A

Thesis

entitled

"Studies on Mould Metabolites and

the Biosynthesis of Coumarins"

submitted in part fulfilment of the

requirements for admittance to the Degree

of

Doctor of Philosophy

in the

University of Glasgow

by

Douglas J. Austin.

University of Glasgow 1965

I dedicate this thesis,

the only tangible result

of all her pride and

concern for me, to

MY DEAR MOTHER.

Acknowledgements.

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CONTENTS.

Part 1. THE METABOLITES OF PENICILLIUM PUBERULUM BAINIER.

1.1 Introduction.

For millennia the fungi have contributed to the ease and disease of mankind. The action of yeast in promoting the fermentation of diverse carbohydrate sources to yield beer was known to the Philistines ca.1100 B.C. while the 'unleavened bread' of the Israelites was produced by the deliberate omission of yeast from the baking mixture. It is certain that the controlled use of fungi has played a major part in food production from the earliest periods of civilisation.

Baking and brewing still represent the most significant applications of microorganisms but, in recent years, great advances have been made in the field of chemotherapeutics by the direct use of fungal metabolites e.g. penicillin, griseofulvin and the tetracyclines. The potential uses for new metabolites are so important that the investigation of fungal cultures by modern analytical techniques is of more than theoretical interest.

The Fungi (Phylum Mycophyta) form one of the larger groups within the plant kingdom. Although

the more familiar 'mushroom' members of this group have macroscopic fruiting bodies, most species are inconspicuous and may in fact only be visible under the microscope. The essential difference between the Fungi and the true plants is that the Fungi lack chlorophyll and thus do not possess the ability to carry out photosynthesis. For this reason, they are found in nature either as Saprophytes (on dead plant or animal tissue) or as Parasites (on living tissue). By these adaptations, they are able to secure a supply of essential carbohydrates which they further transform as required.

The fundamental unit of fungal structure is a tube-like element or hypha which consists of an elongated cylindrical wall of polysaccharide material containing a mass of cytoplasm and many nuclei which may or may not be separated by cross-walls, the septa. The mass of hyphae that makes up a fungal growth is termed a mycelium.

The classification of fungi is based on morphological criteria and need not be detailed here. However, the four main groups are:

Phycomycetes (tube fungi)

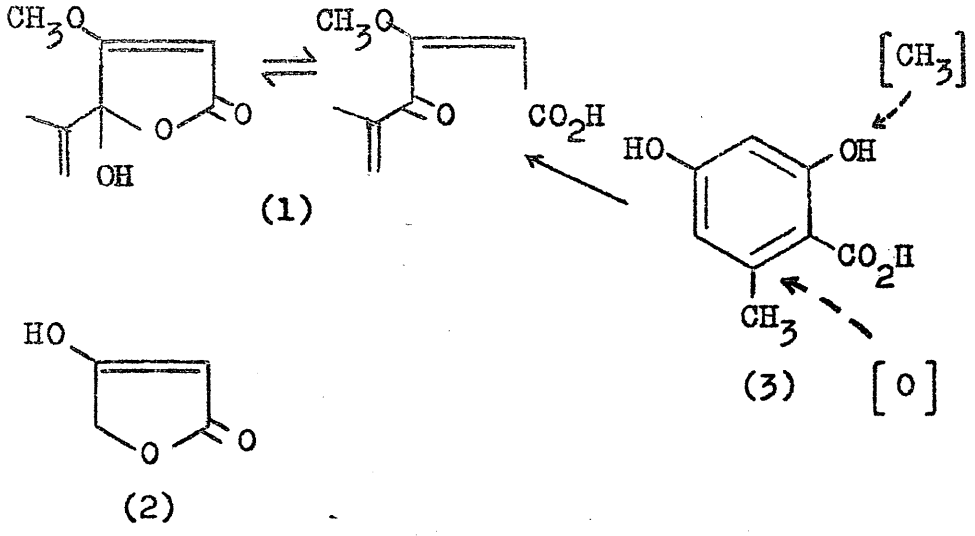
Ascomycetes (sac fungi)

Basidiomycetes (club fungi)

Fungi Imperfecti.

Penicillium puberulum Bainier can be placed in the Ascomycete class and is distinguished, like all the Penicillia, by the characteristic penicillus (Lat. 'little brush') form of the modified hypha which carries the organism's asexual spores. The detailed classification of the whole genus has been described by Raper and Thom[1] but it must be pointed out that the interspecific differences are often very slight. Furthermore, great controversy exists on the precise placing of the Penicillia as a whole and many authors prefer to assign them to the Fungi Imperfecti[2].

P.puberulum is regarded, with some reservations, as a member of the Fasciculata sub-section of the Asymmetrica group of the Penicillia[3]. Within that sub-section, it is classified with the P.cyclopium series, a widely distributed family frequently encountered on stored grain. P.puberulum itself was first described by Bainier[4] in 1907 and since then it has been found on maize[5], meat[6],



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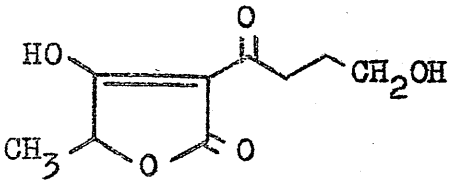

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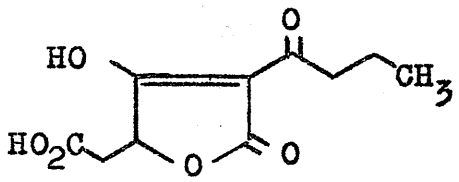

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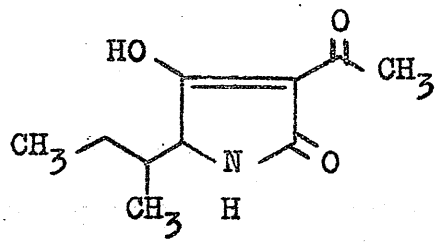

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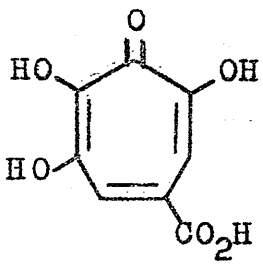

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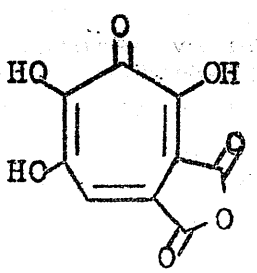

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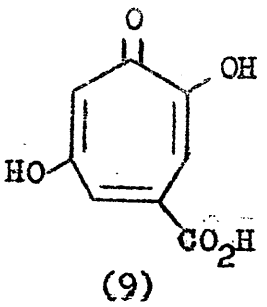
cheese[7], wood[8], and peanuts[9].

The structure of penicillic acid, first isolated by Arlesberg and Black from P.puberulum cultures in 1913[5], was shown to be (1) by Birkinshaw, Oxford and Raistrick more than twenty years later[10]. It has since been isolated from P.cyclopium[11] and P.baarnense[12] as well as P.thomii, P.suavolens and Aspergillus ochraceus[13]. This compound is formally a derivative of tetronic acid (2) and, despite its branched structure it is derived from orsellinic acid (3) by ring cleavage and the loss of carbon dioxide[11,12,14]. Many other tetronic acid derivatives are known microbial products[15] and the biosynthetic routes to several of them e.g. carolic (4) and carlosic (5) acids[16] and tenuazonic acid (6)[17] have been studied.

The fungal tropolones have presented a challenge to many first-rank organic chemists since the original isolation of puberulic (7) and puberulonic (8) acids from P.puberulum cultures in 1932[18]. They are produced by several other Penicillia and the work leading to their structural elucidation has been well reviewed[19]. Related compounds, stipitatic (9)



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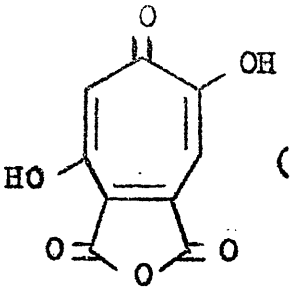

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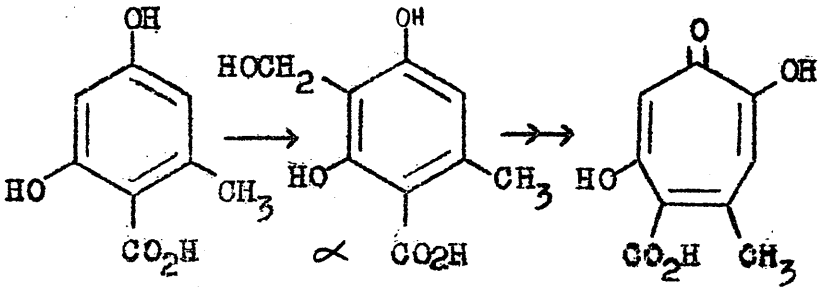
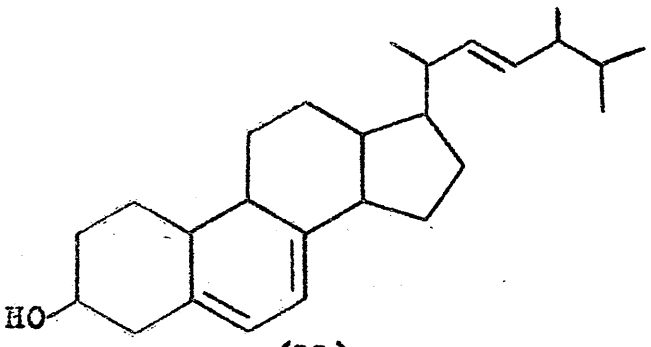


1 acetylCoA + 3 malonylCoA + 1 C_1 → C_9 precursor + 3 CO_2



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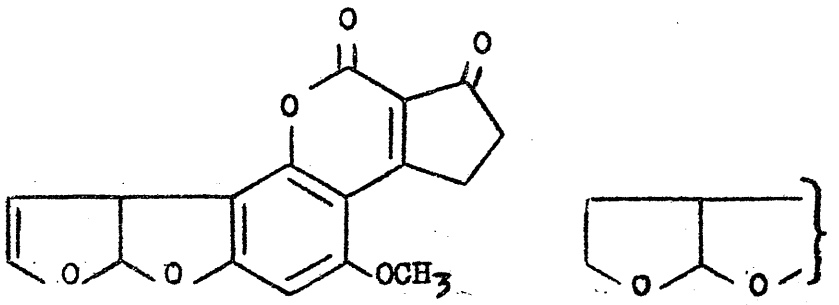
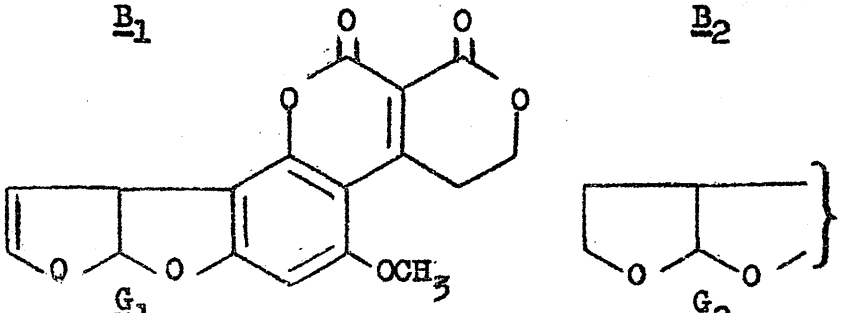


B_1 B_2



G_1 G_2

(12)- Aflatoxins.

and stipitatonic (10) acids, have been isolated from P.stipitatum[20]. The detailed biosynthesis of this class of compound remains obscure although the sophisticated radiotracer experiments of Bentley and others have demonstrated that the tropolone ring is built up from acetate and malonate units with orsellinic acid as a possible intermediate[21] e.g. (3)-α-(10). If this were proved, orsellinic acid might represent a 'branching point' of the secondary metabolism of these fungi, one pathway leading to the penicillic acids and another to the tropolones.

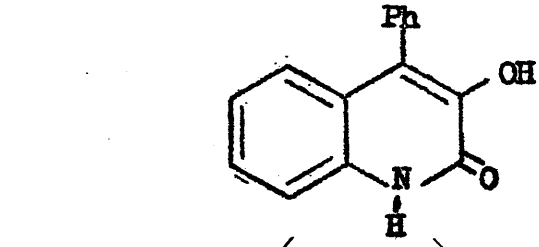
A variety of additional compounds have been isolated from P.puberulum. These include ergosterol (11)[22] (a very common fungal product[15]) and an unidentified photosensitive compound, $C_{17}H_{12}N_2O_2$[23]. The most recent report on the metabolites of this fungus is that of Hodges et al.[9] who have found a strain of P.puberulum which produces the aflatoxins B_1 and G_1 with the respective dihydroderivatives, aflatoxins B_2 and G_2 (12) when grown on a variety of substrates, including shredded wheat. The aflatoxins, originally extracted from toxic peanut meal contaminated with Aspergillus flavus Link ex

Fries[24], give rise to characteristic liver damage in animals. Their structures have been investigated by chemical[25] and by X-ray crystallographic means[26].

In the present study, Penicillium puberulum Bainier has been subjected to further chemical analysis to ascertain whether it has the ability to make quinolone derivatives which is possessed by two genetically related fungi. As will become apparent, the available strain does have this ability.

