1949) showed that o-tyrosine was a substrate for dopa decarboxylase, and Mitoma et al. (1957) found that o-tyrosine could be decarboxylated in the central nervous system, the product being o-tyramine. o-Tyramine is converted by amine oxidase to o-hydroxyphenylacetaldehyde (Randall, 1946) which can then give rise to o-hydroxyphenylacetic acid.

The formation of o-hydroxyphenyllactic acid as a metabolite of coumarin but not of o-tyrosine, may be explained by postulating a different route of degradation to o-hydroxyphenylacetic acid for the two compounds. The pathway for coumarin involving 3-hydroxycoumarin and o-hydroxyphenylpyruvic acid as intermediates is established; it seems likely that for o-tyrosine, intermediate formation of o-tyramine and o-hydroxyphenylacetaldehyde may be the preferred route in vivo.

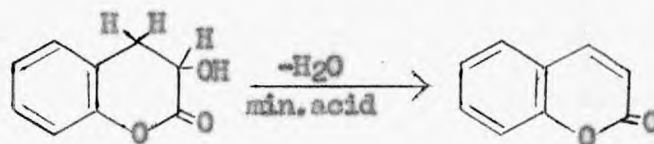


The present findings do not support the assumption made by Armstrong and Shaw (1955) of complete oxidation of some of these hydroxy-acids by rats and rabbits, as no $^{14}\text{CO}_2$ was found to be exhaled by either species. It is possible that o-hydroxyphenylpyruvic acid is formed at a late stage in the breakdown of coumarin, for example in the kidney, and that oxidative decarboxylation occurs here, immediately before excretion. There would then be no time for the hydroxy-acids to be metabolised further, whereas the orally administered compounds may be partly oxidised.

Acid-labile precursors of coumarin. In seeking the identity of the acid-labile precursor (or precursors) of coumarin found as coumarin metabolites in rabbit and rat (pp. 71, 72), a number of compounds could be considered.

o-Coumaric acid is converted into coumarin by strong acid treatment (p. 5) but neither free o-coumaric acid nor its conjugate, o-coumaryl-glycine were found as metabolites.

Dihydro-mono-ols have been postulated (cf. Young, 1950) as precursors of various polycyclic hydrocarbons (naphthalene, anthracene, and phenanthrene) which were obtained when the urines from animals which had been dosed with the hydrocarbon were acidified and heated. A similar type of compound could be postulated as a metabolite and precursor of coumarin. The only evidence which can be cited for or against this is

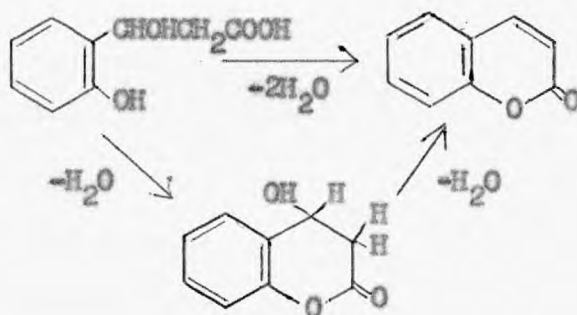


indirect. Eight isomeric compounds of this type are theoretically possible, 3,4-dihydro-3-hydroxycoumarin being shown above. The 3,4-substituted

compound would be most expected on the grounds of the reactivity of the 3,4-bond.

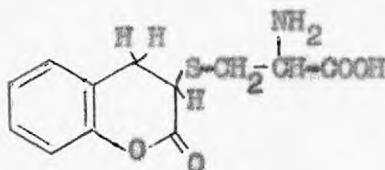
If the hydroxyl were attached to carbon 3 as shown, the compound would be the lactone of o-hydroxyphenyllactic acid, the free acid actually being found as a coumarin metabolite. This acid might be expected to cyclise to the lactone on strong acid treatment and this should then form coumarin, but it was found to be extremely stable to boiling with 5*N* HCl for 3 hours, and no coumarin was detected.

If the hydroxyl were attached at position 4, the compound would be the lactone of o-hydroxyphenylhydracrylic acid. Booth et al. (1959) reported that this acid was converted into o-coumaric acid by mild acid



treatment. It was confirmed in the present work that it was converted into coumarin by strong acid treatment but no trace of the acid was found in coumarin-urine by chromatography.

It is unlikely that the urinary coumarin precursor can be identical with any of the above compounds, but it does seem likely that the 3,4 bond of coumarin is involved in a labile linkage with some other molecule. It is now suggested that, for example, a cysteine molecule may have added on to the coumarin molecule at this point to form a compound of the following type:



It has been shown that naphthalene gives rise to N-acetyl-S-(2-hydroxy-1,2-dihydro-1-naphthyl)cysteine when administered to several species (Knight and Young, 1957; Boyland and Sims, 1958; Boyland et al., 1957).

Unidentified coumarin metabolites in the rat. It remains as a major problem that the fate of a large part of the coumarin administered to rats is unexplained.

The unidentified compounds in rat faeces could have been formed by the animal and subsequently excreted into the intestine via the bile, or by intestinal bacteria. The fact that two of the compounds found in faeces also occurred in urine might suggest the former route, but it is also possible that the major unknown compound might have been produced by bacteria, absorbed from the gut and then excreted in the urine (pp. 66, 73; Table 11).

Part of the unidentified radioactivity in rat urine could be due to compounds which have not been detected on paper chromatograms by colour reactions. The possibility of metabolic dimerisation of coumarin should not be overlooked, bearing in mind the ease of self-combination of the coumarin molecule (pp. 3-4). Also, a number of suggestions in earlier sections of the Discussion (dihydroxycoumarins, dihydroxyphenylacetic acids) were tentatively advanced mainly with the unidentified rat metabolites in mind.

Small oral doses of ^{14}C -coumarin. The approximate similarity between the pattern of elimination of small doses and larger doses in rabbits and rats (p. 69) suggests that the mode of metabolism found for larger doses

may be normally operative when the animal ingests small amounts of coumarin with its food. This is especially likely in the case of the rabbit, which feeds on the types of plant in which coumarin is widespread.

The ingestion of foreign compounds in nature by animals favours the view (Brodie, 1956) that certain enzymes exist in the organism for the principal purpose of metabolising these compounds, and the biotransformation of coumarin is a good example of this.

PART II. THE METABOLISM OF INDOLE.

Chapter 7. Introduction.

This part of the thesis describes a study of the metabolism of indole in rabbits. An understanding of the metabolic fate of indole in animals is important because the indole nucleus forms part of the structure of a large number of drugs in current use and of the essential amino acid tryptophan, and some light may be thrown on the metabolism and mode of action of these derivatives by a study of the parent compound. Indole derivatives occurring in plants, and in some animal products, are also likely to be ingested by animals, and in addition, indole itself is formed in the animal and problems of auto-intoxication may arise.

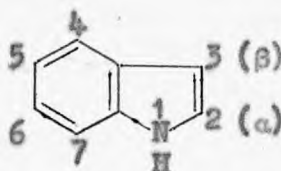
Indole has been found in jasmine flowers, orange blossoms, some citrus plants, *Robinia pseudacacia*, and a few other plants, and is also present in coal tar in the fraction boiling between 240° and 260°. It is found as a product of putrefaction of many biological materials, such as blood, milk, and albumin, and this is due to decomposition of tryptophan in the protein. 3-Methylindole (skatole), the odoriferous constituent of faeces, is derived from the same source. Other indole derivatives prominent in the biological field are urinary indican; indoleacetic acid (hetero-auxin), a plant growth hormone; serotonin (5-hydroxytryptamine), the vasoconstrictor substance occurring in brain and intestine; and a large number of plant alkaloids. Another substance, also known as indican, is the glucoside of 3-hydroxyindole (indoxyl), which occurs in the leaves and stems of the plant *Indigofera* (order Leguminosae) and which has been the source of indigo since the earliest periods of history. A different glucoside of indoxyl is present in the woad plant which was also used as a dye.

Chemistry of Indole.

The chemistry of indole has been reviewed by Van Order and Lindwall (1942), Morton (1946), and Julian et al. (1952), and references will not be given for information taken directly from these reviews.

The chemistry of indole was investigated intensively during the second half of the nineteenth century owing to the necessity of finding a synthetic route to the important dye, indigo, hitherto obtained from plant sources. Baeyer (1866) obtained indole by reduction of oxindole with zinc dust and the formula accepted today was proposed in 1869 by Baeyer and Emmerling.

Indole is 1-benzo[b]pyrrole, having the structure and numbering shown below.



The reactions of indole are similar to those of pyrrole, modified by the presence of the benzene ring. Indole is very feebly basic, and forms a picrate, but generally it tends to form resin-like materials when heated with acids. However, under certain conditions the hydrogen on the nitrogen atom is weakly acidic. Thus, with metallic sodium or sodamide, a sodium salt is formed, and with KOH, a potassium salt.

The 3-position is the most reactive of the molecule and appears to be exclusively attacked by many reagents. Mild oxidising agents usually convert indole into 3-hydroxyindole, which is then converted to indigo. Thus action of air in presence of sulphite or bisulphite, monopersulphuric acid, hydrogen peroxide, or ozone lead to formation of indigo from indole.

Chromic acid and ferric chloride oxidise indole to unidentified complex products. The Elbs alkaline persulphate oxidation gives rise to potassium indoxyl sulphate. Oxidation with permanganate opens the pyrrole ring, provided the N atom is benzoylated, otherwise amorphous masses are produced. The product is benzoylantranilic acid.

Indole undergoes few substitution reactions and these take place mainly at position 3. Direct nitration or halogenation of indole to yield derivatives substituted in the benzene nucleus is not feasible: indirect syntheses have to be used. 3-Nitroindole can be made by the action of ethyl nitrate and sodium ethylate on indole, or by oxidation of the nitroso derivative. Sulphuryl chloride reacts with indole to form 2-chloro- or 2,3-dichloroindole and iodine in potassium iodide yields 3-iodoindole. Alkylation of indole gives rise to the 3-alkyl- or the 2,3-dialkyl derivative, and treatment with acetic anhydride yields 1-acetyl- and 1,3-diacetylindoles.

Many of the reactions of indole appear to be due to the indolenine form, with a reactive methylene group at position 3. Thus, compounds that contain an active carbonyl group, such as aldehydes, will condense with indole at this position.

3-Nitrosoindole is obtained by the action of sodium nitrite and acetic acid or of amyl nitrite and sodium ethoxide. This product exists largely as the isomeric 3-oxime. The action of chloroform and alkali introduces an aldehyde group into position 3, and ring expansion also takes place with formation of 3-chloroquinoline.