Viridicatin (13) was first isolated by Cunningham and Freeman from the mycelium of P. viridicatum Westling[27]. They established its molecular formula, $C_{15}H_{11}O_2N$, and prepared seven crystalline derivatives, including a monomethyl ether m.p. 239°. One of the oxygen atoms of the parent compound was shown to be present in a 'phenolic' hydroxyl group and the other probably in a cyclic amide. Insufficient material was available for degradative studies since their fungal strain ceased to produce viridicatin in culture. Such losses of synthetic ability are frequently encountered and can only rarely be reversed.

The structure of viridicatin was shown to be (13) by Bracken, Pocker and Raistrick, who obtained



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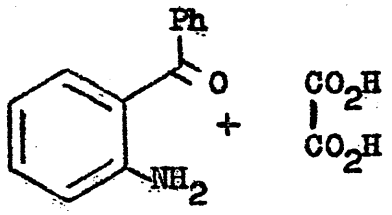

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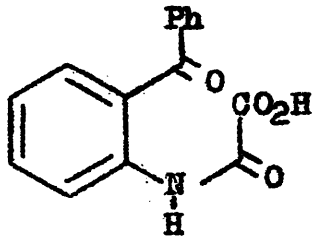

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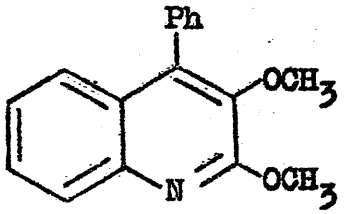

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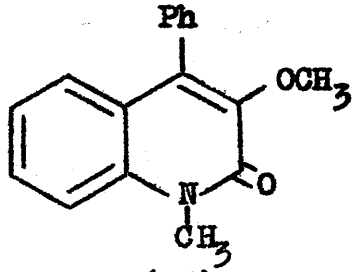

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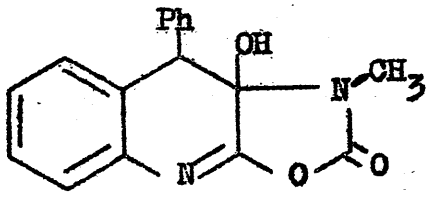

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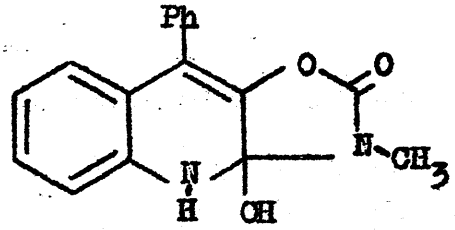
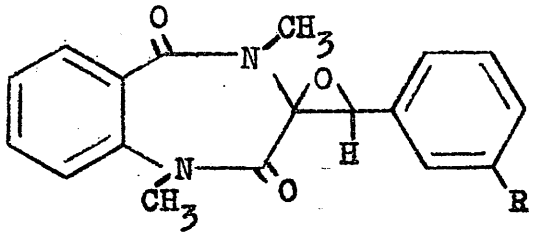


(19)

it from the mycelium of five strains of P.cyclopium Westling[28]. Proof of this structure was achieved by alkaline oxidation of the compound to o-amino-benzophenone (14) and oxalic acid. Oxidation of viridicatin with potassium permanganate in boiling acetone or with hydrogen peroxide in glacial acetic acid at 100°, gave good yields of oxalyl-o-amino-benzophenone (15). These results, combined with the proven identity of synthetic (13) with the naturally-occurring material, demonstrated that viridicatin possesses the unusual 3-hydroxy-4-phenyl-2-quinol-one system. Bracken, Pocker and Raistrick prepared several new derivatives, including O,O-dimethyl- (16) and O,N-dimethylviridicatin (17). A signif-icant part of their work was concerned with the structural elucidation of cyclopenin, $C_{17}H_{14}O_3N_2$, which they isolated from the culture filtrates of a sixth strain of P.cyclopium. Cyclopenin is readily decomposed by dilute mineral acids yielding one mole-equivalent each of carbon dioxide, methy-lamine and viridicatin. Using this fact, they proposed alternative structures (18) and (19) for cyclopenin.



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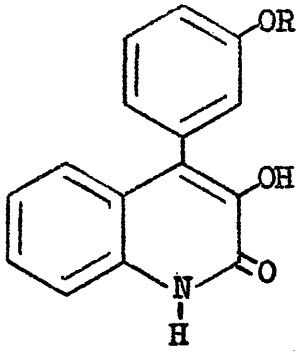

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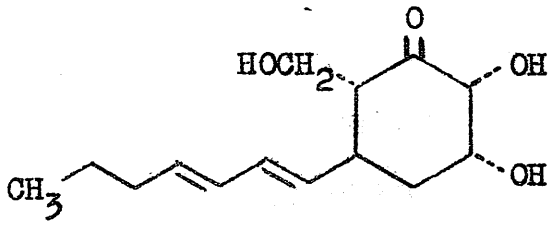

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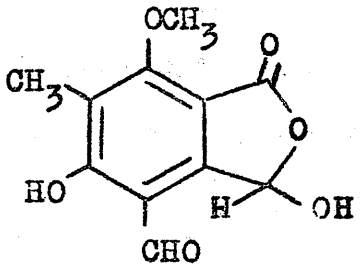

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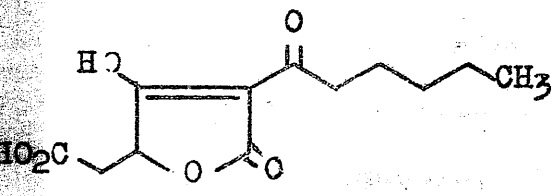

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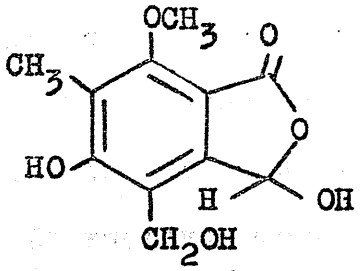


(24)

In addition to viridicatin and cyclopenin, the meta-hydroxylated analogues have been found in Penicillium cultures. The viridicatin analogue was first reported, as 'alkaloid X' by Luckner and Mothes [29,30]. It was found, together with viridicatin itself, in mycelial extracts of P.viridicatum. The culture filtrates (the liquid on which the mycelium had grown) were shown to contain cyclopenin and its hydroxy-analogue 'cyclopenin B' now termed cyclopenol. 'Alkaloid X' has been shown to have the structure (20), by degradative studies and unambiguous synthesis [31,32], and has been named viridicatol.

A new proposal for the structure of cyclopenin has been made by Mohammed and Luckner [33], who question the previous suggestions of Bracken, Pocker and Raistrick [28], (18) and (19), and suggest on the basis of spectroscopic and further degradative studies that (21),R=H, better represents cyclopenin. Since its congenor, cyclopenol, yields viridicatol on treatment with dilute mineral acid it would have structure (21),R=OH.

A very convenient synthesis of viridicatin from phenyldiazomethane and isatin, together with the

preparation of N-methylviridicatin, has been described by Eistert and Selzer[34].

The biosynthetic route to the unusual viridicatin system has received attention[29,30,33] and will be discussed in a later section.

The connection between these known metabolites of P.cyclopium and P.viridicatum and the present study should now be considered. On chemotaxonomic grounds, it seemed possible that P.puberulum might produce viridicatin or a derivative of a related quinolone since a survey of all the reported metabolites of the P.cyclopium series of the Penicillia (to which P.puberulum belongs) indicated certain correspondences with those of the P.viridicatum series.

P.cyclopium series.[15]

1. P.cyclopium produces penicillic acid (1), palitantin (22) cyclopaldic (23) and cyclopoldic (24) acids (related to the tropolone precursor∝?), viridicatin (13), viridicatol (XX), cyclopenin (21), R=H, and cyclopenol (21), R=OH.

2,3. P.aurantio-virens and P.johannioli (syn.P. martensii) produce puberulic (7) and puberulonic (8)

acids.

4. P.puberulum produces puberulic (7) and puberulonic (8) acids, as well as penicillic acid (1).

Intermediate form.[15]

1. P.cyclopium-viridicatum produces puberulic (7) and puberulonic (8) acids.[36]

P.viridicatum series.[15]

1. P.viridicatum produces a tetronic acid-viridicatic acid (25), viridicatin (13), viridicatol (20), cyclopenin (21), R=H, and cyclopenol (21), R=OH.

2. P.olivino-viride has apparently not been studied.

3. P.palitans produces palitantin (22).

A comparative analysis of these patterns prompts several questions. Thus - do all of these fungi make tetronic acids? Do the members of the P. viridicatum series produce tropolones ? Finally, is the ability to produce viridicatin derivatives confined to the fungi P.cyclopium and P.viridicatum?

The examination of P.puberulum metabolites detailed in the following section has resulted in the identification of a new viridicatin derivative as well as the partial characterisation of a series of novel long-chain amides.

This confirms the indirect, chemotaxonomic, evidence that P.puberulum might produce quinolones and suggests that tropolones and quinolones may be formed by the same fungus.

1.2. RESULTS AND DISCUSSION.

The metabolites of Penicillium puberulum Bainier were examined with the specific intention of detecting quinolone derivatives. The fungal strain employed (LSHTMP.47) was obtained from the Department of Biochemistry, London School of Hygiene and Tropical Medicine and was cultured here in the Joint Mycological Laboratories, Departments of Botany and Chemistry.

The seed required for the main culture was grown on malt agar and the mycelium inoculated into 150 'Glaxo' bottles each containing 500 ml. of Czapek-Dox medium (see[1]) with added 'Corn Steep Liquor' (10 ml./litre). After five weeks, the mycelium had fragmented and its colour had begun to change from green to brown. Accordingly, the mycelium was filtered off, dried, and weighed before extraction with

chloroform. The examination of the mycelial extracts will be detailed below.

1.2.1. METABOLITES IN THE CULTURE FILTRATE.

The culture filtrate, amounting to 75 litres, was treated in batches with 10gm./litre of bone charcoal for two hours. The charcoal was then filtered off and continuously extracted with acetone for two days. Reduction of the acetone extracts gave a brown tar which was partitioned between water and ethyl acetate.

Column chromatography of the water-soluble fraction over deactivated alumina did not yield any promising material.

The organic phase from the partition was dried over sodium sulphate and the solvent removed under vacuum. The residue was dissolved in acetone and the solution reduced to give an oil which was triturated with petroleum ether ($60-80^{o}$). The portion insoluble in petroleum ether only contained material resembling a polypeptide and was not further examined.

The portion soluble in petroleum ether gave an intense green colour with methanolic ferric chloride

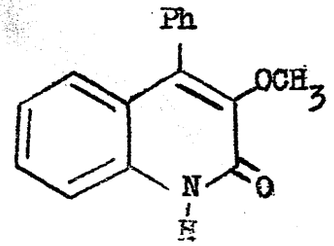
solution. Since this is one characteristic of viridicatin [28] (and, of course, many other phenolic/enolic systems), the yellow solution was reduced in bulk to 50 ml. and an equal volume of ether added. Over a period of three weeks at 0°C, two crops of colourless prismatic crystals were deposited. The first crop had melting point 245-50° and gave the intense green ferric chloride reaction. It was analysed by thin-layer chromatography on Kieselgel G_5 using 9:1 chloroform/methanol as the eluting solvent. The compounds present were made visible by oxidation with ceric ammonium nitrate and by ferric chloride colour reactions.

Two trace components (Rf 0.26 and 0.53) giving positive green ferric chloride reactions were detected in the first crop. The component with Rf.0.53 was shown to have an ultra-violet spectrum and base shift characteristics identical with thos of viridicatin. Insufficient material was available to permit more detailed study both of this compound and the component with Rf 0.26.

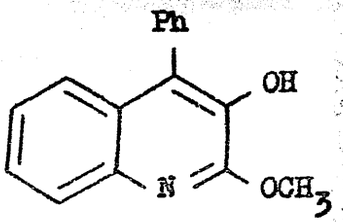
By far the major constituent of the first crop of crystals was the material with Rf 0.71,

which may be termed 'A'. In sharp contrast to the other metabolites, 'A' did not give a colour with ferric chloride. This, coupled with its position on the plate, suggested that if it were a viridicatin derivative, at least one of the polar functions of the fundamental 3-hydroxy-4-phenyl-2-quinolone system must be masked. As a reasonable first step towards the identification of this metabolite, it was decided to accumulate as much as possible of pure 'A'.

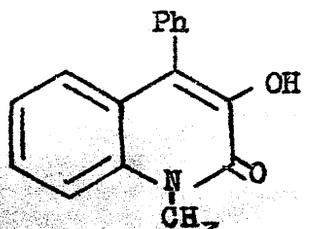
The second crop of crystals from the culture filtrate petroleum ether-soluble material proved to be pure 'A' and a further larger quantity of the compound was obtained by column chromatography of the mother liquors over deactivated alumina using gradient elution techniques. The earlier fractions were eluted with petroleum ether (40-60^{o}) progressively enriched with benzene and merely contained large quantities of lipids. However, fractions 42-47, eluted with a 1:4 ether/benzene mixture, contained a crystalline metabolite 'B' that has been further examined. For the purpose in view, the important result of this chromatogram was that



α



β



γ

Viridicatin Derivatives.

Compound.	Structure.	Rf on Kieselgel G with 9:1 $CHCl_3$-MeOH, $CHCl_3$.	Colour $FeCl_3$.
Viridicatin.	(13)	0.54	0.05	+ve green
3-O-methyl-viridicatin	α	0.71	0.35	-ve
N-methyl-viridicatin	(13), $N-CH_3$	0.90	0.39	+ve green
O,N-dimethyl-viridicatin	(17)	0.92	0.41	-ve
O,O-dimethyl-viridicatin	(16)	0.90	0.40	-ve
Viridicatol	(20), R=H	0.12	0.00	+ve green
3'-O-methyl-viridicatol	(20), $R=CH_3$	0.40	0.00	+ve green

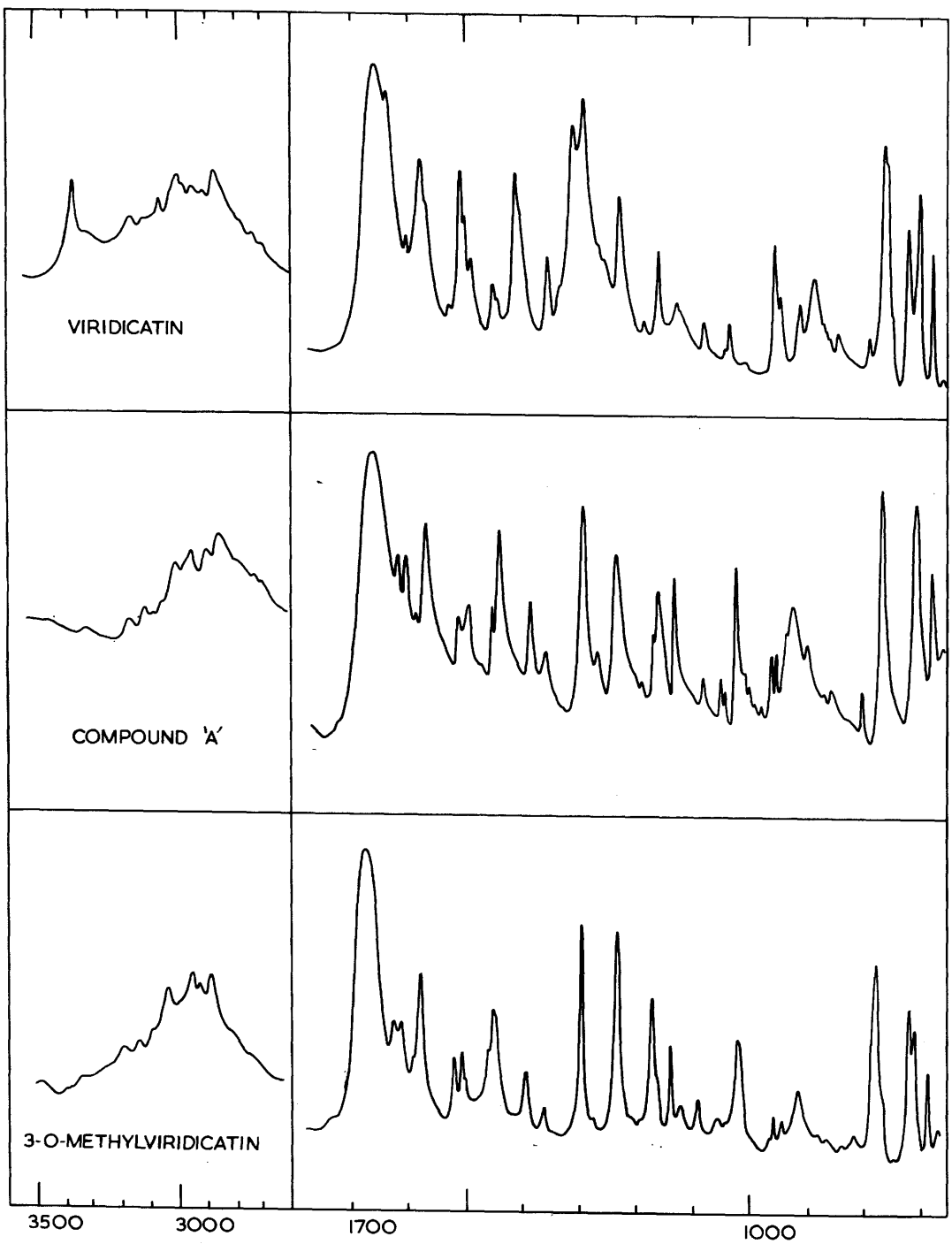
This compound was the generous gift of Professor B.Eistert of Saarbrücken.

Δ This was identical with natural and synthetic viridicatol kindly provided by Dr.M.Luckner of Haale.

fractions 51-55, eluted with pure ether, contained 147 mgm. of crude 'A'. This was decolourised with animal charcoal and recrystallised three times from methanol. Combined with the twice-recrystallised material from the two crops from the petroleum ether solubles, the total quantity of pure 'A' available was 158 mgm.

Viridicatin[34] and its O,O- and O,N-dimethyl derivatives[28] were synthesised by procedures already reported and proved to be useful standards for the analysis of P. puberulum quinolones. The structures, mobilities and colour reactions of the viridicatin derivatives produced for this study are shown in the table.

Microanalysis of 'A' indicated that its empirical formula is $C_{16}H_{13}O_2N$. This immediately suggested that it might be one of the three theoretically possible mono-methylated viridicatins (α, β, γ). This general hypothesis was supported by the close apparent similarities between the infra-red spectra of 'A' and authentic viridicatin shown in the diagrams. Thus both exhibit an intense broad band near 1655 cm^{-1} which is one characteristic of a


VIRIDICATIN
COMPOUND 'A'
3-O-METHYLVIRIDICATIN
3500
3000
1700
1000

2-quinolone.[37] In addition, both have strong absorption bands in the region 1570-1560 cm^{-1}. These two bands may be assigned with some confidence to the C=O stretching (Amide I) and N-H bending (Amide II) modes of a secondary amide group. Further, viridicatin has a sharp band at 3359 cm^{-1} which is not paralleled by 'A' but is rather replaced by bands, at 2850-30 cm^{-1}, 1123 cm^{-1} and 1017 cm^{-1}, characteristic of a methoxyl grouping.[38]

Since N-methylviridicatin does not show absorption in the 1560 cm^{-1} or 1000 cm^{-1} regions, but does have a sharp band at 3360 cm^{-1}, certain inferences seem justified.

The fact that 'A' exhibits absorption characteristic of a secondary amide is evidence that structures β and γ are not preferred for 'A'. This is reinforced by the fact that 'A' does not show bands for the hydroxyl stretching mode in the region 3600-3200 cm^{-1}. The strong probability that 'A' possesses a methoxyl group is indicated and thus the preferred structure for 'A' is α, which may now be named as 3-O-methylviridicatin. This accords well with the observation that 'A' does not

Ultra-violet Spectra of Viridicatin and 'A'.

Viridicatin.	'A'
205 (26,400)	205 (36,600)
223 (37,900)	223 (40,500)
234 (21,100)	-
241 (18,500)	-
282 (7,900)	281 (8,200)
289 (6,800)	-
308 (7,400)	313 (7,300)
319 (9,900)	324.5(9,100)
331 (8,300)	337 (6,400)
-	-
Viridicatin + Base.	**'A' + Base.**
207 (n.m.)	207 (n.m.)
218 (33,600)	-
226 (31,000)	230 (42,200)
253 (17,300)	-
284 (7,900)	278 (7,300)
293 (8,400)	-
-	313 (6,100)
-	326 (8,400)
331 (13,300)	338 (7,300)
341 (14,800)	-

- = no peak in this region. n.m. = not measured.

All peaks in mμ, with epsilon values in brackets.

give a colour with ferric chloride solution and permits the suggestion that chelation between the amide carbonyl and a free 3-hydroxyl group is essential for a positive reaction.

Further evidence that 'A' is a viridicatin derivative and that its likely structure is~~α~~ was obtained by a comparative study of the ultra-violet spectra of 'A' and viridicatin. As shown in the table, there are close similarities between the spectra of these compounds in neutral methanolic solution, particularly in the region 305-340 mμ, where a triplet of bands (possibly characteristic of the 3-oxygenated-4-phenyl-2quinolone system) is evident. The effect of base on the spectrum of viridicatin is immediate and striking since the triplet is replaced by two high intensity bands at longer wavelength and the two bands at 234 and 241 mμ are replaced by one at 253 mμ. Acidification of the solution restores the original spectrum. The addition of base to a methanolic solution of 'A' causes no significant change in its ultra-violet spectrum apart from slight alterations in the relative intensities of the bands.

From these observations, it is clear that

viridicatin possesses an acidic ionisable grouping not present in 'A' and also that the absorption bands of viridicatin which are not paralleled in 'A' probably result from the electronic transitions associated with this ionisable grouping. The structure of viridicatin is such that only the 'phenolic' 3-hydroxyl function can meet the requirements of this data. Therefore the structure of 'A' must be such as to exclude the ionisation of this grouping. This reinforces the assignment of structure α to 'A' arrived at from infra-red data.

Final proof that this is indeed the structure of 'A' was achieved by comparison of the natural material with synthetic 3-O-methylviridicatin.

The work of Cunningham and Freeman[27] did not permit a definition of the structure of viridicatin but, as has already been described, among the seven crystalline derivatives prepared by them was a monomethyl ether with melting point 239°. This is somewhat lower than that of pure 'A' (248-9°) but the possibility existed that their compound was the 3-O-methyl ether of viridicatin especially since Eistert and Selzer[34] had prepared the N-methyl compound and reported its melting point as 208-9°.

Accordingly, the procedure of Cunningham and Freeman, involving reaction of methyl iodide with the silver salt of viridicatin, was followed in repeated attempts to obtain their monomethyl ether. In our hands, however, none of the reported monomethyl ether was produced, almost all of the product being O,N-dimethylviridicatin m.pt.195-7°, identified with an authentic sample. Column chromatography of the total product of this attempted monomethylation over alumina did in fact produce a small amount of a crystalline viridicatin derivative m.pt. 204°, giving a positive green ferric chloride test. That this was N-methylviridicatin was confirmed by later comparison of its infra-red spectrum with that of an authentic sample*.

Since it was deemed essential to obtain a sample of 3-O-methylviridicatin, several further attempts were made to prepare it by standard methylation procedures using the established thin-layer chromatographic systems for evaluation of the products. The major product in each case was the O,N-dimethylated compound, little or none of the required product being observed.

* This was the generous gift of Professor B.Eistert. of Saarbrücken.

The desired monomethyl ether was ultimately prepared in low yield by a method analogous to that employed by Fales and Wildman for the selective methylation of secondary hydroxyl groups in Amaryllidaceae alkaloids[39]. Their technique requires that a potassium salt of the compound be dissolved in benzene and methyl-p-toluenesulphonate added. This was not applicable to the viridicatin series since the parent compound and its salts are almost totally insoluble in benzene. Accordingly, a search was made for an inert solvent capable of dissolving both viridicatin and the methylating agent. Dioxan proved to be suitable and the amount of methyl-p-toluenesulphonate calculated to give monomethylation was added to a solution of viridicatin. The mixture was heated for four hours under reflux and the solvent removed at the water pump with heating. The residue was dissolved in aqueous base and O,N-dimethylviridicatin extracted with ether and chloroform. The alkaline aqueous solution was acidified with dilute mineral acid and the resulting suspension extracted with ether. The purified material obtained in low yield from this ether extract proved to be 3-O-methylviridicatin

(mp. 247-9°) as judged by its chromatographic mobility, lack of ferric chloride colour reaction, microanalysis, infra-red and ultraviolet spectra.

In all of these properties, as well as mixed melting point, 3-O-methylviridicatin was identical to compound 'A' isolated from P. puberulum. The structural proof of this hitherto unknown metabolite has been published.[40]

The evidence presented above demonstrates that P. puberulum shares the ability of P. cyclopium and P. viridicatum to make quinolones. The biosynthetic implications of this will be discussed later.

Reference has already been made to a previous crystalline fraction 'B' from the column chromatogram of the extractives of P. puberulum culture filtrate. Compound 'B' was obtained in small quantity from the material eluted with 4:1 benzene-ether. The crude material (166 mgm.) was crystallised twice from 1:1 ether-petroleum ether (40-60°) to give colourless needles m.pt 178-80°. Although TLC of this compound showed only one spot (Rf 0.09 with $CHCl_3$) it almost certainly contained a nitrogenous contaminant. Thus, microanalysis indicated the possible empirical formulae $C_{59-63}H_{89-97}O_6N$, or,

neglecting nitrogen, $C_{28-30}H_{42}O_3$. The nuclear magnetic resonance spectrum of 'B' was so strongly reminiscent of steroidal compounds that the C_{28-30} formulation is absolutely preferred. This is borne out, to a certain extent by the infra-red spectrum which shows only small hydroxyl stretching bands in the region 3600-3200 cm^{-1}, large peaks attributable to methyl and methylene stretching and bending modes in the regions 3000-2850 cm^{-1} and 1480-1350 cm^{-1}. No significant absorption is seen in the region 1800-1500 cm^{-1}, suggesting that there are neither carbonyl nor olefinic double bonds in this molecule. The ultra-violet spectrum shows only end absorption. Since this material was isolated before the advent of preparative layer chromatography, it was not further examined.