The 2,3-bond of the pyrrole ring of indole can be reduced more readily, electrolytically or by catalytic hydrogenation, than the bonds of the benzene ring.

Pharmacology of Indole.

Indole is toxic to animals, acting as a cirrhotic poison of the liver, although Hervieux (1907) stated that indole and some alkylindoles had no toxic effect on the dog or rabbit. Indole causes convulsions and may be lethal when injected into lymphatic circulation of frogs, but skatole is less toxic (Gautier, 1913a and b).

Intravenous injection of indole into dogs leads to apnoea followed by an increase in respiration and a fall in blood pressure (Ets and Feinberg, 1942). Death from large doses results from dilatation and arrest of the heart. Indole and skatole affect the hearts of cold-blooded (frogs and turtles) more than warm-blooded animals (Waddell and Calhoun, 1927).

The motility of isolated intestine of frog (Danilevski, 1909) or rabbit (García-Blanco et al., 1941) is also inhibited by indole. Skatole is said to be the most powerful anti-histamine substance found in the intestinal tract (Bunyatyan and Matinyan, 1948).

Especially important from the pathological point of view is the effect of continued ingestion of subacute doses of indole over long periods. The symptoms in man include headache, sleepiness, diuresis, hunger, thirst and sore lower extremities (Griggs, 1916). Many of the mice dosed with indole by Büngeler (1932) died within a few months or developed haemolytic anaemias, leucopenias or leukaemias, and blood disturbances also result in rabbits (Le Calvé, 1921). The haemolytic anaemia induced by indole in dogs can be cured by liver extract (Rhoads et al., 1938a, b).

Indole is a weak haemolysin for erythrocytes of rabbits (Ponder, 1938), sheep and man (Gnesda, 1932). Skatole and 2-methylindole are also active, especially the latter.

Indole and skatole produce histological changes in the central nervous system (Crile, 1924), such as proliferation of the brain neuroglia (Wladyczko, 1912), and regressive changes of the blood vessels of the brain. Hardening of the arteries is another chronic effect of these compounds (Biebl, 1933).

Injection of indole and skatole into the knee joints of rabbits leads to chronic arthritis (Forbes and Neale, 1937) and indole has been found in the freshly voided urine from rheumatoid arthritis patients (Neuwirth, 1941). These long-term effects of indole may help to explain the naturally occurring disorders, as indole is normally produced in the body. Phocas (1927) suggested that indole produced in normal human metabolism tends to lead to nephritis over a long period of time.

The Formation of Indole in the Organism.

Interest in the metabolism of indole arose at the time when indigo was being intensively investigated, due to the presence in many normal and pathological urines of substances which gave rise to indigoid pigments on oxidation. These contain both indigotin and indirubin (Rimington, 1946). Indican was the name given to the precursors of the pigments and was detected by treating the urine with an oxidising agent and mineral acid.

It became clear from observations on fasting animals and those kept on various diets that digestion products of dietary proteins are broken down by intestinal bacteria to indole, which is absorbed by the animal and converted to indican (Underwood and Simpson, 1920; Brandeis, 1910; Gorter, 1909). No indican was found in the urine of new-born babies, and this was taken to indicate that there are no bacteria in the intestine at that time (Porcher, 1910).

Many bacteria, such as *B. coli* of the large intestine, form indole from breakdown products of proteins, but not from undigested proteins (Wollman et al., 1924). Addition of sugars to the medium inhibits the formation of indole, if they are fermentable by the organism (Rougantsoff, 1913; Applemans, 1921).

The source of indican in the case of fasting animals is probably due to the breakdown of tissue proteins (Klumenthal and Jacoby, 1910; Kishi, 1928).

The Metabolism of Indole and Some of its Derivatives in Animals.

Indole. It was early established that indole is oxidised by the organism to indoxyl, which is conjugated and excreted as glucuronide and ethereal sulphate (Baumann, 1977; Mester, 1888). Administration of indole to man (Porcher and Hervieux, 1907) and to rabbits and frogs (Gautier, 1910) gave rise to indican in the urine, and former workers found, after larger doses, another chromogen (presumably the glucuronide) which could form indigo by the action of bacteria. The main conjugate appears to be the ethereal sulphate. Ethereal sulphate excretion was increased when indole was fed to man, but not apparently glucuronide excretion (Tollens, 1910; Kauffmann, 1911), but output of both conjugates was raised in animals (Stern, 1910).

The first of these conjugates to be isolated was potassium indoxyl sulphate, which was obtained from the urine of dogs fed indole (Baumann and Brieger, 1879), from normal dog urine, and from urine of humans with intestinal tuberculosis (Hoppe-Seyler, 1884; 1916). Indoxyl glucuronide was demonstrated in the urine of rabbits fed 6-nitrophenylpropionic acid (Hoppe-Seyler, 1882-3) and in normal human urine (Mayer and Neuberg, 1900)

on the basis of its reducing properties and laevorotation. However more definite proof of the glucuronide was not obtained until Neuberg and Schwenk (1917) isolated the double barium salt of indoxyl sulphate and indoxyl glucuronide from the urine of a dog fed indole.

The indican content of blood is reported to vary from 0.001 to 0.004 per 100 ml. in horses and cattle, and from 0.08 to 0.35 g. per 100 ml. in urine (Spisni and Cappa, 1955).

Removal of the liver from dogs and toads resulted in considerable decreases in the amounts of indole converted to indoxyl (Barac, 1940; Stoppani, 1943b), and Laroche and Desbordes (1932) found that indole injected into the portal vein of a dog appeared in the hepatic vein as indoxyl. The latter workers reported that indole was converted to indoxyl in vitro by liver and lung tissue of dogs but not by kidney or brain, but García-Blanco and Naele (1935) state that liver, kidney, muscle and brain of rabbits all effect the conversion, with kidney being the most active. There is apparently a slight formation of indican in the intestinal wall (Nicolai, 1942) but the evidence indicates that the liver is the main site of detoxication of indole. The oxidation is rapid: García-Blanco and Vidal (1934) could not detect indole circulating in the blood 5-10 minutes after its injection into rabbits, but did detect indoxyl.

Indole inhibits the growth of young rats when administered over long periods, the effect being abolished by cystine, cysteine, methionine, and glutathione, but not by sodium sulphate, sulphite, thiosulphate, sulphide, or thiocyanate, elemental sulphur or cysteinamine (Wellers, 1953a, b; 1954). Thus the growth effect appears to be due to the use of sulphur-containing amino acids as a source of sulphate for conjugation.

Methylindoles. 3-Methylindole (skatole) does not give rise to indican excretion when administered to animals (Böhm, 1937) in common with other indoles substituted in position 3. It appears to be metabolised by oxidation of the methyl group to form indole-3-carboxylic acid (Porcher, 1909), distillation of which gives indole. The latter was found in distillates of normal urine of several species, and in increased amount after administration of skatole to rabbits (Jaffe, 1908; Blumenthal and Jacoby, 1910). More recently hydroxylation of the benzene ring has been shown by Delgliesh et al. (1958) and Decker (1957), who isolated the ethereal sulphates of 5- and 7-hydroxyskatoles from rat urine. After feeding skatole to animals, the parent substance of "skatole red" appears in the urine; the latter is a red pigment produced by treatment of the urine with strong acid (Salkowski, 1904). The precursor also occurs in association with urochrome (Rangier and de Traverse, 1938).

2-Methylindole fed to rabbits increases ethereal sulphate and glucuronide output, claimed to be due to formation of 2-methylindoxyl (Furuta et al., 1956), but Böhm (1937) states that this compound does not form an indican-like substance. 1-, 4-, 5-, and 7-methylindoles yield the corresponding methylindoxyls in man and rabbit (Böhm, 1937).

Other derivatives. Indole-3-aldehyde forms an indican in vivo, being apparently the only 3-substituted derivative which does this (Böhm, 1937). Indole-2-carboxylic and indole-3-carboxylic acids do not form indican.

A number of o-nitrophenyl derivatives, notably o-nitrophenylpropionic acid, form indican in the animal, but o-aminophenyl derivatives, except o-aminophenylethanol, do not. This seems to indicate that an intermediate

hydroxylamine is involved in the cyclisation (Böhm, 1938, 1939a, b; Stoppani, 1945).

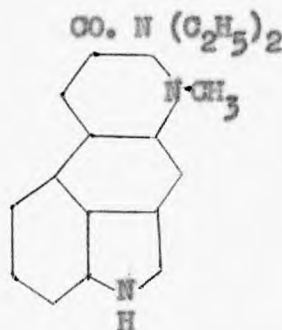
The remainder of this Chapter is devoted to a discussion of selected indole derivatives of biological importance.

Indolelactic acid. Dl- β -3-indolelactic acid, but not the l-isomer, is said to be capable of replacing tryptophan in the diet of rats and rabbits (Okagawa and Tatsui, 1931), and indolepyruvic acid can also be utilised (Jackson, 1929).

Indoleacetic acid. Indole-3-acetic acid was isolated from human urine obtained from hospitals, by Kogl *et al.* (1934) and was named hetero-auxin on account of its marked plant-growth effects. It causes growth by cell-elongation and promotes root-formation (e.g. Thimann and Koeplli, 1935).

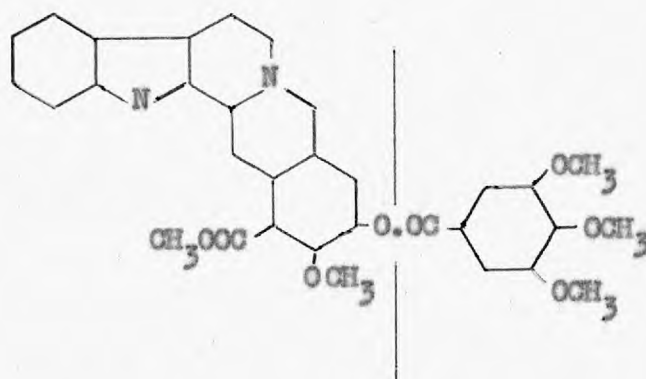
Indole-3-acetic acid is conjugated with glycine in the dog to form indolylaceturic acid (Swins and Laidlaw, 1913) and in man, it forms indolylacetylglutamine and indolylacetylglucuronide (Jepson, 1958).

d-Lysergic acid diethylamide (LSD) is a hallucinogenic drug exerting its effects in very small concentrations. It markedly depresses blood pressure and elicits characteristic behaviour responses in animals (e.g. Gogerty and Dille, 1959). It also effectively antagonises the action of serotonin.



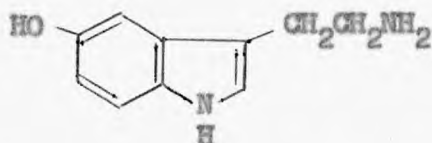
ISD is hydroxylated in position 2 of the indole nucleus by liver microsomes in the presence of TPNH and oxygen (Axelrod et al., 1956).

Reserpine. This is one of the group of alkaloids obtained from the root of the Indian shrub, *Rauwolfia serpentina*. It has a depressant effect on the central nervous system, being active in very small amounts, and is used as a tranquillising drug. It is a hypnotic for dogs, cats, and rabbits (Bein, 1953).



The metabolism is mainly by hydrolysis to methyl reserpate and trimethoxybenzoic acid (cleavage at the point indicated) (e.g. Numerof et al., 1955). No metabolic reactions of the indole moiety have been reported.

5-Hydroxytryptamine (serotonin). The highly active vasoconstrictor substance obtained from beef serum by Rapport et al. (1948), was found to be 5-hydroxytryptamine (Rapport, 1949), also occurring in salivary glands of Gastropods (enteramine) and in the intestinal mucosa of mammals (e.g. Erspamer and Boretti, 1951). It is associated in plasma with the blood platelets (Rand and Reid, 1951) from which it can be liberated by administration of reserpine to the animal (Shore et al., 1956). More recently, serotonin has been implicated in mental disorder, particularly schizophrenia (discussion by Woolley and Shaw, 1954).



5-Hydroxytryptamine is formed in animals from tryptophan via 5-hydroxytryptophan, and is converted to its excretory product, 5-hydroxyindoleacetic acid (Udenfriend et al., 1956a, b).

Melanins. Benzene ring-substituted hydroxyindoles are of importance as postulated intermediates in the formation of melanin pigments from tyrosine and 3,4-dihydroxyphenylalanine. A number of mechanisms have been proposed, one of which is self-condensation of indole-5,6-quinone at the 3- and 7-positions with cross-links involving reaction at the 2-position (J.D. Bu'Lock and J. Harley-Mason, 1954). 5,6-Dihydroxyindole is said to be a urinary metabolite of ^{14}C -labelled 3,4-dihydroxyphenylalanine in rats (Pellerin and D'Iorio, 1955).

Chapter 8. Materials and Methods.

Reference Compounds. The following compounds were purchased and purified where necessary. Indole (B.D.H. Ltd.), m.p. 52°; indoxyl-O-acetate (Light and Co.), m.p. 126° white needles from water (charcoal); isatin (B.D.H. Ltd.), m.p. 201° (sublimes); anthranilic acid (B.D.H. Ltd.), m.p. 145°.

5-Hydroxy-2-methylindole, m.p. 133°, 5-methoxy-6-hydroxyindole, m.p. 111°, and 5,6-dihydroxyindole-2-carboxylic acid, m.p. 231° (decomp.) were donated by Glaxo Ltd.

Potassium indoxyl sulphate (indican was obtained by alkaline persulphate oxidation of indole.

An attempt was made to prepare indoxyl by alkaline fusion of potassium phenylglycine-o-carboxylate and decarboxylation of the resulting indoxyllic acid (Heumann, 1890; cf. Gattermann, 1937, p. 369), but only indigo could be isolated. Hydrolysis of indoxyl acetate in the cold with an air-free solution of NaOH under hydrogen gave similar results.

Attempts to prepare 5-hydroxyindole from 2-nitro-5-hydroxybenzaldehyde (Light and Co.), m.p. 160°, via 2-nitro-5-hydroxyphenylpyruvic acid (Beer et al., 1948) yielded only resinous material. 2-Nitro-5-hydroxybenzaldehyde was then prepared from m-hydroxybenzaldehyde by conversion into the m-aldehydophenylcarbonate followed by nitration (Mason, 1925). The procedure was modified as follows: m-hydroxybenzaldehyde (12 g.) and 6 g. of Na₂CO₃ were dissolved in 15 ml. of warm 20% NaOH, cooled below 0°, and shaken with 80 ml. of 12.5% phosgene in toluene in a 250 ml. centrifuge bottle stoppered with a rubber bung. The m-aldehydophenylcarbonate soon

separated and was filtered off, washed with water and alcohol, and dried; m.p. 132° from glacial acetic acid. Yield quantitative. 2-Nitro-5-hydroxybenzaldehyde (2.5 g.) was converted into 5-hydroxyindole via 2:β-dinitro-5-acetoxystyrene according to Beer et al. (1948). 5-Hydroxyindole formed white needles, m.p. 107° from petroleum ether (80-100°), yield 0.2 g.

2-Nitro-3-hydroxytoluene, yellow needles, m.p. 41°, was prepared by the method of Gibson (1923), and this was converted into 2-nitro-3-benzoyloxytoluene, white plates, m.p. 36°, but the synthesis of 7-hydroxyindole from the latter (Beer et al., 1948) could not be realised.

2-Nitro-6-hydroxybenzaldehyde, pale yellow prisms, m.p. 54° from petroleum ether (40-60°), was prepared by the method of Ashley et al. (1930). Attempted synthesis of 4-hydroxyindole from this via the nitrostyrene (Beer et al., 1948) resulted in the formation of unworkable resinous material.

Indirubin was prepared from indoxyl acetate and isatin according to Drum (1951) and was purified by sublimation, separated from a little contaminating indigotin on a column of activated alumina, with chloroform as developing solvent, and re-sublimated. A small yield of red-brown needles was obtained.

Analytical Methods.

Glucuronides in urine were estimated by the method of Hanson et al. (1944); ethereal sulphates were estimated turbidimetrically according to Sperber (1948); and mercapturic acids according to Stekol (1936).

Indole in urine was estimated by measuring the violet colour produced when xanthydrol is boiled with indole in glacial acetic acid

(Fearon and Drum, 1951). Indole was isolated from urine by steam distillation, using the Markham apparatus. The steam volatility of indole is greatest over the pH range 8.0-10.5, the optimum pH being 9.2 (Zoller, 1920). The pH of the urine was adjusted to 9.0 with 10% NaOH and 10 ml. aliquots were steam distilled, 30 ml. of distillate being collected. 2 ml. of distillate were taken, 0.2 ml. of 5% xanthydrol in methanol was added and the volume was made up to 8 ml. with glacial acetic acid. The solution was heated at 100° for 10 minutes to develop the colour, cooled, and the extinction was measured at 550 m μ . in the Unicam S.P. 600 spectrophotometer. The standard curve was constructed using 0.5 ml. of a standard solution containing 0.25 to 10.0 mg. of indole per 100 ml.; a linear relationship was obtained for this concentration range ($\epsilon_{550 \text{ m}\mu}$, 890). Three points on the calibration curve were determined each day on account of deterioration of the xanthydrol. Recovery from urine containing 2.5 and 5.0 mg. of indole per 100 ml. was 90%, when 30 ml. of distillate were collected.

Indoxyl in urine was estimated by the method of Drum (1951). A standard curve was constructed by boiling 2.5 ml. of a solution containing 0.5 to 6 mg. of indoxyl acetate per 100 ml. with 2 ml. of saturated isatin solution and 1 ml. 50% H₂SO₄. The indirubin was extracted into chloroform, free isatin removed by shaking with alkali, and the absorption was measured at 525 m μ . in the Unicam S.P. 600 ($\epsilon_{525 \text{ m}\mu}$, 2660). The curve obtained was compared with the absorption of a standard solution of pure indirubin at corresponding dilutions. Good agreement was found. The recovery of indoxyl acetate added to normal urine was inconsistently low. Urines containing 1.0, 2.0, 3.0, and 5.0 mg. per 100 ml. gave recoveries of 77, 55, 59, and 67% respectively, average 65%. Drum encountered this difficulty when HCl was used as condensing agent, but overcame it by using sulphuric

acid, with potassium indoxyl sulphate as standard. Values for excretion of indoxyl were calculated on the assumption that the recovery of indoxyl from urine was 65%.

Spectrophotofluorometric Assay of Indican and 5-Hydroxyindole.

The fluorescent properties of potassium indoxyl sulphate and 5-hydroxyindole were investigated using the Aminco-Bowman Spectrophotofluorometer. Since the fluorescence characteristics of compounds vary with pH, measurements were made at a range of pH values. Buffer solutions used are listed in Table 8.1.

Table 8.1.

<u>pH</u>	<u>Solution</u>
1	0.1 N HCl
2-8	Na ₂ HPO ₄ - citric acid buffers (McIlvaine, 1921)
10	500 ml. 0.2 M H ₃ BO ₃ } 500 ml. 0.2 M KCl } diluted to 439 ml. 0.2 M NaOH } 2 litres
13	0.1 N NaOH

Calibration of Spectrophotofluorometer. As a preliminary to the investigations, the instrument was calibrated using a solution of quinine in 0.1 N H₂SO₄ (Sprince and Rowley, 1957). Within the concentration range 0.01-0.1 µg./ml. the activation maximum wavelengths were 265 mµ. (minor) and 360 mµ. (major), and the fluorescence maximum was 460 mµ. Calibration curves were constructed by plotting photomultiplier readings against concentration in µg./ml. and were linear over the range 0.01-0.5 µg./ml. Above 0.5 µg./ml. for activation wavelength 360 mµ., and above 0.8 µg./ml. for activation wavelength 265 mµ., the curves flattened out ("concentration quenching"). Curves were plotted at meter multiplier settings 1 and 0.3.

Three or four points on the calibration curve were determined daily, in order to check diurnal variations of the instrument.

Fluorescence of Indoles. A very pure product is required for spectrophotofluorometric work, as minute amounts of impurities can interfere by causing scattering. Repeated crystallisation from methanol of synthetic potassium indoxyl sulphate gave pale buff plates, showing one spot on paper chromatography in several solvent systems, and which proved to be satisfactory. The sample of 5-hydroxyindole available was also suitable.