1.2.2. Metabolites in the mycelial extracts.

The crumbly, green-brown mycelium obtained from the main fungal culture was dried and weighed (200 gm.). It was then crushed and extracted with chloroform for 24 hours in a Soxhlet extractor. The dark brown solution obtained was reduced to give a dark brown oil, This oil was itself extracted with petroleum ether to give a dark brown solution

and a brown gum which proved to contain mainly polypeptide material not of interest for the present study.

The petroleum-ether soluble material was chromatographed over deactivated alumina. By far the main component, eluted with chloroform, proved to be ergosterol (11), a common mould product,[15] which was present to the extent of 0.8% of the mycelial dry weight. This was established by comparison of the isolated compound with authentic ergosterol. The criteria of identity were: melting point and mixed melting point, IR and UV spectra and TLC comparison. The UV spectrum of the compound showed a quartet of peaks at 262, 272, 283 and 294 mu. Such a pattern is highly characteristic of ergosterol and other sterols containing the Δ-5,7 diene grouping. This observation confirms the previous work of Birkinshaw, Callow and Fischmann[22] who found 0.13% of ergosterol in the dried mycelium of P. puberulum.

Later fractions from this chromatogram, those eluted with 19:1 $CHCl_3$/MeOH, contained 685 mgm. (crude weight) of a metabolite which has proved to be of great interest and will be discussed as

compound 'C'.

The crude material 'C' was purified by a rather unusual procedure making use of the remarkable properties of this compound. It was observed that, although 'C' was soluble in all the common organic solvents on heating, the effect of allowing the solution to cool was to precipitate the compound as more or less white spherical particles. Since the impurities present tended to remain in the solvent, 'C' was purified by dissolving the total crude material in 1:1 MeOH/$CHCl_3$ and cooling the solution. The white gel was collected and submitted to the same treatment twice more finally yielding 280 mgm. of 'C' as pure white spherical particles m.pt.139-41°, which crystallise on the Kofler block and remelt at 144-6°. Much later it was found that 'C' does in fact crystallise slowly from dilute pyridine solution yielding colourless needles m.pt. 146-8°.

The analytical figures for 'C' suggest that its empirical formula is $C_{36}H_{67}O_5N$. Those for the acetylated compound correspond with the formula $C_{40}H_{71}O_7N$, with a molecular weight of 677 mass units, and indicate that a diacetate of 'C' has

Molecular Species in diacetyl 'C'.

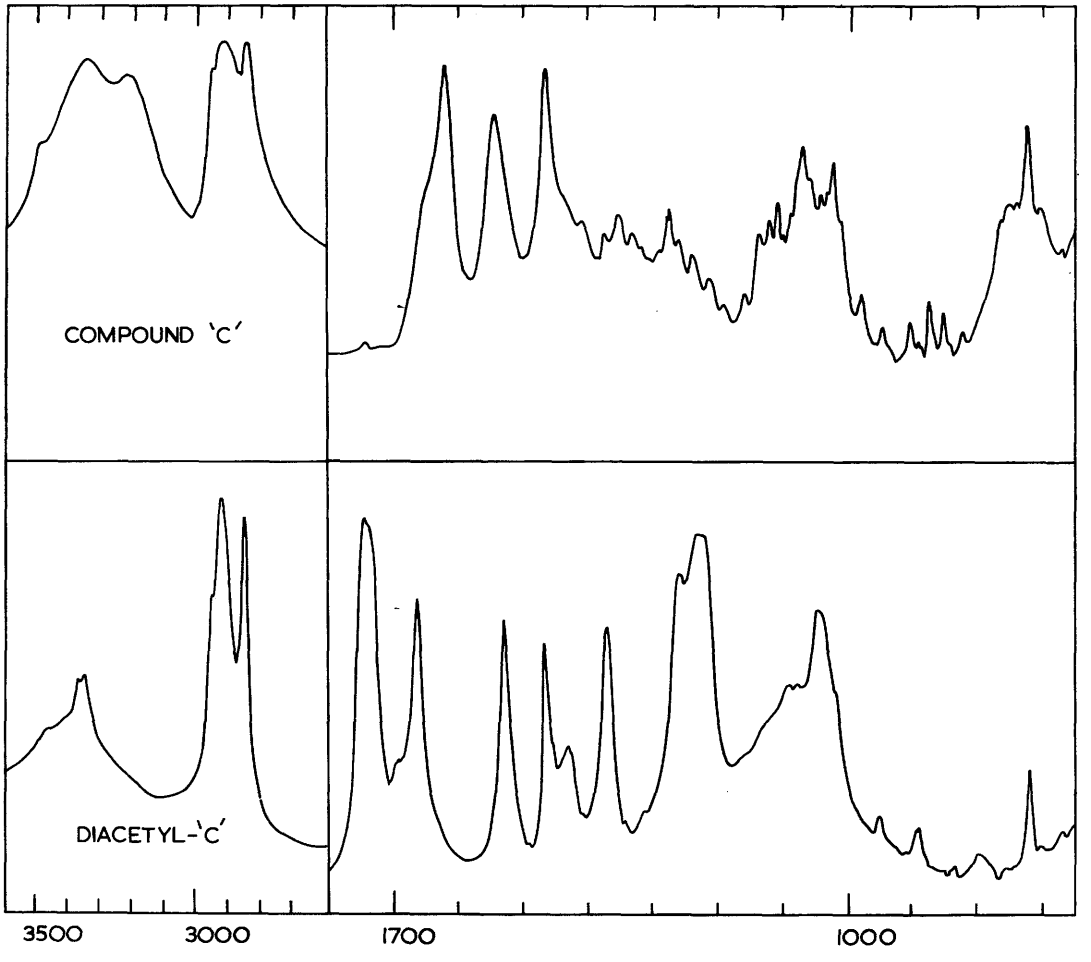
MW.645	-	$C_{40}H_{71}O_5N$
MW.647	-	$C_{40}H_{73}O_5N$
MW.659	-	$C_{40}H_{69}O_6N$
MW.661	-	$C_{40}H_{71}O_6N$
MW.665	-	$C_{40}H_{75}O_6N$
MW.675	-	$C_{40}H_{69}O_7N$
MW.679	-	$C_{40}H_{73}O_7N$
MW.691	-	$C_{40}H_{69}O_8N$
MW.693	-	$C_{40}H_{71}O_8N$

Full details of the mass spectrum of diacetyl 'C' are set out in the Experimental section - 1.4.

been formed under the mild conditions employed. Acetylation of 'C' under forcing conditions gave only the diacetate together with traces of more polar compounds.

It proved impossible to obtain a useful mass spectrum of 'C' itself due to the rapid decomposition of the molecular ions. Therefore, the general formulations given above were confirmed by the mass spectral data obtained for diacetyl-'C', shown in the table. For this compound, it is clear that the major high mass peaks lie between 645 and 693 mass units. Detailed analysis of this information will be given below.

Analysis of this compound and diacetate by TLC on Kieselgel G suggested that they are homogeneous since single spots were observed for each (Rf values; chloroform - 0.06 and 0.50; 9:1 chloroform-methanol - 0.17 and 0.90 for 'C' and its diacetate respectively). The mass spectrum of the acetyl derivative shows that 'C' is actually a mixture of nine closely related compounds. However, the conclusions regarding the fundamental structural features of 'C' are not affected by this fact. Attempted gas-liquid analysis of 'C',


COMPOUND 'C'
DIACETYL-'C'
3500
3000
1700
1000

its diacetate, and its trimethyl silyl ether was of no value because of the rapid decomposition of these compounds at the high temperatures necessary.

The first real clues as to the nature of 'C' came from consideration of its infra-red spectrum and that of its diacetate. In passing, it should be noted that these compounds gave rise to end absorption only in ultra-violet analysis.

The infra-red spectrum of 'C' reproduced here, shows large bands due to methyl and methylene groups in the regions 3000-2800 cm^{-1} and 1500-1400 cm^{-1}. In addition, the strong band at 720 cm^{-1} is one characteristic of long-chain aliphatic compounds. There are no signs of aromatic character. The complex patterns of bands seen in the spectra of 'C' and its diacetate in the region 1800-1500 cm^{-1} are of great interest. Thus, 'C' has two bands at 1620 and 1545 cm^{-1} which may be assigned to the Amide I (C=O stretch) and Amide II (N-H deformation) modes of a secondary acyclic amide. It is a striking fact that the band assigned as Amide I is at a lower frequency than that normally observed for this system (1680-1630 cm^{-1}). This suggests that the normal

stretching mode of the amide carbonyl function of 'C' is altered by hydrogen bonding between the carbonyl and a nearby hydroxyl or other group. This hypothesis is supported by the presence of three bands in the appropriate region in the spectrum of diacetyl-'C'. Thus, the intense band at 1742 cm^{-1} may be due to the presence of one or more O-acetyl groups while the two bands at 1663 and 1530 cm^{-1} represent normal secondary amide absorption. Therefore, the acetylation of a hydroxyl function has apparently removed the hydrogen bonding effect on the carbonyl absorption of 'C'. The region 3500-3200 cm^{-1} in the spectrum of diacetyl-'C' contains one sharp peak at 3373 cm^{-1} due to the N-H stretching mode of a hydrogen bonded secondary amide. This is to be compared with the same region in the spectrum of 'C' itself which contains three peaks, at 3500, 3340 and 3210 cm^{-1} respectively. The peaks at 3500 and 3210 cm^{-1} can be attributed to the hydroxyl functions that are acylated in diacetyl 'C'. Clearly, one of these may be 'free' (3500) while the other is strongly hydrogen bonded (3210 cm^{-1}) within the molecule of 'C'. The shoulder at 1650 cm^{-1} in the spectrum

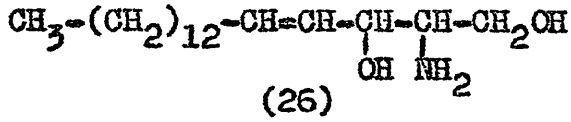
of 'C', combined with the peak that is apparent in the region 980-960 cm^{-1} for both compounds, suggests that a trans-disubstituted double bond is present in the molecule. The strong bands in the region 1280-60 cm^{-1} seen in both spectra may be due to the amide III band of a secondary amide and an interesting possibility is that the weak bands at 1260 cm^{-1} represent the C-O stretching modes of one or more epoxide functions. This possibility is reinforced by the presence of a band at 853 cm^{-1} in the spectrum of 'C' and at 838 cm^{-1} in that of diacetyl-'C'. These frequencies are very close to those previously assigned to the cis-epoxide function in long-chain aliphatic acids, esters and alcohols by Shreve and his co-workers[41].

The nuclear magnetic resonance (NMR) spectra of 'C' and its diacetate were measured in pyridine and carbon tetrachloride solutions respectively. Because of the high molecular weight and the tendency of these compounds to form gels in concentrated solution, the spectra were not completely satisfactory and, of course, the necessity of using pyridine as the solvent for 'C' eliminated the region from $\tau 1$ to $\tau 3$ by completely swamping any signals

due to 'C'. However, the spectrum of the diacetate yielded some valuable quantitative results.

The dominating feature of both spectra is an extremely large peak at τ8.72. Using the integrated areas of the peaks at τ7.94 and τ7.99 in the spectrum of diacetyl-'C' (ascribed to the six acetate CH_3-C protons) as an internal standard, the τ8.72 peak may represent as many as fifty protons! Since this chemical shift value is associated with the methylene protons of linear chains,[42] it is abundantly clear that 'C' must essentially consist of linear hydrocarbon chains. Only one peak associated with the protons of C-CH_3 groups is present in both spectra. It appears as an asymmetric triplet centred at τ9.1 corresponding to six protons and may be assigned to the terminal methyl groups of aliphatic chains. Therefore 'C' appears to consist of two aliphatic chains possibly linked through the observed secondary amide function.

The only other peak that can be assigned in these spectra corresponds to four protons and appears at approximately τ7.80. One possible assignment is to the protons attached to two disubstituted epoxide rings. Fair analogy for



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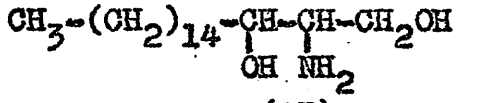

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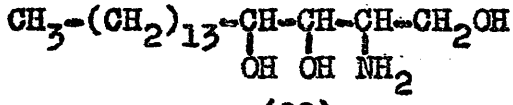
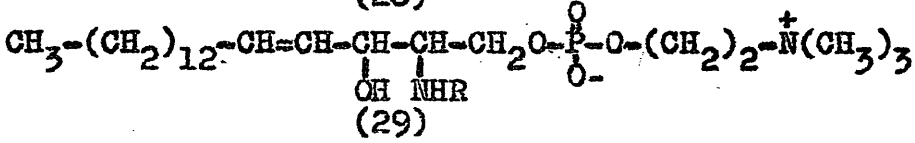


(28)



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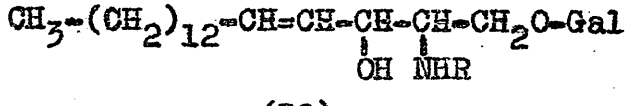

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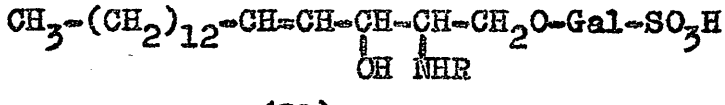

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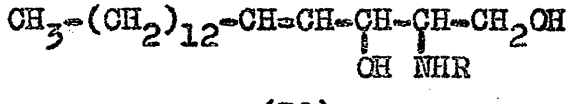

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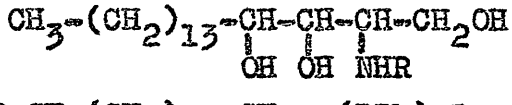
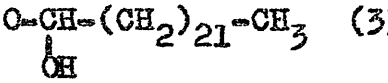
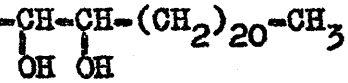
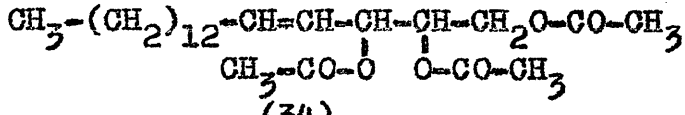


(33a) R=  (33b) R= 



(34)

this is available in recent work[43] although the peaks for sugar and steroid epoxides are usually found in the range τ 6.5-7.2[44].