Fluorescence measurements were made by dissolving about 1.00 mg. of the compound in 1 ml. distilled water (indican) or ethanol (5-hydroxyindole), then diluting to the desired concentration with buffer solution. The main activation and fluorescence maxima of indican and 5-hydroxyindole at various pHs are shown in Table 8.2. These values did not vary within the

Table 8.2.

pH	Potassium indoxyl sulphate		5-hydroxyindole	
	Activation Max.	Fluor. max. (m μ .)	Activation Max.	Fluor. max. (m μ .)
1	290	400	295 (290)	350 (355)
7	290	400	295	350
Distilled water	290	400	295	350
10			295	350
13			-	-

concentration range 0.01-10.0 μ g./ml. Calibration curves were constructed at meter multiplier settings 1, 0.3, and 0.1. These were linear over the range 0.01-0.1 μ g./ml. Above 1.0 μ g./ml., concentration quenching was appreciable. The maxima quoted for 5-hydroxyindole differ slightly from those listed by the American Instrument Co. at pH 1 (given in parentheses above).

Stability of 5-Hydroxyindole. Since 5-hydroxyindole is not stable, particularly in alkali, it was necessary to investigate its stability over a range of pHs, so that the pH of maximum stability could be chosen for estimations. The fluorescent intensity of a solution of 5-hydroxyindole in buffer was measured at intervals over a period of about 20 hours, and a curve of intensity against time was plotted for each pH. The fluorescent intensity (obtained by multiplying the photometer reading by the meter multiplier setting) was taken to be a measure of the concentration of 5-hydroxyindole in the solution. It was impracticable to take readings over the whole 20 hours, so readings were taken for 4-7 hours, the solutions were left overnight, then a further reading was taken. The instrument appeared to become "fatigued" if kept working for too long a time.

The concentrations of the 5-hydroxyindole solutions were corrected by an appropriate factor to make the graphs (Figures 4, 5 and 6) comparable.

Isolation from Urine and Assay. For the quantitative isolation of these indoles from urine, a paper chromatographic method was employed. A number of different solvent systems were tried, in order to find one which gave clear separation from other urinary components, a convenient R_f value, and a discrete spot. Solvent A, E, and F were found to be best for indican and solvent G for 5-hydroxyindole (Table 8.5).

Recoveries of indican were made from water or urine, by separation in the above solvents. Whatman No. 4 paper and the descending technique were used. A measured volume (usually 0.04 ml.) of a solution of potassium indoxyl sulphate in water or urine (0.5-0.1 mg./ml.) was applied to the chromatogram in the form of a band extending half the width of the paper (2.5 in.). The other half was used as a control blank. A space of 0.5 in.

was left on either side of the band to allow for irregularities in its movement. After development of the chromatogram, the band was located by its fluorescence in u.v. light, cut out, and eluted with water side by side with the control strip. Recovery experiments showed that the first 1-2 drops of eluate contained all the indican, so the first 3 drops were collected, diluted with buffer solution (pH 7), and the fluorescence intensity of the solution determined. Standard solutions of the compound were used for reference, a concentration of standard being chosen which gave a reading close to the test reading. Readings were made at at least two meter multiplier settings, and further dilutions were made to test the linearity of the response. Solvent system A was found to be most satisfactory in this respect, and it also gave best recoveries, so it was used in subsequent work. The recoveries of indican from urine are summarised in Table 8.3.

Recoveries of 5-hydroxyindole from urine were carried out as follows: 1.0 ml. of normal urine, 0.5 ml. of gastric juice of *Helix pomatia*, 0.5 mg. of 5-hydroxyindole and 7.5 mg. of indican (the approximate amount expected in indole urine) were mixed with 1 ml. of buffer (pH 6) and incubated at 37° for 1 hour. The hydrolysate (0.04 ml.) was separated on paper as described for indican but using solvent G. The band was located by means of a marker spot of hydrolysate applied to the centre of the chromatogram between the band and the control strip. This was detected by spraying with α -nitroso- β -naphthol reagent. The band and control strip were cut out and eluted with ethanol. The first 3 drops of eluate were diluted with buffer (pH 7) and the fluorescence was measured. Recoveries of 5-hydroxyindole from ethanol and urine are shown in Table 8.4.

Table 8.3.

Recoveries of potassium indoxyl sulphate from urine (Solvent A).

Indican added to urine (mg./ml.)	Indican found (mg./ml.)	Recovery %	Meter multiplier	Dilution of eluate
1.0	0.83	83	1	x 20
1.0	0.863	86	1	x 20
1.0	0.868	87	1	x 40
1.0	0.895	90	1	x 80
1.0	1.11	111	0.3	x 80

Table 8.4.

Recoveries of 5-hydroxyindole from ethanol (Solvent G).

5-Hydroxyindole added (mg./ml.)	5-hydroxyindole found (mg./ml.)	Recovery %	Meter multiplier	Dilution of eluate
0.5	0.239	48	1	x 50
0.5	0.249	50	1	x 100
0.5	0.162	32	1	x 200
0.5	0.178	36	0.3	x 100

Recoveries of 5-hydroxyindole from urine after incubation 1 hour at 37°.

0.2	0.135	68	1	x 50
0.2	0.140	70	1	x 100
0.2	0.124	62	0.3	x 100

Animals. Chinchilla doe rabbits were maintained as described in Chapter 2, p. 28. Indole was administered orally as a solution in the minimum arachis oil. Urine was collected as previously described.

Qualitative Tests.

Tests for pH, glucuronide, ethereal sulphate, and reducing substances, and Gibbs's test and the ferric chloride test were carried out as described in Chapter 2, pp. 31-32. The following tests were also used.

Legal's test: a few drops of a 0.1% solution of sodium nitroprusside were added to 0.5 ml. of test solution, which was made alkaline with 10% NaOH, and then acidified with glacial acetic acid. Indole gives a blue-violet colour, changing to blue on acidification.

Ehrlich's test: p-dimethylaminobenzaldehyde (2% in 2N HCl) was added to the test solution. Indole and many of its derivatives give a red colour.

Obermayer's test: 0.5 ml. of a 2% solution of ferric chloride was added to an equal volume of urine followed by a few drops of concentrated HCl, and the mixture was boiled for 0.5 minute. A blue colour or precipitate shows the presence of indican.

Xanthydrol: 2-3 drops of a 5% solution of xanthydrol in methanol were added to 0.5 ml. of urine, 1 ml. of glacial acetic acid was added and the mixture was boiled for 1 minute. Indole gives a violet colour.

All of the above tests, except Gibbs and xanthydrol tests were used as a routine for urine.

Paper Chromatography.

Paper chromatography was carried out as described for coumarin

derivatives (p. 33) except that Whatman No. 1 paper was usually used. The following solvent systems were employed, all proportions being by volume:

- A. Benzene - glacial acetic acid - water (1:1:2)
- B. n-Butanol - glacial acetic acid - water (4:1:5 or 4:1:2)
- C. Ethyl methyl ketone saturated with water
- D. Iso-propanol - 28% aqueous ammonia - water (8:1:1) (Stowe and Thimann, 1954)
- E. Benzene - n-butanol - ammonia solution (sp. gr. 0.88) (2:5:2)
- F. n-Butanol - benzene - pyridine - water (5:1:3:3)
- G. Benzene - ethanol - water (2:1:2)

R_F values of some indole derivatives in these solvents are listed in Table 8.5.

Table 8.5.

<u>Compound</u>	<u>R_F value in solvent system</u>					
	<u>A</u>	<u>B</u>	<u>C</u>	<u>E</u>	<u>F</u>	<u>G</u>
Indole	-	0.88	-	-	-	-
5-Hydroxyindole	0.27	0.89	0.97	-	-	0.55
Potassium indoxyl sulphate	0.00	0.45	0.41	0.32	0.62	-
Indoxyl-O-acetate	-	0.83	-	-	-	-
Anthranilic acid	-	0.79	-	-	-	-

The spots were detected on paper by exposure to u.v. light (270 m μ .) and by spraying with reagents, a list of which is given below (all these were not necessarily used in a single experiment).

- (1) Ferric chloride, 0.5M in ethanol.
- (2) Ferric chloride (1 ml. 0.5M) + 3 drops of concentrated H₂SO₄.
- (3) Salkowski reagent (Gordon and Weber, 1951): ferric chloride (1 ml.) mixed with 50 ml. of 30% perchloric acid.
- (4) Gibbs's reagent: cf. p. 34
- (5) Diazotised sulphanilic acid (Ames and Mitchell, 1952): 0.9 g. sulphanilic acid was dissolved in 9 ml. concentrated HCl and diluted to 100 ml. with water. To 5 ml. of this stock solution, 25 ml. of 5% NaNO₂ were added slowly at 0°.

- (6) Diazotised p-nitroaniline was made up in a similar way.
- (7) Modified Ehrlich's reagent (Dalglish, 1955). Two types were employed. Normal (n): p-dimethylaminobenzaldehyde, 2% solution in 1.3N HCl.
- (8) Strong (s): p-dimethylaminobenzaldehyde, 2% solution in 6N HCl.
- (9) α -Nitroso- β -naphthol, 0.1% in 95% ethanol, followed after drying by a mixture of 0.2 ml. of 2.5% NaNO₂ and 5 ml. 2N HCl (Udenfriend et al., 1955).
- (10) Methanol (2 ml.) mixed with fuming HCl (1 ml.).
- (11) Ammoniacal silver nitrate.
- (12) NaNO₂ (5%) followed by HNO₃.

Colour reactions given by some indole derivatives with these reagents on paper are shown in Tables 8.6 and 8.7.

In order to investigate the colour reactions and chromatography of indoxyl, indican was hydrolysed as follows: (1) Boiled with 2N HCl for 3-5 minutes, cooled, neutralised, filtered to remove resin, and extracted with benzene. The concentrated benzene extract was applied to chromatograms. (2) Boiled for 5 minutes with dioxal solution, cooled, and applied to chromatograms.

With method (1), the results show that the indican was not hydrolysed with method (2) a number of unidentified fluorescent spots were obtained (Table 8.8), none of which could have been indoxyl.

Table 8.8.

Paper Chromatography of Hydrolysed Indican (2).

Solvent : Methyl Ethyl Ketone : Water

	<u>R_f Values</u>	<u>Fluorescence in u.v. light</u>	<u>Gibbs's reagent</u> <u>+ NaHCO₃</u>
(1)	0.32	Blue	-
(2)	0.39	Weak blue	-
(3)	0.58	Blue	-
(4)	0.70	Blue	-
(5)	0.98	Green	-

Table 8.6.

Colour reactions on paper of indole derivatives.

	<u>5-Hydroxy-2-methyl- indole</u>	<u>5-Methoxy-6-hydroxy- indole</u>	<u>5:6-dihydroxy-2- carboxylic acid</u>
FeCl_3 (0.5M)	Blue	Brown $\rightarrow$ mauve	Grey $\rightarrow$ purple
FeCl_3 + conc. H_2SO_4 (1 ml. 0.5M- FeCl_3 + 3 drops conc. H_2SO_4)	Slow orange-red	Imm. red $\rightarrow$ purple	Purple-grey (slow)
2:6-dichloroquinonechloroimide (1% in ethanol) (pH 7)	Immed. purple	Slow brown	Slow red-brown
2:6-dichloroquinonechloroimide (pH 9)	Blue	Blue	Bright Blue
(1) Diazot. ^d sulphanilic acid (pH 7)	Immed. Bright Yellow	Immed. Yellow $\rightarrow$ Brown	Red-brown $\rightarrow$ pink
(1) Diazot. ^d sulphanilic acid (pH 9)	Immed. Bright Yellow	Immed. Yellow $\rightarrow$ Brown	Red-brown $\rightarrow$ pink
(2) Diazot. ^d p-nitraniline	Red-brown $\rightarrow$ Orange	Brown	Dark brown
(3) p-Dimethylaminobenzaldehyde (n)	Immed. Bright Crimson	Immed. Crimson	Slow grey
(4) p-Dimethylaminobenzaldehyde (s)	Brown-red	Brown	Brown
(5) Perchloric acid + FeCl_3 (Salkowski)	Brick-red	Violet	Slow grey-mauve
(6) α -Nitro- β -naphthol	Pink violet	Violet	-
(7) Methanol - fuming HCl	Brick red (Carmine)	Pink-violet	-
Ammon. AgNO_3	Black	Black	Black
NaNO_2 - HNO_3	Brown	Brown	Black

See p. 123 for preparation of reagents.

Table 8.7.

Colour Reactions on Paper of Indole Derivatives.

	<u>5-Hydroxyindole</u>	<u>Hydrolysed Indican (1)</u>	<u>Indican</u>
Alcoholic FeCl_3	Olive green	Bluish green	Very slow pale green
$\text{FeCl}_3 + \text{H}_2\text{SO}_4$	Pink	Greenish blue $\rightarrow$ Blue	Slow pale blue $\rightarrow$ darkening
Salkowski reagent	Pink-violet	Green $\rightarrow$ Blue	Pale bluish
2:6-dichloroquinone- chloroimide	Red-violet $\rightarrow$ brown	Pink $\rightarrow$ Brown $\rightarrow$ Blue (slowly)	Brownish slowly $\rightarrow$ Blue
2:6-dichloroquinone- chloroimide + satd. aq. NaHCO_3	Blue	Purple	Purple
Diazot ^d . sulphanilic acid + NaHCO_3	Brown-pink	Brown $\rightarrow$ pink	Brown $\rightarrow$ pink
p-Dimethylaminobenzaldehyde (n)	Immed. Red	V. slowly $\rightarrow$ greenish $\rightarrow$ brown	
p-Dimethylaminobenzaldehyde (s)	Immed. violet	Brown	Brown
α -Nitroso- β -naphthol + nitrite	Violet	Bluish-green $\rightarrow$ Blue	Bluish-green $\rightarrow$ Blue
Ammon. AgNO_3	Black	V. slow greenish	V. slow greenish
Brentamine Fast Red B	Immed. Brown (dark)	Yellow-brown	Yellow-brown
Brentamine Fast Blue 2B	Pinkish-brown v. gradually $\rightarrow$ violet	V. gradually $\rightarrow$ purple after some hours	

See p. 123 for preparation of reagents.

Chapter 9. The Metabolism of Indole in Rabbits.

The only mechanism known by which the organism can metabolise indole is by oxidation to indoxyl and conjugation of the latter with sulphuric acid and glucuronic acid. Therefore it was necessary in a further examination of the problem to determine to what extent the indole molecule is transformed in this way, before looking for other metabolites in an attempt to account for the remainder.

Experimental.

Excretion of indole as conjugates.

Daily determinations of glucuronide and ethereal sulphate were carried out for three rabbits for a week, to establish the average normal excretion. They were then dosed with indole and the determinations were continued for three days. The increases in the excretion of glucuronide and ethereal sulphate were estimated, and the percentage of indole excreted as conjugates was calculated on the assumption that these increases were due to excretion of indoxyl conjugates. The results for two sets of experiments, using three rabbits in each, are summarised in Table 9.1 and set out in detail in Tables 1 and 2, Appendix 2. In the first experiment (dose level, 250 mg./kg.) the whole of the indole fed was excreted, 30% as glucuronide and 70% as ethereal sulphate, therefore the second experiment was carried out at a lower dose level (100 mg./kg.). In the measurement of ethereal sulphate at the higher dose level, there was sometimes slight darkening of the turbid solution due to the oxidation of indican, which may have interfered somewhat with the estimation; at the lower dose level, this difficulty was not encountered. Mercapturic acid excretion was measured at the lower dose level.

Table 9.1.

Excretion of indole as glucuronide, ethereal sulphate, and mercapturic acid.

Rabbit No.	Wt. (kg.)	Dose level (mg./kg.)	Percentage of dose excreted as:			Total
			Glucuronide	Ethereal Sulphate	Mercapturic Acid	
1	3.5	250	25	94	-	119
2	3.3	250	33	69	-	102
3	2.8	250	37	53	-	90
Average			32	73	-	105
4	3.6	100	18	37	1.3	56
5	3.6	100	17	31	0.5	49
6	4.0	100	5	23	0.8	29
Average			13	30	0.9	45

Table 9.2.

Excretion of indole as indoxyl.

Rabbit No.	Wt. (kg.)	Dose level (mg./kg.)	Percentage of dose excreted as indoxyl
7	3.3	100	50
8	3.3	100	42
9	2.8	100	50
Average			47

Table 9.3.

Excretion of indole as potassium indoxyl sulphate.

Wt. 2.5 kg. Dose 0.25 g. Dose level 100 mg./kg.

Time after dosing (hours)	Volume of urine (ml.)	Excretion of indican (mg.)	Increase over normal (mg.)	Indole (mg.)	Percentage of dose
6	80	33.3	32.1	15.0	6.0
24	101	97.2	96.0	44.8	17.9
30	41	8.2	7.0	3.4	1.4
48	81	4.4	3.2	1.5	0.6
54	11	1.2	0	-	-
Total			138.3	64.7	25.9

Excretion of indole in urine.

The fairly sensitive xanthydrol test which gives a violet colouration with solutions containing indole, was negative for urine of rabbits which had received 100 mg. of indole per kg. Assay of the urine by the xanthydrol method indicated that less than 0.02 mg.% of the dose of indole was present.

Excretion of indole as indoxyl.

Indoxyl was determined in the urine of three rabbits which had received 100 mg. of indole per kg. The urine was collected under toluene to minimise loss of indoxyl by oxidation. The results are summarised in Table 9.2, and set out in Table 3, Appendix 2.

Excretion of potassium indoxyl sulphate.

Potassium indoxyl sulphate and 5-hydroxyindole were estimated spectrophotofluorometrically in the urine of a rabbit for 2.5 days after it had been fed indole (100 mg./kg.). The excretion of potassium indoxyl sulphate is shown in Table 9.3. No significant amount of 5-hydroxyindole was present.

Indoxyl glucuronide and indoxyl sulphate: isolation.

Attempts were made to isolate indoxyl glucuronide from the urine of rabbits by the lead acetate precipitation method (e.g. Kamil et al., 1951). The glucuronide gum from the urine of 3 rabbits which had received collectively 3.5 g. of indole rapidly darkened on exposure to air, gave a strong naphthoresorcinol reaction and slightly reduced Benedict's reagent, but did not yield any crystalline material. In another experiment, the glucuronide gum from rabbits which had received 4.5 g. of indole was dissolved in 10 ml. of water and the glucuronide fraction was precipitated

with acetone. The supernatant contained ethereal sulphate; it was taken to dryness and the residue (300 mg.) was dissolved in the minimum amount of water and KHCO_3 (100 mg.) was added. Ethanol was added to the solution until precipitation was complete, the precipitate of inorganic material was removed by filtration, and the solution was taken to dryness in vacuo. The residue was crystallised from hot methanol. Repeated recrystallisation yielded buff plates (80 mg.), behaving identically on paper chromatograms with synthetic potassium indoxyl sulphate.

The glucuronide fraction was dissolved in water and inorganic material precipitated with ethanol. The filtered solution on evaporation yielded a gum. This gave a strong naphthoresorcinol reaction and negative ethereal sulphate test. Potassium, benzylamine or p-toluidine salts, or the triacetylglucuronic acid, could not be prepared from this material.

Qualitative Examination of Urine.

The daily urine of rabbits which had received indole had a volume of about 50-180 ml., and the pH varied between 6 and 9. Benedict's test for reducing substances was negative throughout. The naphthoresorcinol and ethereal sulphate test were strongly positive. Obermayer's test for indican was usually positive or weakly positive for normal urine, with strong positive reactions on experimental days. The sodium nitroprusside test gave turbid orange or red-orange colours or precipitates, which changed to clear green-yellow or green on acidification. With p-dimethylaminobenzaldehyde, the urine generally gave a rather weak red colour. Gibbs's reagent sometimes gave a red-violet colour. The xanthydrol test for indole was negative throughout, and the urine gave no colours with ferric chloride.

The urine was sometimes green or orange after a dose of indole, and

there was sometimes slight darkening in urines containing large amounts of indoxyl. When the urine was collected under toluene, the toluene layer was coloured red-violet in such cases.

Paper chromatography of urine. The 24 and 48 hour urine from rabbits which had received indole was chromatographed in solvent systems A, B, and C (Table 8.2). Indican (potassium indoxyl sulphate was detected by its fluorescence in u.v. light and its colour reactions, but 5-hydroxyindole was not detected.

Urine was adjusted to (a) pH 4 and (b) pH 6, and 2 ml. of each were mixed with 1 ml. of gastric juice of *Helix pomatia* and incubated overnight at 37°. The solutions were filtered from precipitated indigo and chromatographed in A, B, and C. 5-Hydroxyindole could now be detected by its colour reactions with Gibbs's, Salkowski's, Ehrlich's, and α -nitroso- β -naphthol reagents, but the indican spot had disappeared. A similar picture was presented by urines hydrolysed at pH 4 and pH 6, but the former gave more intense spots.

Spraying of papers with 0.1% or 1% ferric chloride or 1% cupric sulphate (to detect chelating compounds) did not reveal any coloured spots.