The signals below τ 7.0 are so weak that they cannot be discussed with confidence. They appear to be associated with olefinic protons as well as those of methylene attached to oxygen functions.

As a result of this information, a somewhat clearer picture of the basic structure of 'C' was beginning to form, but a concept embodying all the known data was still lacking. A study of the sphingolipid class of compounds has provided both this unifying concept and the hoped for correlation with known natural compounds.

The naturally-occurring lipids[45] may be divided into two groups depending on the molecule employed as the back-bone of their extended structures. The first group yields glycerol while the second is distinguished by the release of sphingosine (26) (D-erythro-1,3-dihydroxy-2-amino-trans-octadec-4-ene) on hydrolysis. In some instances dihydrosphingosine (27) or phytosphingosine (28) is found as a constituent in place of the more common sphingosine[46].

The four main groups of the sphingolipids are the sphingomyelins (29), cerebrosides (30), sulphatides (31) and the complex mucolipids.[47]

It is of particular interest that all the sphingolipids have a long-chain fatty acyl substituent on the amino group, thus they are secondary amides. The ceramides, from which all the others are formally derived[47], have only this acyl substituent and therefore the hydroxyl groups flanking the amide function are well situated for hydrogen bonding with the carbonyl. Their basic structure is (32) and many have been synthesised[47a].

As well as differences in the substituents on the primary alcoholic group of sphingosine, variation can occur in the acyl portion of the amide. Thus C_{24} straight-chain acids are frequently found as well as their α-hydroxy derivatives. Palmitic (C_{16}) and stearic (C_{18}) acids have also been found[48].

Sphingolipids containing sphingosine and dihydrosphingosine have been found so far only in animal tissue, in particularly high concentration in brain and nerve tissue. A large body of literature exists on their structures and functions.

Phytosphingosine and its derivatives[46] have been reported as occurring only in plants but a recent report on the stereospecific synthesis of this compound[49] is an excellent source of leading references on its occurrence in various yeasts and fungi as well as human kidney and brain tissue. Of particular interest in this section is the fact that Oda[50] has shown that a Penicillium mould produces two ceramide phytosphingolipids with the structures (33a) and (33b). The fatty acid group has a chain length of 24 carbon units but the C_{26} homologue of (33a) has been isolated from various yeasts[51].

A total analysis of the data available on the structural features of 'C' shows compelling analogy with the properties to be expected for a member of the ceramide sphingolipids and, on the basis of this and the work to be described, it is proposed that 'C' is indeed a member of this class and that the multiple peaks in the mass spectrum of its di-acetate are due to real differences in the 18-carbon acyl group.

A comparison of the published IR spectrum of N-lignoceryl-sphingosines (32), R=-CO-$(CH_2)_{24}$-CH_3[52],

Comparison of Infra-Red Spectra-(Nujol Mulls).

N-lignoceryl-dihydrosphingosine	'C'	N-lignoceryl-sphingosine
-	*3500	-
*3344	*3340	-
-	*3210	*3278
*1634	1647	*1650
-	*1620	*1615
*1577	1565	*1568
*1550	*1545	*1550
1278	*1277	1271
1254	1260	-
1233	1238	1239
1211	1215	-
-	1160	-
1133	1140	*1133
1124	1121	-
1092	1110	1103
*1074	*1070	*1064
*1049	1041	1051
-	*1022	*1034
-	-	1009
-	977	982
-	948	*962
899	903	904
-	*872	*874
-	850	-
-	825	823
-	-	800
*720	*720	*720 (not Nujol)

Major peaks marked thus - *. Lignoceryl data from Ref.52.

with that of 'C' is shown in the table. Their striking similarity constitutes evidence in favour of the proposed formulation of 'C'. Comparison of the spectra of triacetylsphingosine (34)[53] and diacetyl-'C' reinforces the hypothesis since they too are closely similar.

The assumption that 'C' is indeed a ceramide sphingolipid is borne out by the coincident data available on its molecular formulation and spectroscopic properties. It is therefore possible to calculate the acyl functions in the mixture by analysis of the mass spectrum of diacetyl-'C'. The mass spectrum obtained for 'C' itself shows no definite 'parent ions' (because of their rapid decomposition) although the highest mass peak observed was at 541 units.

For diacetyl-'C', then, the major high mass peaks, which are all due to the 'parent ions' of different species, are tabulated with the molecular formula for each diacetyl derivative. These formulae were obtained by detailed consideration of the possible structures and are highly favoured assignments. In ascribing structural formulae

to certain of these molecular species, the fact that 'C' forms only a diacetate, even under forcing conditions, has been of value. This suggests that, although the backbone of the structures can be either sphingosine or dihyrosphingosine, it cannot be phytosphingosine which would yield triacetyl derivatives. Furthermore, it suggests that the only hydroxyl functions in 'C' are those flanking the amide grouping since any present on the fatty acyl side chain would give an overall triacetyl derivative. The mass spectrum of diacetyl-'C' up to ca.760 mass units shows no trace of higher acetylated species.

The extreme rarity of octadecatetraenoic acids and derivatives has been taken into account to exclude them from detailed consideration in the structural hypotheses. They have been found only in a few plant seed oils besides whale and herring oils[45].

The spectroscopic evidence for epoxide functions in 'C' and its diacetate has also been employed in the analysis of this complex mass spectrum.

On the basis of the known structures of the naturally-occurring C_{18} mono-,di- and tri-enoic

Ceramides from Penicillium puberulum Bainier.

A. Dihydrosphingosine series - (27)+ C_{18} acyl group R.

(oleate): R= $-CO-(CH_2)_7-CH\!-\!\underset{O}{}\!-CH-(CH_2)_7-CH_3$ MW.665*

(linoleate): = $-CO-(CH_2)_7-(CH=CH-CH_2)_2-(CH_2)_3-CH_3$ MW.647

= $-CO-(CH_2)_7-(CH\!-\!\underset{O}{}\!-CH-CH_2)_2-(CH_2)_3-CH_3$ MW.679*

(linolenate):= $-CO-(CH_2)_7-(CH=CH-CH_2)_3-CH_3$ MW.645

= monoepoxylinolenyl- MW.661

= $-CO-(CH_2)_7-(CH\!-\!\underset{O}{}\!-CH-CH_2)_3-CH_3$ MW.693*

B. Sphingosine series - (26)+ C_{18} acyl group R.

(linolenate):= monoepoxylinolenyl- MW.659*

= diepoxylinolenyl- MW.675*

= $-CO-(CH_2)_7-(CH\!-\!\underset{O}{}\!-CH-CH_2)_3-CH_3$ MW.693*

The terms in brackets thus (oleate) indicate the possible origin of each acylating moiety. All of these assignments are highly favoured. Those marked * appear to be unambiguous.

acids and their epoxy derivatives [45], it has proved to be possible to attempt the formulation of six of the components of 'C' and to give alternatives for the other three. It must be emphasised that, although degradative studies confirm the overall structural features, further work on 'C' appears essential.

The peaks at 665, 679 and 693 mass units may represent acyl dihydrosphingosines since no formulation using sphingosine itself can satisfy the structural requirements set out above. By analogy with the known C_{18}-epoxy-acids[45], and in accordance with the results of degradative experiments on 'C', their most likely structures are mono-, di-, and tri-epoxystearoyldihydrosphingosine respectively. From analogous considerations, it is probable that a second series of compounds is present in this mixture. They are the mono-, di- , and triepoxylinoleylsphingosines with molecular weights of 659, 675 and 691 respectively.

It is more difficult to allot structures to the species of mass 645, 647 and 661 since they may belong to either series. However, from a consideration of the likely biosynthetic routes

to the higher mass epoxides, it has proved possible to assign them as linolenyl-, linoleyl-, and mono-epoxylinoleyldihydrosphingosine respectively. This was done by the following analysis of the situation. The three acyl groups unambigously assigned to the sphingosine series are logically derivable from linolenic acid by successive epoxidation steps. This implies that the ultimate precursor for this series is in fact N-linolenyl-sphingosine, (26), $R=-CO-(CH_2)_7-(CH=CH-CH_2)_3-CH_3$ with molecular weight 643.

The uncertain peaks at 645, 647 and 661 do not fit naturally into this sequence but can be successfully placed in the dihydrosphingosine series in which the known acyl groups are mono-, di-, and tri-epoxy-stearic acids. The olefinic acyl derivatives from which each of these may be derived would have molecular weights of 645, 647 and 649 respectively. Only that at 649 is absent. If the derivation of the poly-epoxy compounds of the sphingosine series is indeed as stated - a successive epoxidation of olefinic precursors - then one would also expect to find the intermediates in the sequence to the dihydrosphingosines. The peak at

661 may be due to a monoepoxylinoleyldihydrosphingosine precursor for the triepoxy compound at 693. No significant peaks are present in the mass spectrum at 663 or 677 corresponding to the precursors for the mono and di-epoxystearoyldihydrosphingosines. Accurate analysis of the lower mass peaks of this spectrum is well-nigh impossible because of the complexity of the mixture. It is reassuring, however, to note the presence of a large peak at 384 mass units which may correspond to the diacetyldihydrosphingosyl fragment anticipated from a fission of the secondary amide linkage[54]. Only a small 382 peak is apparent, suggesting that dihydrosphingosine derivatives may be the major components of 'C'.

These hypotheses as to the structural formulae of the components of 'C' are supported by the degradative experiments carried out on the mixture. In essence, 'C' has been hydrolysed and the fragments correlated with known compounds.

The sphingolipids have been intensively studied and a great volume of experimental data is available on their identification. For the present purposes, 'C' was hydrolysed with methanolic sulphuric acid

by the method of Carter et al[55]. This treatment would convert any epoxide rings present to glycols. The fatty acid methyl esters produced were separated from basic (nitrogenous) material and subjected to periodate-permanganate oxidation[56] in order to cleave the chains into indentifiable acid fragments. Direct comparison of the methyl esters of these fragments with authentic compounds was easily achieved by a gas liquid chromatographic technique and the oxidation products of methyl linoleate were analysed in an identical manner to provide a standard for the analysis of 'C'.

The results show that the acids present after oxidation of 'C' conform to the pattern expected from the breakdown of an effective octadeca-9,12, 15-trienoic acid i.e. a linolenic acid.

As anticipated, methyl linoleate (Δ-9, 12-C_{18}) gave rise to methyl azeleate (C9), one of methyl malonate (C_3) and one of methyl caproate (C_6). Comparison of the traces obtained for the 'C' esters with those for the linoleate esters demonstrates that the acyl side chains of 'C' break down to give one mole of methyl azeleate (C_9) and

two of methyl malonate (C_3). Only a trace of methyl caproate (C_6) is present and there is no methyl caprylate (C_8).

It was not possible to analyse the 'C' esters for methyl propionate at that time and thus the overall chain length of the acyl side chain was not proved.

However, the breakdown products that have been found prove that the overall double bond pattern in this side chain is Δ-9, 12, 15 and this is precisely the pattern found in the ubiquitous C_{18}-trienoic acids and their derivatives. In particular, this is the pattern found for the C_{18} acids of the yeasts and the ascomycete and basidiomycete fungi[45]. A recent study of the fungal acids by Shaw has demonstrated that octadeca-9,12,-15-trienoic acid (Linolenic acid) is the characteristic fatty acid of various species of Penicillium[57].

The fact that C6 and C8 mono acids are practically nonexistent in the 'C' acyl side-chain breakdown products greatly strengthens the previous assignment of the 645, 647 and 661 mass peaks to the dihydrosphingosine series since the acylsphingosines of the same molecular weight would give rise

to these acids on oxidative cleavage. The trace of C_6 acid observed may be due to the 647 and the 679 mass dihydrosphingosines.

The analysis of the free nitrogenous base produced by the hydrolysis of 'C' indicates that 'C' is essentially a mixture of dihydrosphingolipids and thus that the acyl side chains are eighteen carbon units long. Direct comparison of the free base with commercially available sphingosine has been possible by both spectroscopic and chromatographic means. The infra-red spectra of sphingosine and its triacetate are similar but not identical to those of the 'C' free base and its triacetyl derivative respectively. As already stated, the spectrum of diacetyl- 'C' is comparable to that of triacetyl sphingosine.

A tentative correlation of the major free base with dihydrosphingosine has been obtained by TLC since the recent work of Sambasivarao and McCluer[58] has established a convenient procedure for the preparation of sphingosine bases from lipids and also TLC conditions for their identification, (Kieselgel G, Eluent $CHCl_3$: CH_3OH: 2NNH_4OH-40:10:1). The material from 'C' gave rise to one spot Rf 0.21

with a positive pink colour reaction with ninhydrin-pyridine-n-butanol spray, characteristic of all the sphingosine bases. An additional substance present with Rf 0.57 gave a brown colour with this spray after heating and may be due to residual hydroxyacid esters. Commercially obtained sphingosine had an Rf value of 0.38 in this system and, although the ratio of the Rf values of sphingosine and its dihydro derivative has been quoted as equal to 1.31[58] and the equivalent value for sphingosine and the major free base from 'C' is 1.81, the possibility exists that the major base from 'C' is in fact dihydrosphingosine, especially since structural alterations of this class of compound during isolation are well documented.

This evidence that P.puberulum is capable of producing ceramides with C_{18} unsaturated acyl functions is of great phylogenetic interest. Lipids containing these bases have apparently been reported solely from animal nervous tissue and only phytosphingosine has been found in microorganisms[46]. Furthermore, the C_{18} side chain possesses unsaturation

and oxidation patterns hitherto observed in the fatty acids of plants[45]. In fact, neither di- epoxy- nor triepoxy-fatty acids have been previously isolated from natural sources.

Thus the components of this fungal product are molecules one half of which is 'characteristic' of animal tissue and the other of plants! The implications of this fact, if proven, are of such interest that a second large culture of P.puberulum was carried out in an effort to obtain more '^{13}C'. The growing conditions were slightly different from the previous culture and, to our chagrin, only a trace of 3-O-methylviridicatin and no sphingolipid could be isolated from the fungal culture by the established techniques. It is possible that the sphingolipids were present in a different form, not isolable by the methods previously employed.

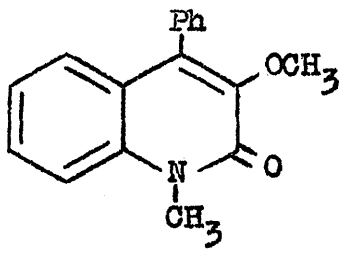
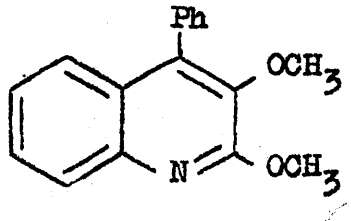
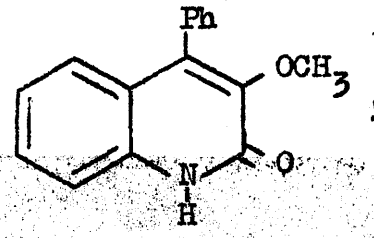
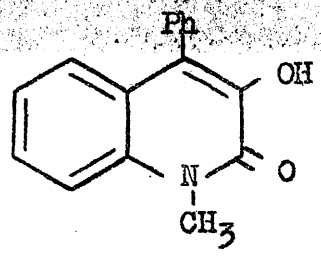
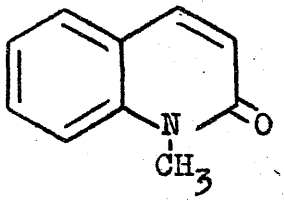
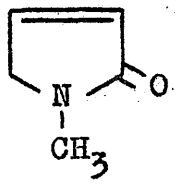
No further metabolites of interest were isolated from P.puberulum Bainier in the course of this work, but two further projects have been carried out on topics arising from this investigation of fungal metabolites.

1.2.3.a. The Synthesis of Viridicatol.

The reason for undertaking this synthesis was that the structure allotted to viridicatol (20), R=H, by Birkinshaw and his co-workers[31] contains the extremely rare meta-hydroxylated phenylpropanoid system. Proof of this structure seemed of interest in view of the possible biosynthetic implications. Thus - is the hydroxyl group introduced early or late in the sequence? Is it perhaps the metabolite of a 3,4-dihydroxylated viridicatin? These questions have not yet been answered.

In association with Mr. C.M. Stewart, a fourth year student, 3'-methoxyviridicatin (20), R=CH_3, was prepared from meta-methoxy-benzaldehyde by a route precisely analogous to that used by Eistert and Selzer for their synthesis of viridicatin[34]. The methyl ether so produced was refluxed with 45% aqueous hydriodic acid for two hours and the product extracted with ether. It was sublimed and crystallised to give a low yield of 3'-hydroxyviridicatin. This was later shown to be identical to natural and synthetic viridicatol, the generous gift of Dr. M. Luckner of Halle.

NMR Spectra of Viridioatin Derivatives and other Cyclic Amides.

Compound.		$CDCl_3$.	$(CH_3)_2CO$.	D_6DMSO.	CF_3CO_2H.	C_6H_6.	$\Delta\nu$.	Assignment.
	a.	5.78,	6.18	6.23,	5.53,	6.66,	+0.88	$N-CH_3$.
		6.32	6.22	6.29	6.34	6.22	-0.10	O_3-CH_3.
	b.	6.16,	5.84,	5.86,	5.27,	6.50,	+0.34	O_2-CH_3.
		6.21	6.31	6.36	6.18	5.96	-0.25	O_3-CH_3.
	c.	6.13	6.20	6.27	6.35	6.12	-0.01	O_3-CH_3.
	d.	6.13	6.12	6.20	5.57	6.75	+0.62	$N-CH_3$.
3'-O-methyl-viridicatol. (20), R=CH_3	e.	insol.	insol.	6.15	5.88	insol.	-	O_3,$-CH_3$.
	f.	6.29	6.30	6.34	5.67	6.76	+0.47	$N-CH_3$.
	g.	7.15	7.24	7.24	6.83	7.45	+0.30	$N-CH_3$.

All spectra were obtained using ca. 0.25 M solutions. Each methyl peak appeared as a sharp singlet. The aromatic protons are not detailed.

The synthesis of viridicatol that has been reported since the completion of this work[32] employed the same reaction scheme.

1.2.3.b. Nuclear Magnetic Resonance (NMR) Studies of Methylated Viridicatins.

The NMR spectra of the viridicatins are uninformative at first sight since they consist merely of an ill-resolved group of peaks due to the aromatic protons in the region τ 1.5-3.5. and one or more sharp singlets representing the methyl resonances. However this simplicity is deceptive, since a detailed examination of the variation in the positions of the methyl signals with change of solvent reveals evidence for interesting solvent-solute interactions. In addition to the five methylated viridicatins, a-e N-methyl-2-quinolone f and N-methyl-2-pyrrolidone g were studied in this connection. The author is sincerely grateful to Mr. D.D. MacNicol for his practical advice and for valuable discussions.

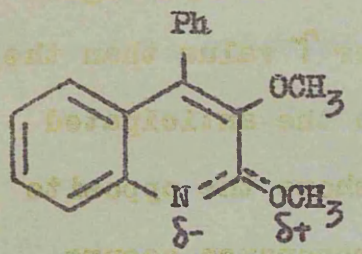
The table shows the results obtained using five solvents and includes assignments of the peaks in the dimethylated members of the series. In this

connection, it is noteworthy that a is closely approximated by a summation of the resonance values for the pairs of spectra c-d and c-f. The assignments for b are based on those for a as well as the comparison of the pair b-c. The peak τ-value shifts between solvents are entirely self-consistent for all these compounds. In general terms, the signals assigned to the 3-O-methyl groups in the spectra of a, b, c, e, lie in the range τ5.88-6.36. This is not significantly different from the usual range found for phenolic methyl ethers[42,60] and analysis of the individual spectra shows that the 3-O-methyl signals are not subject to large variations with change in solvent. However, those from the N-methyl functions in the spectra of a, d, f, g are in the range τ5.53-6.76 and analysis of the shifts for the individual compounds demonstrates that large characteristic solvent variations do occur. The normal region for cyclic tertiary N-methyl group signals is ca. τ7.8[42] and there seems little reason to doubt that the presence of a carbonyl oxygen function adjacent to the N-methyl causes a strong deshielding of the methyl protons. However, it is of interest to note that the methyl resonance

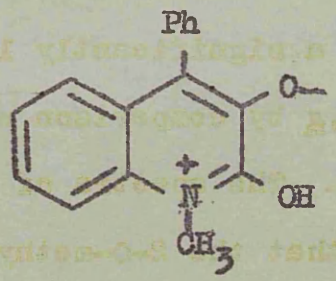
of N-methyl-2-pyrrolidone g occurs in the range τ6.83-7.45, permitting the suggestion that the 'styryl' group round the amide function of the other N-methyl amides (a,d,f) is responsible for the greater part of the observed deshielding effect. The 'N-phenyl' substituent in these amides is ideally situated to cause a large deshielding of any other groups attached to the nitrogen. Johnson and Bovey[61] have carried out valuable theoretical studies on this topic and it appears that such a deshielding effect may well account for a large part of the observed difference beteeen the spectra of g and a,d,f.

The spectrum of b is of interest since, although the 3-O-methyl group resonates in the 'normal' range, τ5.96-6.36, the 2-O-methyl group signals are in the range τ5.27-6.50 and the solvent variations for this methyl group are comparable to those for the N-methyl groups in this series. This will be discussed further.

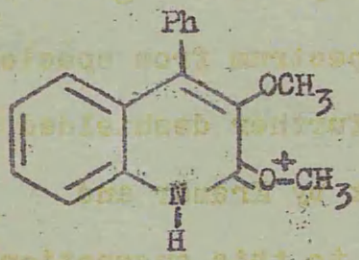
The spectra of these compounds in deutero-chloroform, acetone and hexadeuterodimethylsulph-oxide show only small overall solvent variations. It is of interest that the N- and O-methyl signals



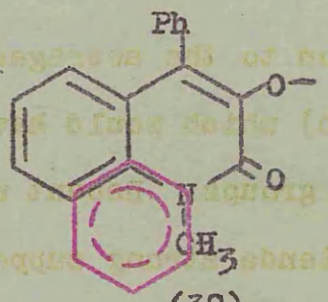
(35)



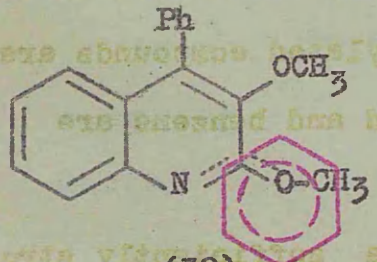
(36)



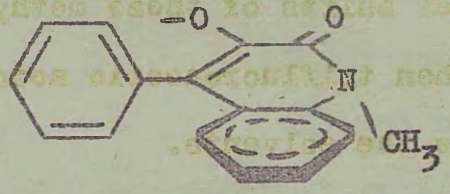
(37)



(38)



(39)



(40)

of a more widely separated in the first solvent than in the other two and in fact the N-methyl signal of a is at a significantly lower τ value than the others d,f,g by comparison with the anticipated τ6.2 value. The spectra of b shows the opposite effect in that the 2-O-methyl resonance occurs at lower field in acetone and hexadeuterodimethylsulphoxide suggesting that an increase in the dielectric constant of the solvent permits a greater contribution to the averaged spectrum from species such as (35) which would have further deshielded 2-O-methyl groups. Recent work by Kramer and Gompper[62] lends strong support to this suggestion on the basis of their studies on β-dimethylaminoacrolein and related compounds.

By far the most interesting variations in the chemical shifts of these methylated compounds are seen when trifluoroacetic acid and benzene are used as the solvents.

Trifluoroacetic acid is a sufficiently strong organic acid[63] to cause protonation of the amide carbonyl groups of compounds a,c,d,e,f,g. This general statement is supported by the wide range of

evidence available from previous study of analogous systems by NMR[64,65,65a] and by IR[66] spectroscopic techniques. The small 3-O-methyl peak variation between deuterochloroform and trifluoroacetic acid for a, b and c demonstrates that protonation of these compounds has little effect on the overall environment of the 3-O-methyl functions. However, this protonation has caused a strong deshielding to the N-methyl groups of a, d, f,g which may be attributed to the greater contribution to the averaged spectra of species such as (36) in which the nitrogen atom carries a formal positive charge, leading to a paramagnetic shift of the N-methyl signals in each case. Once again, the spectrum of b is of interest since protonation would be expected to be directed to the nitrogen. This will permit the formation of species such as (37) in which the 2-O-methyl group will experience a strong paramagnetic shift.

Recent studies of the effects of aromatic solvents on the signals due to methyl groups in amides[67], aromatic aldehydes[68], mesityl oxide[69], steroids[44,70] and alicylic ketones[71], coupled with

the interest attached to the suggestion of solvent-solute 'collision complexes' to explain the observed variations, prompted the examination of the NMR spectra of the methylated compounds in benzene solution. One criterion of such complexes is a significant difference between the τ values for a group in deuterochloroform and benzene solutions. These are tabulated for each assigned methyl resonance as shown: $C_6H_6 - CHCl_3 = \Delta\tau$.

It is possible to account for the effects of the collision complex in terms of the alteration in the magnetic field around the solute molecules by the solvent. Although this type of field alteration may well account for certain of the shifts observed for other solvents, the very strong anisotropy of benzene gives rise to particularly marked shifts. Possible models for this type of complex at the molecular level have been described in 44,67,71.