The R_f values of spots obtained for non-hydrolysed and hydrolysed urine (solvent C) are shown in Table 9.4. Spot 1 corresponds to indican and spot 7 to 5-hydroxyindole. Spot 3 does not correspond to 5-hydroxyindole, which only appears after hydrolysis, and which gives a red Ehrlich's colour. Of the fluorescent spots obtained from hydrolysed indican (Table 8) spots 1, 4 and 5 appear to correspond to spots 8,9 and 10 (Table 9.4) when run on the same chromatogram. Anthranilic acid was not detected in urine or hydrolysates.

Table 9.4.

Chromatography of rabbit indole urine.

Non-hydrolysed urine.Solvent C.

	<u>R_F value</u> <u>(solvent C)</u>	<u>Gibbs's</u> <u>reagent</u>	<u>Ehrlich's</u> <u>reagent (s)</u>	<u>Fluorescence in</u> <u>u.v. light</u>
(1)	0.43	Violet	Brown	Strong blue
(2)	0.85	Purple	-	-
(3)	0.98	Blue	Green-yellow	-

Hydrolysed urine.

	<u>R_F value</u>	<u>Gibbs's</u> <u>reagent</u>	<u>Salkowski</u> <u>reagent</u>	<u>α-Nitroso-</u> <u>β-naphthol</u>	<u>Fluorescence in</u> <u>u.v. light</u>
(4)	0.39	Blue	-	-	-
(5)	0.61	Weak blue	-	-	-
(6)	0.85	Purple	-	-	-
(7)	0.97	Blue	Red	Violet	Q
(8)	0.32	-	-	-	Blue
(9)	0.73	-	-	-	Blue
(10)	0.95	-	-	-	Green

Q means quenching of the background fluorescence.

Table 9.5.

Chromatography of rabbit indole urine.

All spots except 1a appeared before hydrolysis.

Solvent B.

	<u>R_F value</u>	<u>Gibbs's reagent</u>	<u>Ehrlich's</u> <u>reagent (n)</u>	<u>Fluorescence in</u> <u>u.v. light</u>
(1)	0.51	Violet	Brown	Blue
(2)	0.77	Purple	-	-
(3)	0.93	Blue	-	-
(1a)	0.55	Purple	Brown	-

In one experiment, after development of chromatograms in solvent B, substances were hydrolysed on the paper by (a) spreading snail juice on one paper with a brush and allowing it to dry; (b) spraying another paper with 0.1N HCl and drying at 50°. Spraying with Gibbs's reagent then revealed a purple spot (1a) in the lower part of the indican spot (1) (Table 9.5). Diazotisation on the paper with acidified nitrite followed by coupling with α -naphthol in alkali gave a pink colour at 1a. Thus the newly revealed spot may have been a hydroxyamine.

Discussion.

The present work has provided some quantitative data for the excretion of indole as indoxyl conjugates, but the metabolism of a large part of the dose remains obscure. Approximately half of the indole administered to rabbits (100 mg./kg.) is excreted as indoxyl compounds, of which approximately 40% appears to be indoxyl glucuronide and 60% indoxyl sulphate. Excretion of mercapturic acids (less than 1% of a dose of indole) is of little importance.

Previous estimations of indoxyl conjugates have been indirect, by measuring the increase in excretion of glucuronides and ethereal sulphates after a dose of indole. These results could also include conjugates of other metabolites formed from indole. A check on this was provided in this work by estimation of indoxyl itself. Determinations of indole metabolites by measurement of indigoid pigments have been used by other workers, and on this basis it seems that about half of a dose of indole is converted to precursors of indigo (Wang, 1889; Kauffmann, 1911). These methods, involving oxidation of indoxyl conjugates by variations of the Obermayer reaction, are less specific for indoxyl than the method used in this work,

which involves condensation of indoxyl with isatin. However, the values obtained are similar.

Previous evidence (Tollens, 1910) has indicated that conjugation of indoxyl with ethereal sulphate is quantitatively more important than conjugation with glucuronide, and this is supported by the present results, which show that about 30% of the indole appears as ethereal sulphate and rather less than 20% as glucuronide at a dose level of 100 mg./kg. (Table 9.1). The indirect determinations of ethereal sulphate were confirmed by direct estimation of potassium indoxyl sulphate (26%) in the urine of a rabbit fed indole at the same dose level (Table 9.3). Although results for only one rabbit could be obtained, as the method is time-consuming, it is also specific, and provides good evidence that the increase in ethereal sulphates is actually due to excretion of potassium indoxyl sulphate. Unfortunately, it was not found possible to isolate indoxyl glucuronide, so that a similar direct estimation could not be developed for this metabolite.

The question of whether all the conjugates are due to excretion of indoxyl is of importance, as if they are, it would seem unlikely that the other hydroxy-isomers of indole occur as metabolites to a significant extent.

It is remarkable that a much larger dose of indole (250 mg./kg.) should all be recoverable as conjugates. Some interference in the ethereal sulphate estimation by formation of indigo may have raised the values slightly, but no gross darkening of the solutions was observed, and it would still appear that the values obtained (70%) gave a reasonably accurate picture. It would be expected that at the higher dose level, supplies of sulphur available to the organism for conjugates being limited, glucuronide formation might become more important. In fact, the ethereal sulphate:

glucuronide ratios at the two dose levels are similar (2.28, 2.34). It would appear from the work of Wellers (1953a) on the stoppage of the growth of young rats by indole, that glucuronide conjugation does not intervene to exert a sparing effect on sulphur amino acids when these are in short supply. It may be that indoxyl glucuronide cannot be formed to a large extent because of its instability, and it is worthy of note that it has never been isolated as such.

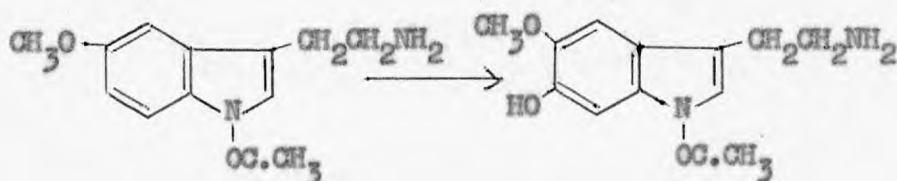
It is not known whether all the conjugates at the higher dose level are those of indoxyl. The most conspicuous feature of the chemistry of indole is the high reactivity of position 3. Almost all reactions take place at this point and this also could hold true for its behaviour in the organism.

Other hydroxyindoles which could be considered most likely as metabolites are 5-hydroxy- and 7-hydroxyindoles, oxindole, and N-hydroxy-indole. 5-Hydroxyindole has been shown on paper chromatographic evidence to be a metabolite of indole, but the amount formed is small, and the compound readily decomposes, so that it could not be estimated. Of the hydroxyindoles substituted in the benzene ring, 5-hydroxyindole would be expected to be formed to the greatest extent as position 5 is para to the hetero atom (Williams, 1959, p. 546).

It seems possible that 7-hydroxyindole may form a coloured chelate compound with metal ions, in a manner analogous with 8-hydroxyquinoline, and if this were so it should be detectable on paper chromatograms by spraying with an appropriate metal salt solution. No coloured spots were detected on chromatograms, however, using ferric chloride or cupric sulphate.

It may be noted that when position 3 is substituted by an alkyl

group or fatty acid residue, being thus no longer available for hydroxylation, other points of the molecule are more readily attacked. Thus, Dalgliesh et al. (1958) have found that skatole is hydroxylated by rats at positions 5 and 7. Tryptophan is oxidised at position 5 to form 5-hydroxytryptophan (Udenfriend et al., 1956a, b), and it appears that an appreciable fraction of the daily intake of tryptophan by animals is metabolised by this route (Udenfriend and Titus, 1955). Hydroxylation of melatonin, the N-acetyl derivative of 5-methoxytryptamine, proceeds in position 6 in rats, and the ethereal sulphate of 6-hydroxymelatonin is the main metabolite, with less of the glucuronide (Kopin et al., 1960). Thus substitution of position 5 by methoxyl favours the introduction of a further hydroxy group into position 6.



7-Hydroxylation of the indole nucleus in a derivative having position 3 substituted was reported by Ichihara and Sakamoto (1956) who identified 7-hydroxyindoleacetic acid in the urine of a rabbit fed skatolylhydantoin.

Complex indoles may be hydroxylated at position 5 of the nucleus. Thus Loo and Reidenberg (1959) isolated 10-hydroxy-yohimbine from cultures of *Streptomyces platensis*, grown in presence of yohimbine.

The consideration of the extent of formation of benzene ring-substituted hydroxyindoles raises the question whether these compounds, if formed, are oxidised further before being excreted. This could account for

the fact that only half the dose (100 mg./kg.) appears as conjugates. Certain indole derivatives bearing a hydroxyl group at positions 4, 5, 6 or 7 are oxidised by an oxidase present in the gill plates of *Mytilus edulis* and by caeruloplasmin, the copper-containing oxidase of mammalian plasma, with formation of blue or brown pigments (Blaschko and Levine, 1960). Although 5-hydroxyindole itself is not oxidised by the plasma enzyme, 7-hydroxytryptamine is oxidised to a much greater extent than 5-hydroxytryptamine, so that oxidation of 7-hydroxyindole is feasible. In any case, 5-hydroxyindole may be oxidised to 5,6-dihydroxyindole by another enzyme, bearing in mind the ready conversion of indole to the 5,6-quinone under certain conditions, for example by X-rays (Allsopp and Wilson, 1952), and also the 6-hydroxylation of melatonin above. The 5,6-quinone could then polymerise to melanin. It is of interest that rabbits injected with indole or skatole showed increased pigmentation around the site of injection (Introszi, 1926).

There is no evidence concerning the formation of oxindole or N-hydroxyindole. There is a possibility of dihydroxylation at positions 2 and 3: related diols have been prepared by treating 1-acetylindoles with osmium tetroxide and pyridine (Ockenden and Schofield, 1954), and this reagent often parallels the effects produced in vivo (Schoental, 1950).

The heterocyclic ring of indole is fairly stable in vitro, and it appears that it is not opened in the body. Anthranilic acid was not detected chromatographically in urine or in hydrolysates, and if it were present, even in small amounts, it should be revealed by its strong u.v. fluorescence. If hydroxylation of the benzene ring were to make the pyrrole ring less stable, hydroxyanthranilic acids might be expected as metabolites.

No evidence was obtained for the presence of 3-hydroxyanthranilic acid, using the R_f values and colour reactions given by Dalglish (1955).

Figure 4. Rate of disappearance of 5-hydroxyindole
measured by fluorescence intensity.

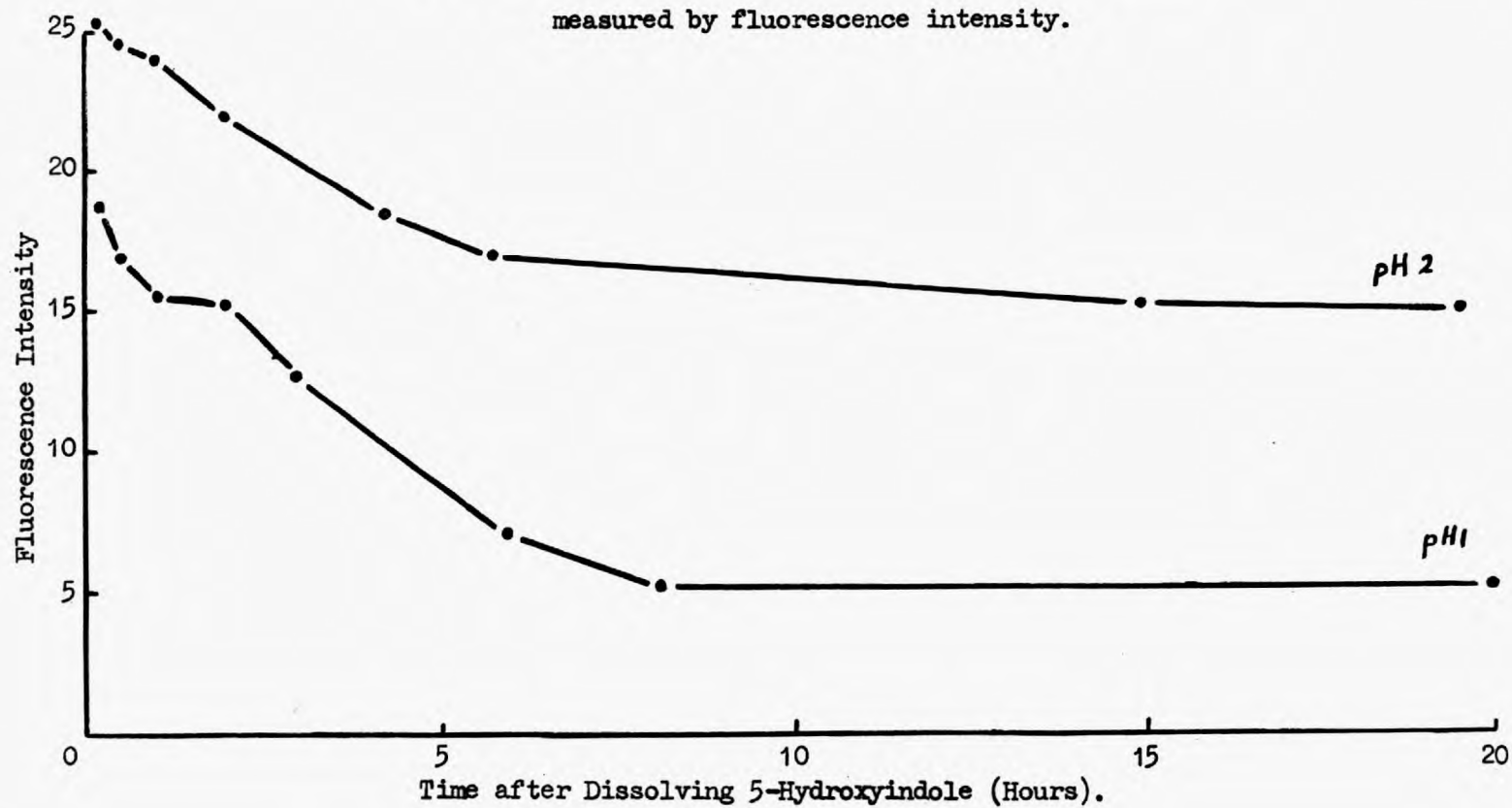


Figure 5. Rate of disappearance of 5-hydroxyindole
measured by fluorescence intensity.



Figure 6. Rate of disappearance of 5-hydroxyindole
measured by fluorescence intensity.



APPENDIX 1.

Table 1.

Recovery of metabolites from a rabbit dosed
with 3-hydroxycoumarin.

0.84 g. 3-hydroxycoumarin fed to a rabbit (3.4 kg.)

	Isolated (mg.)	As 3-hydroxy- coumarin (mg.)	Percentage of dose
3-Hydroxycoumarin	10	10	1.2
	190	190	22.6
3-Hydroxycoumarin glucuronide	680	325.9	38.8
Hydroxyacids	22.6	24.1	2.9
	93.5	99.7	11.9
Total		649.7	77.4

The amounts of 3-hydroxycoumarin equivalent to hydroxyacids were calculated on the assumption that all the acid was o-hydroxyphenyl-acetic acid.

Table 2.

Chromatography of ether extract of acid-hydrolysed urine
from rabbits receiving 4-hydroxycoumarin.

Rf value (Solvent A)	Gibbs	Fluorescence in u.v. light (270 m μ .)
0.02	-	Blue
0.15	Red-Purple	Purple (4-hydroxycoumarin)
0.35	Green	-
0.43	Red	-
0.87	Green	-
0.94	-	Blue-green

Table 3.

Excretion of 4-hydroxycoumarin free
and as glucuronide.

Rabbit No.	Wt. (kg.)	Dose (mg./kg.)	4-Hydroxy- coumarin %	Glucuronide %
1	3.7	200	30	28.5
2	2.9	200	51	32
3	2.4	200	27	30
Average			36	30
4	3.1	500	40.7	
5	3.0	500	37	
6	3.0	500	44	
7	3.0	500	50.4	
			43	

Table 4.

Excretion of glucuronide after administration of 4-hydroxycoumarin.

Dose level 200 mg./kg.

Rabbit No.	1			2			3		
Wt. kg.	3.7			2.9			2.4		
Dose (mg.)	740			580			500		
Time after dosing (hours)	Gluc. (mg.)	4-HO-C. (mg.)	% of dose	Gluc. (mg.)	4-HO-C. (mg.)	% of dose	Gluc. (mg.)	4-HO-C. (mg.)	% of dose
24	430	206	27.9	373	179	30.8	218	104.5	21
48	10	4.8	0.6	0	0	0	98	47	9
72	0	0	0	17	8	1.4	0	0	0
Total	440	210.8	28.5	390	187	32	316	151.5	30

Table 5.

Excretion of Radioactivity in Urine of Rabbits after Dosing with ¹⁴C-Coumarin.

<u>Experiment No.</u>	<u>1</u>	<u>2</u>	<u>3</u>					
Radioactivity fed (μc)	1.345	58.42	47.65					
Dose of Coumarin (mg./kg)	40	50	50					
Column a: Time after dosing (hours); column b: volume of urine (ml.); column c: percentage of radioactivity recovered in urine.								
<u>a</u>	<u>b</u>	<u>c</u>	<u>a</u>	<u>b</u>	<u>c</u>	<u>a</u>	<u>b</u>	<u>c</u>
16	23.5	19.0	22	87	78.4	17	171	78.6
42	55.5	13.4	48	136	11.8	24	38	0.5
66	41.0	10.0	72	96	0.8	41	122	1.1
88	268	50.0	95	111	1.2	48	14	0.2
112	68	1.6	-	-	-	65	110	0.6
136	95	1.9	-	-	-	72	13	0.1
160	163	1.1	-	-	-	-	-	-
184	156	0.6	-	-	-	-	-	-
210	211	1.2	-	-	-	-	-	-
235	56	0.7	-	-	-	-	-	-
256	108	1.1	-	-	-	-	-	-
282	184	0.7	-	-	-	-	-	-
<u>Total</u>		<u>101.3</u>			<u>92.2</u>			<u>81.1</u>

Table 6.

Excretion of Radioactivity in Urine of Rats after Dosing with ^{14}C -Coumarin.

<u>Experiment No.</u>	<u>4</u>	<u>5</u>
Radioactivity fed (μc)	9.977	14.49
Dose of Coumarin (mg./kg.)	100	100

Column a: Time after dosing (hours); column b: volume of urine (ml.)

Column c: percentage of radioactivity recovered in urine.

	<u>a</u>	<u>b</u>	<u>c</u>		<u>a</u>	<u>b</u>	<u>c</u>
	24	24.4	15.4		5	3.7	15.25
	40	5.6	22.2		17	2.75	20.3
	48	3.5	5.4		30	3.85	8.2
	64	8.2	3.3		41	9.4	8.5
	88	20.5	0.7		42	4.75	1.4
	-	-	-		53	2.25	1.7
	-	-	-		65	9.4	1.8
	-	-	-		71	2.4	0.3
	-	-	-		73	1.3	0.2
	-	-	-		89	7.5	0.8
	-	-	-		115	1.5	0.46
<u>Total</u>			<u>47.0</u>				<u>58.9</u>

Table 7

Excretion of Radioactivity in Faeces of a Rat after Dosing with
 ^{14}C -Coumarin.

Experiment No.4Radioactivity fed (μc)

9.977

Dose of Coumarin (mg./kg.)

100

Column a: Time after dosing (hours); column b: weight of
 faeces (g); column c: percentage of radioactivity rec-
 overed in faeces.

<u>a</u>	<u>b</u>	<u>c</u>
24	3.5	6.8
48	0.94	7.4
64	2.15	22.1
88	5.48	2.4
<u>Total</u>		<u>38.7</u>

Table 8Fractionation of Radioactivity in Rat Faeces.

<u>Experiment No.</u>	<u>4</u>	<u>5</u>
Dose of Coumarin (mg./kg.)	100	100
Dose of ^{14}C (μc /animal)	9.97	14.49

<u>Radioactivity in:</u>	<u>(μc)</u>	<u>(% of dose)</u>	<u>(μc)</u>	<u>(% of dose)</u>
Faeces	3.88	38.8	4.84	33.4
Extract (1)	0.108	1.08	0.107	0.74
Extract (2)	2.05	20.5	0.966	6.67
Total Extracts	2.16	21.6	1.07	7.4
Remaining in Faeces				
Calcd.	1.72	17.2	3.77	26.0
Found	0.62	6.2		
Ether Extract of Acid Hydrolysate	-	-	0.08	0.6
Carbon Residue	0.58	5.8	0.31	2.1

Table 9

Radioactivity in Tissues of Rabbits and Rats after Dosing with [¹⁴C] Coumarin.