In a study of the spectrum of N,N-dimethylformamide in various solvents[67], it has been proposed that the amide molecule associates with the molecule of benzene so that the nitrogen atom with its fractional positive charge (due to mesomerism

of the amide function) is situated close to the region of high π-electron density in the aromatic ring. The negatively charged carbonyl oxygen atom is as far away from the centre of the benzene ring as possible. Application of these principles to the available series suggests that the collision complexes may have preferred orientations such as (38). This readily accounts for the observed strong shielding of the N-methyl groups in this series and also for the small interference with the positions of the 3-O-methyl signals since the N-methyl group is oriented below the benzene ring while the 3-O-methyl is removed from its direct influence e.g. in a.

Compound b, as the only true quinoline examined, is again unusual in its behaviour, and the 2- and 3-O-methyl groups show respectively a strong diamagnetic shift (+0.34τ) and a strong paramagnetic shift (-0.25τ). This may indicate that the collision complex has a structure such as (39), in which the benzene ring has associated with the fractional positive charge on the 2-oxygen and is in such an orientation that it simultaneously shields the 2-O-methyl and deshields the

3-O-methyl group. This is based on the work of Johnson and Bovey[61] previously described, since they were able to show that the benzene ring has a strong shielding zone above and below it and a deshielding 'equatorial' zone.

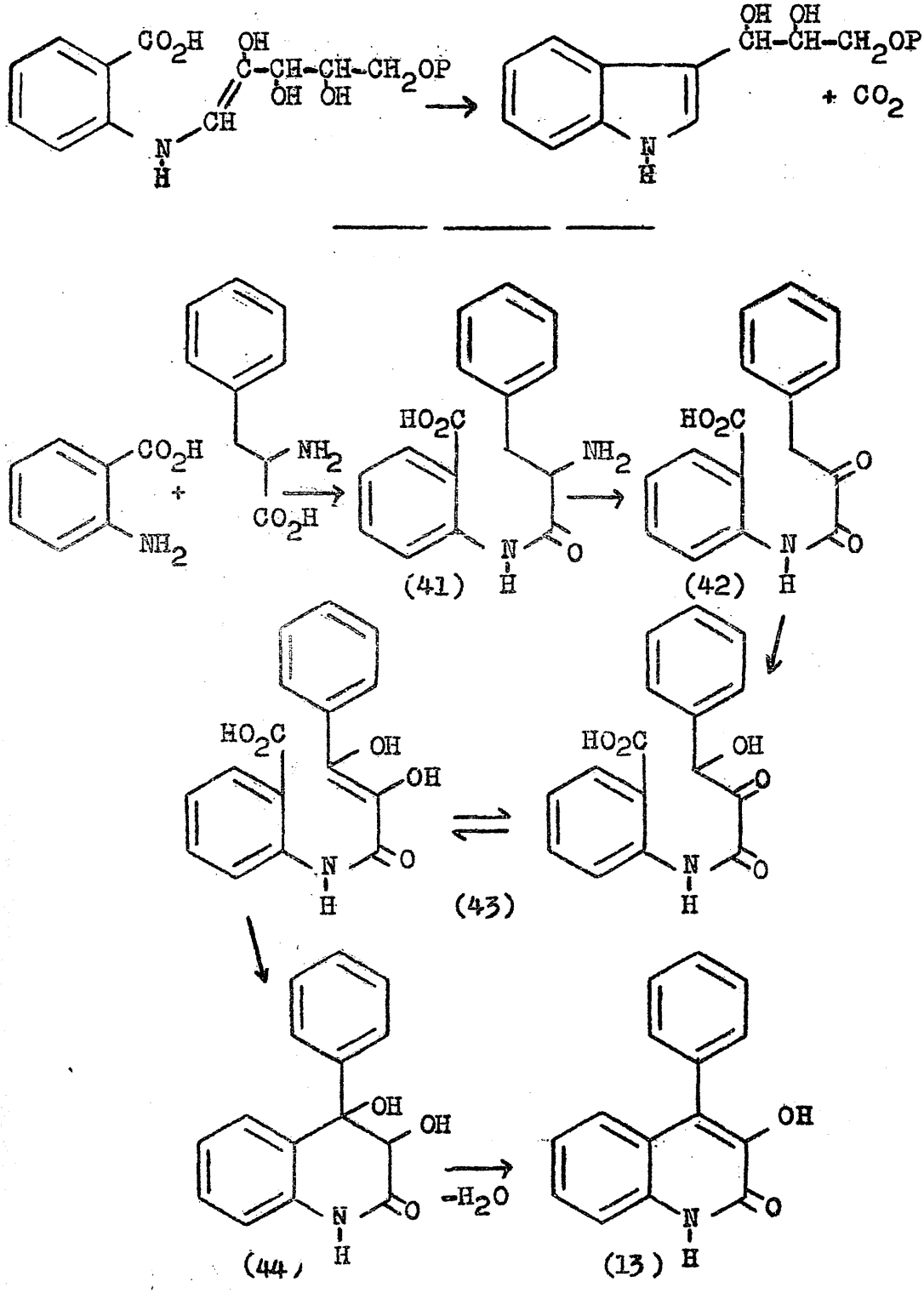
In this connection, it may be significant that the N-methyl group of compounds a and d resonate at τ values consistently lower than that of N-methyl-2-quinolone f in all five solvents. The average difference is ca. 0.12τ (neglecting the anomalous τ value for the N-methyl of a in $CDCl_3$). Since the presence of the 3-oxygen function in all the viridicatin derivatives will tend to restrict the rotation of the phenyl group at the 4-position, the situation can be visualised as in the diagram (40). Making the basic assumption that the N-methyl group is quasi-axial to the amide ring, an assumption supported by recent studies on analogous systems[71,72], the anticipated deshielding of the N-methyl group by the 4-phenyl can be calculated as ca. 0.17τ from a consideration of the ring-methyl distance[61] as judged from Dreiding models. In the quasi-axial conformation, the ring-methyl

distance is ca. 5.58 Å, while in the alternative quasi-planar conformation this is increased to ca. 6.93 Å. By calculation, the N-methyl group in the quasi-planar conformation would only be deshielded to a very small extent. The similarity of the observed and theoretical shifts implies that the N-methyl groups in compounds a and d may in fact be quasi-axial and that the theoretical long-range deshielding may be operative.

The satisfying qualitative explanation of the solvent-solute interactions observed for this series permits the suggestion that similar studies on other series of compounds will prove of value.

1.3a. The Biosynthesis of Quinoline Derivatives.

One of the reasons for undertaking the study of the metabolites of Penicillium puberulum Bainier was that the biosynthesis of the naturally-occurring quinolines and their derivatives had not been examined in great detail. It was therefore hoped to carry out an examination of the biosynthesis of the viridicatin 3-hydroxy-4-phenyl-2-quinolone system. This was however anticipated by the work of Luckner and Mothes[29,30,33] who have used a strain of P.viridicatum as the subject for radio-tracer experiments. Their overall results[30] may be best explained by the diagram. They found that tritium-labelled anthranilic acid was incorporated into viridicatin to a moderate extent but that carboxyl-^{14}C- labelled anthranilic acid was a very poor precursor. Successive experiments with generally- and specifically- ^{14}C-labelled phenylalanine demonstrated that phenylalanine was incorporated into the viridicatin skeleton en bloc, it is therefore clear that the carboxyl group of anthranilic acid is lost in the course of the biosynthetic sequence. To this extent, the biosynthetic route to viridicatin



The postulated biosynthesis of viridicatin - Luckner and Mothes[30].

parallels that to the indole ring system of tryptophan[74] since, in the formation of tryptophan, the cyclisation of the intermediate anthranilic-1-deoxyribonuncleotide to indole-3-glycerol phosphate is accompanied by the elimination of the carboxyl group ultimately derived from anthranilic acid - see the diagram.

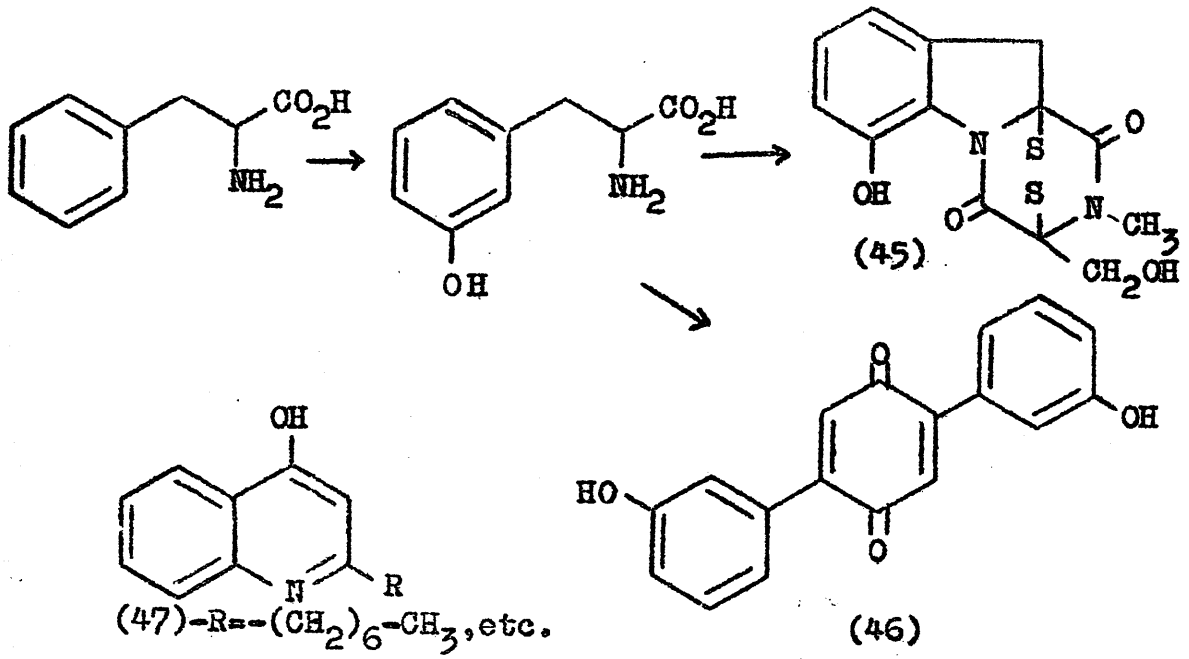
Luckner and Mothes[30] have suggested that the first step in the formation of viridicatin is the production of phenylalanine anthranilide (41) followed by a transamination step to give phenyl-pyruvic anthranilide (42). They further propose the benzylic oxidation of the phenylpyruvic side chain resulting in an ene-diol form (43). By analogy with the known pathway to the indole ring system of tryptophan, the next stage may involve the loss of the carboxyl group and cyclisation to a compound such as (44). Loss of water from this will produce viridicatin. However, other routes from phenylpyruvic anthranilide can be visualised. Thus, merely by enolisation of the 2-carbonyl function and cyclisation of the open structure with loss of carbon dioxide, it is possible to arrive at

3,4-dihydroviridicatin. Oxidation of this compound rather than the initial anthranilide can therefore give rise to viridicatin. Alternatively, the formation of phenylpyruvic acid as such may be the first step. The detailed sequence remains obscure.

The place of cyclopenin in this scheme is uncertain. The fact that it is readily transformed to viridicatin by treatment with dilute acid[28] suggests the possibility that it is a precursor for viridicatin rather than the reverse. This idea is supported by the observation[33] that an enzyme obtained from the mycelium of P. viridicatum converts cyclopenin, with proposed formula (21), to viridicatin with the evolution of carbon dioxide. On the basis of previous work, methylamine would also be an expected product of this transformation.