	<u>Rabbit 2</u>		<u>Rabbit 3</u>		<u>Rat 1</u>		<u>Rat 2</u>
Dose of [¹⁴ C] Coumarin:							
mg./kg.	50		50		100		100
μg/animal	58.4		47.7		10.0		14.5
Duration of Experiment (days)	4		3		4		5
<u>Tissue:</u>	<u>Wet Wt.(g)</u>	<u>% Dose</u>	<u>Wet Wt.(g.)</u>	<u>% Dose</u>	<u>Wet Wt.(g.)</u>	<u>% Dose</u>	<u>% Dose</u>
Liver	75.0	0.09	80.5	0.07	6.4	0.3	whole rat 205 g.
Kidneys	16.2	0	14.9	0	1.1	(0.02)	
Lungs	20.3	0	8.1	0	0.8	0	2.9
Heart (whole)	20.4	0	-	0	1.4	(0.03)	
Blood	-	-	-	0	-	0	
Body fat	-	-	19.4	0	-	-	
Spleen	-	-	0.4	0	0.4	(0.01)	
Visceral fat	-	-	-	-	0.8	0	
Gastro-intestinal tract + contents	-	-	-	-	15.25	0	
Stomach (contents)	-	-	139	0.7	4.0	0	
Small intestine (+ con- tents)	-	-	44.1	0.05	1.9	0	

continued.....

Continued:

Table 9

	<u>Wet Wt.(g)</u>	<u>% Dose</u>	<u>Wet Wt.(g)</u>	<u>% Dose</u>	<u>Wet Wt.(g.)</u>	<u>% Dose</u>	<u>% Dose.</u>
Caecum (contents)	-	-	80	0.3	1.9	0	
Colon (contents)	-	-	10	0.06	4.3	0	
Rectum (contents)	-	-	6.8	0.4	-	0	
<u>Total</u>		<u>0.1</u>		<u>1.2</u>		<u>0.3</u>	<u>2.9</u>

Table 10.

Chromatography of urine from rats dosed with
[^{14}C]coumarin: unidentified spots.

A	<u>R_F value in solvent:</u>			Gibbs's reagent
	B	C	E	
0	(0.27 0.05)	0.06-0.30	0	Red-purple
-	(0.21 0.51)	(0.59 0.07)	0.28	Blue
-	0.17	0.09	0	Orange-yellow

Table 11.

Chromatography of extract of faeces from rats
dosed with [^{14}C]coumarin.

Found in extract:	<u>R_F value in solvent:</u>				Gibbs	Autocatalytic Fluorescence
	A	B	C	E		
1,2	0	0.5 -0.8	0.53	0.27	Blue	-
2	0	0.10-0.32	0.06	0.05	Purple (greenish in centre)	-
2	-	-	0	0	Green- brown (centre)	-
1	0.38		0.77	0.74	Blue	- (o-Hydroxyphenyl- acetic acid)
1,2	0.91	0.93	0.89	0.91	-	Green (Coumarin)
1,2	-	0.09	-	0.14	-	Purple

Specimen Data Sheets.Isotope Dilution Recrystallisations.Rabbit No. 2

Coumarin (free)	Net c/min.	Net c/min. corrected for dilution	Net c/min. corrected for mol. wt.	Wt. on planchette (mg.)	Standard 0.1 μ c./g. net c/min.	m.p. °C.
1st day: 3rd recryst ⁿ . from n-hexane	8.1	-	-	32.2	120.1	70.5
2nd day: 3rd recryst ⁿ . from n-hexane	4.0	-	-	37.9	120.1	70.5
<u>Coumarin (total)</u>						
1st day: 5th recryst ⁿ . from hexane	175.5	-	-	93.8	122.8	70.5
1st recryst ⁿ . from pet. ether (b.p. 120°)	181.1	-	-	83.8	114.3	70.5
2nd day: 5th recryst ⁿ . from n-hexane	25.8	-	-	80.5	122.8	70.5
1st recryst ⁿ . from pet. ether	21.9	-	-	62.8	114.3	70.5
<u>3-Hydroxy- coumarin (total)</u>						
1st day: 5th recryst ⁿ . from water	662.4	-	-	17.6	121.2	152.5
2-oxo-3-phenyl- hydrazono- chroman, 2nd recryst ⁿ . from ethanol-water	71.0	342.1	532.1	29.0	99.4	174
2nd day: 5th recryst ⁿ . from water	195.0	-	-	40.3	121.2	153
Deriv., 2nd recryst ⁿ . from ethanol-water	52.9	114.8	178.4	34.1	120.1	174

Coumarin (free)1st Day.

$$\text{Specific activity of coumarin} = \frac{8.1}{120.1} \times 0.1 = 0.006745 \mu\text{c./g.}$$

$$\text{Specific activity of material fed} = 389.2 \mu\text{c./g.}$$

$$\text{Ratio of activities (Z)} = \frac{389.2}{0.006745} = 57690$$

$$\text{Wt. of coumarin added (b)} = 0.5165 \times 100 = 51.65 \text{ g. in whole urine}$$

$$a = \frac{b}{Z-1} = \frac{51.65}{57689} = 0.0008952 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.0008952 \times 100}{0.1501} = 0.6\%$$

2nd Day

$$\text{Specific activity} = \frac{4.0}{120.1} \times 0.1 = 0.003332 \mu\text{c./g.}$$

$$Z = \frac{389.2}{0.003332} = 116800$$

$$\text{Wt. of coumarin added} = 0.5178 \times 34.4 = 17.79 \text{ g. in whole urine}$$

$$a = \frac{b}{Z-1} = \frac{17.79}{116800} = 0.0001523 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.0001523 \times 100}{0.1501} = 0.1\%$$

$$\text{Total } 0.6 + 0.1 = 0.7\% \text{ of dose}$$

Continued

Continued:Coumarin (total)1st Day.

$$\text{Specific activity of coumarin} = \frac{181.1}{114.3} \times 0.1 = 0.1584 \mu\text{c./g.}$$

$$Z = \frac{389.2}{0.1584} = 2456$$

$$\text{Wt. of coumarin added} = 0.5236 \times 100 = 52.36 \text{ g. in whole urine}$$

$$a = \frac{b}{Z-1} = \frac{52.36}{2455} = 0.02132 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.02132 \times 100}{0.1501} = 14.2\%$$

2nd Day.

$$\text{Specific activity} = \frac{21.9}{114.3} \times 0.1 = 0.01916 \mu\text{c./g.}$$

$$Z = \frac{389.2}{0.01916} = 20310$$

$$\text{Wt. of coumarin added} = 0.5144 \times 34.4 = 17.70 \text{ g. in whole urine}$$

$$a = \frac{17.70}{20310} = 0.0008714 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.0008714 \times 100}{0.1501} = 0.6\%$$

$$\text{Total } 14.2 + 0.6 = 14.8\% \text{ of dose.}$$

3-Hydroxycoumarin (total)1st Day.

$$\text{Specific activity of derivative} = \frac{532.1}{99.4} \times 0.1 \times \frac{162}{146} = 0.5939 \mu\text{c./g. as coumarin}$$

$$Z = \frac{389.2}{0.5939} = 655.2$$

$$\text{Wt. of hydroxycoumarin added} = 0.199 \times 100 \times \frac{146}{162} = 17.94 \text{ g. as coumarin to whole urine}$$

$$a = \frac{17.94}{655.2} = 0.02738 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.02738 \times 100}{0.1501} = 18.2\%$$

2nd Day.

$$\text{Specific activity} = \frac{178.4}{120.1} \times 0.1 \times \frac{162}{146} = 0.1648 \mu\text{c./g. as coumarin}$$

$$Z = \frac{389.2}{0.1648} = 2361$$

$$\text{Wt. of hydroxycoumarin added} = 0.1997 \times 34.4 \times \frac{146}{162} = 6.194 \text{ g. as coumarin to whole urine}$$

$$a = \frac{6.194}{2360} = 0.002625 \text{ g.}$$

$$\% \text{ Dose} = \frac{0.002625 \times 100}{0.1501} = 1.7\%$$

$$\text{Total } 18.2 + 1.7 = 19.9\% \text{ of dose.}$$

APPENDIX 2.

Table 1.

Excretion of Glucuronic acid and Ethereal Sulphate
after Administration of Indole I.

Rabbit No.	1			2			3			
Wt. (kg.)	3.5			3.3			2.8			
Dose level (mg./kg.)	250			250			250			
Excretion % Dose as:	Gluc.	E.S.	Total	Gluc.	E.S.	Total	Gluc.	E.S.	Total	
1st Day	0	32	32	30	66	96	37	50	87	
2nd Day	25	62	87	3	3	6	0	3	3	
3rd Day	0	0	0	0	0	0	0	0	0	
Total	25	94	119	33	69	102	37	53	90	

Average Glucuronides 32%
 Average Ethereal Sulphate 73%
 Average Total Recovery 105%

Table 2.

Excretion of Glucuronic Acid, Etheral Sulphate and
Mercapturic Acids after Administration of Indole II.

Rabbit No.	Dose level (mg./kg.)	Day No.	Percentage of Dose		
			Gluc. Acid	Ether. Sulphate	Mercapt. Acid
4	100	1	14	37	0.36
		2	4	0	0.36
		3	0	0	0.09
		4	0	0	0.49
		Total	18	37	1.3
5	100	1	17	31	0.22
		2	0	0	0.15
		3	0	0	0.13
		Total	17	31	0.5
6	100	1	5	16	0.35
		2	0	7	0.20
		3	0	0	0.20
		Total	5	23	0.75

Table 3.

Excretion of Indoxyl after Administration of Indole.

Rabbit No.	Dose level (mg./kg.)	Day No.	mg. Indoxyl (corr.)	mg. Indole	Percentage
7	100	1	0	0	0
		2	185	163	49
		3	0	0	0
		4	1.2	1.0	0.3
		5	0.6	0.5	0.2
		Total	187	165	50
8	100	1	0	0	0
		2	0	0	0
		3*	80	70	21
		4	80	70	21
		Total	160	140	42
9	100	1	157	138	49.3
		2	0	0	0
		3	1	0.9	0.3
		4	0	0	0
		Total	158	139	50

* On the 3rd day, urines were not collected under toluene.

Loss of indoxyl due to this would appreciably affect No. 8 only.

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* denotes that the date is that of C.A. in which the abstract appeared.

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