What bearing does the isolation of viridicatin and 3-O-methyl viridicatin from P. puberulum in the present study have on these schemes? While the trace component (Rf 0.26) observed in the extracts of the culture filtrate of P. puberulum could well have been cyclo-penin or an analogous compound, it is difficult to reconcile the proposed structures

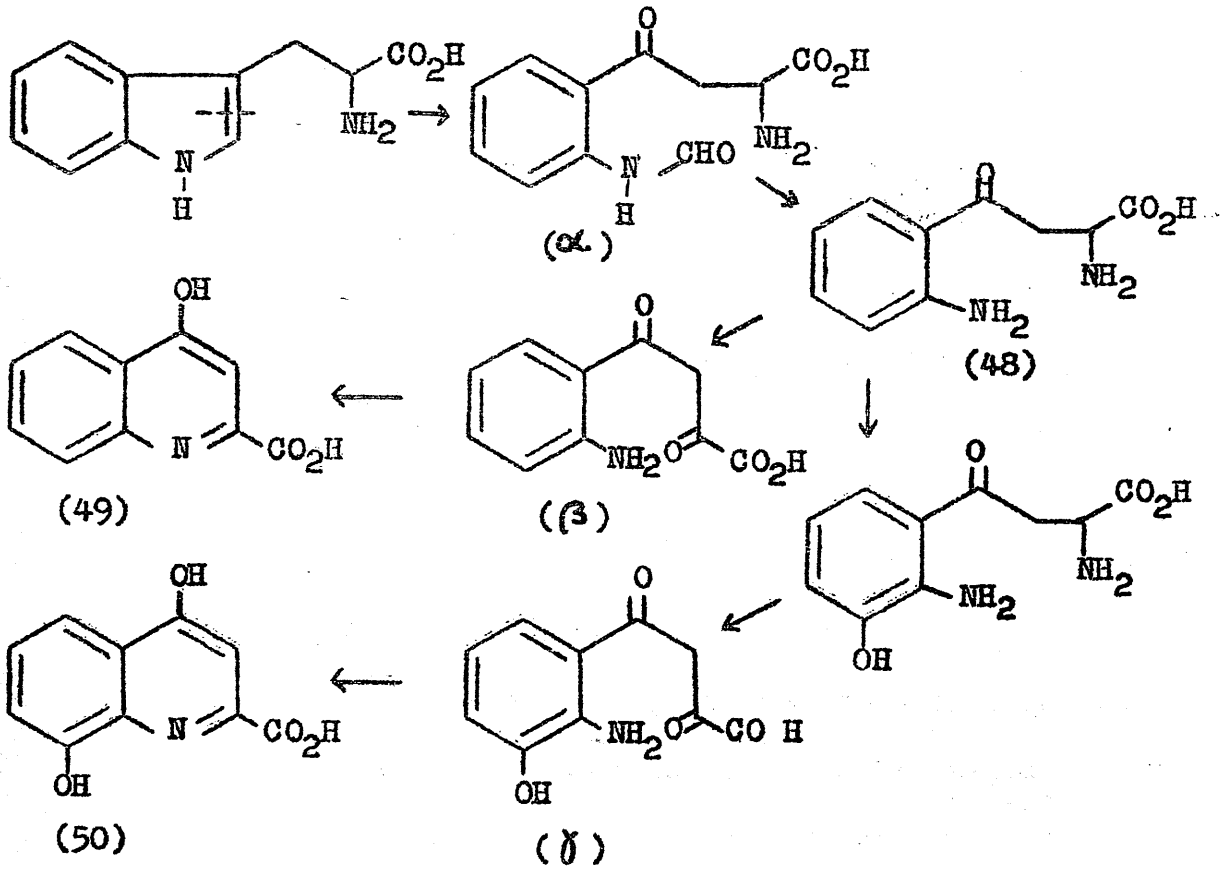
for cyclopenin with a feasible precursor for 3-O-methylviridicatin. Alternative solutions to this problem exist. 3-O-Methylviridicatin may be formed by a direct methylation of the viridicatin nucleus, an interesting possibility in view of the extreme ease of dimethylation found in the chemical synthetic studies. Alternatively, the precursor for this compound may be a methylated cyclopenin in which the oxygen function that appears as the 3-hydroxyl group in viridicatin carries a methyl substituent. Because biosynthetic studies on 3-O-methylviridicatin have not yet been carried out and since the precise structure of cyclopenin remains in doubt, a distinction between these alternatives is not possible. An unusual fact is the existence of meta-hydroxylated analogues of viridicatin and cyclopenin. They may arise by direct hydroxylation of the compounds, by dehydroxylation of a 3,4-dihydroxylated precursor or by the formation of a meta-hydroxylated precursor at an early stage in the overall pathway. By analogy with the known route[75] to the fungal metabolite gliotoxin (45), the last hypothesis is preferred since the efficient



(45)

(47)-R=-$(CH_2)_6$-CH_3, etc.

(46)



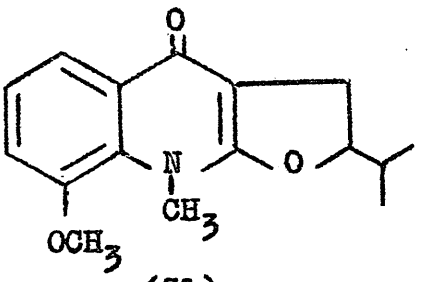
(49)

(50)

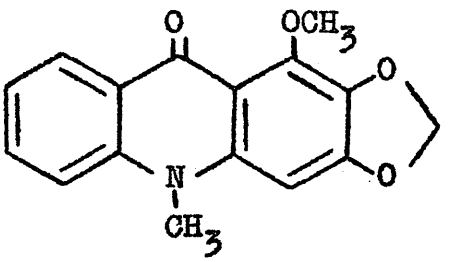
incorporation of tritium-labelled m-tyrosine into this compound, coupled with the results of feedings of phenylalanine and serine, demonstrates that meta-hydroxylation of a phenylpropanoid precursor is part of the operating sequence[95]. This is also the case in the sequence leading to volucrisporin (46).[96]

The work of Luckner and Ritter[97] on the 2-n-alkyl-4-hydroxyquinolines (47), (the so-called 'pyo compounds'), produced by strains of Pseudomonas aeruginosa has demonstrated that, in contrast to the pathways to tryptophan and viridicatin, the carboxyl group of the anthranilic precursor is retained in the biosynthesis of these compounds.

Yet another pathway to the quinoline system is known. In the biosynthesis of kynurenic and xanthurenic acids from tryptophan[78, 79], the indole ring of tryptophan is cleaved to give formylkynurenin (α). Loss of this formyl group yields kynurenin (48) and subsequent transamination of the α-amino-acid yields the postulated intermediate (β), which cyclises spontaneously with the elimination of the elements of water to produce kynurenic acid (49). Hydroxylation of kynurenin, followed by a parallel



(51)



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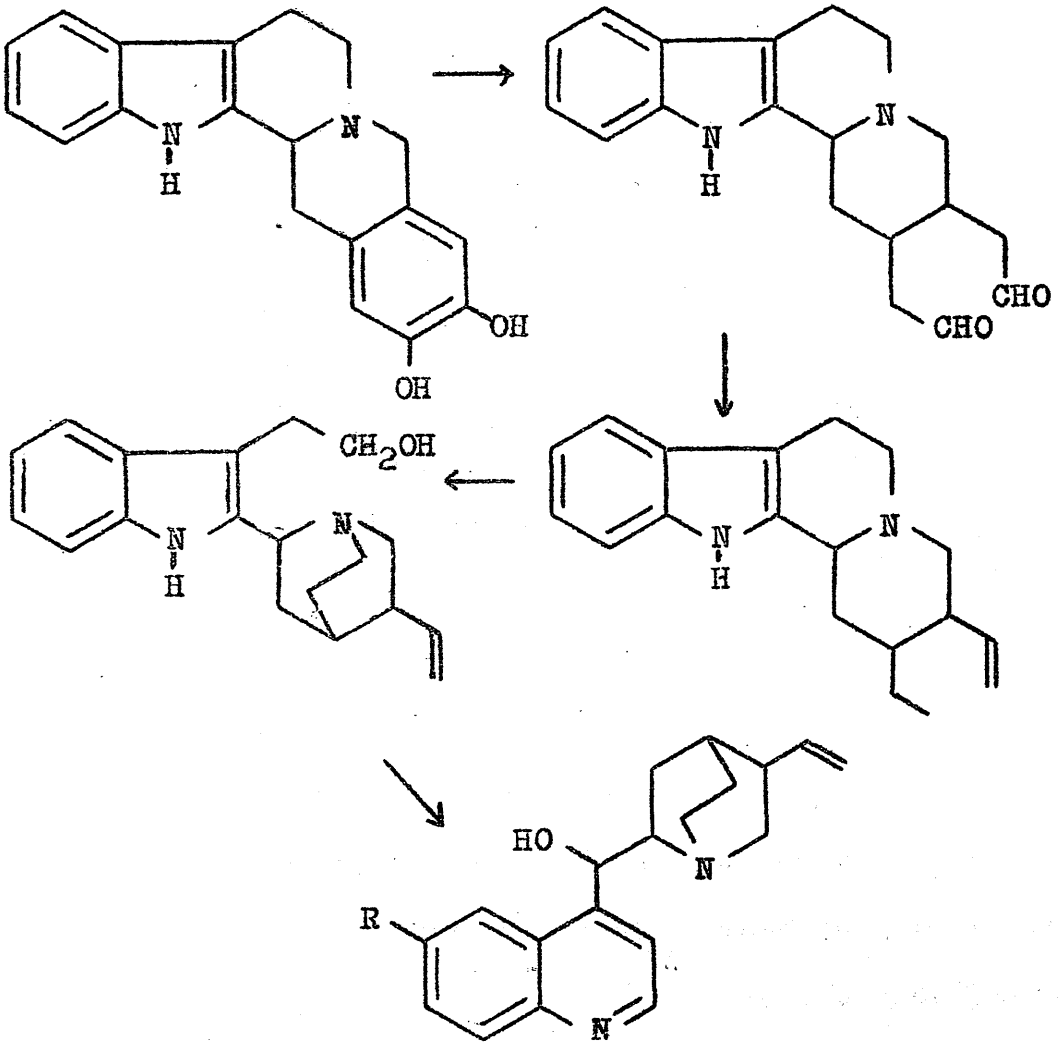
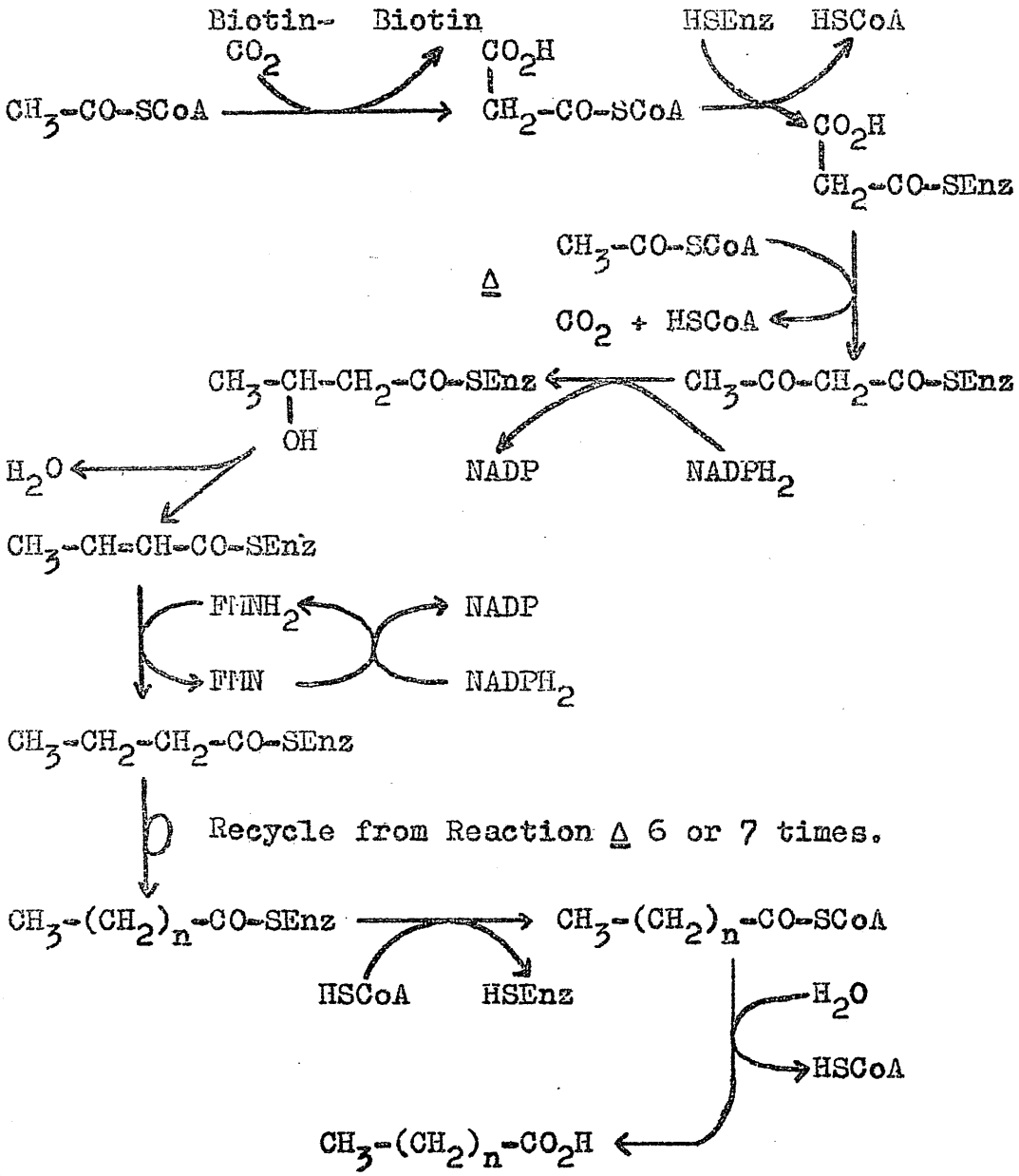


Cinchonine, R =H; Quinine, R =OCH_3.

transamination and dehydration, yield xanthurenic acid (50).

Hypothetical schemes for the formation of the Lunasia, Dictamnine and Acridine groups of alkaloids[80] have been proposed by Leete[81]. Implicit in these schemes is the condensation of 2-carbon units onto the carboxyl group of anthranilic acid followed by cyclisation to form the oxygenated quinoline ring system. Simultaneous or subsequent condensations with isopentenyl or cinnamic acid units, accompanied by further elaborations of the products, may give rise to characteristic members of these groups of alkaloids, e.g. lunacrine (51) and evoxanthine (52). A postulated formation of the Cinchona alkaloids from pentacyclic indoles as shown has been supported by tracer experiments (see[81a]).

In summary, it is clear that the naturally-occurring quinoline derivatives are of diverse origins. At present, the detailed steps in the biosynthesis of many of these compounds remain as ill-defined as their function in the living cell.



In the final products of this pathway, n is frequently equal to ~~fourteen~~ (palmitic acid) or sixteen (stearic acid).

1.3.b. The Biosynthesis of Sphingolipids.

In amplification of the previous discussion of possible routes to the ceramide sphingolipids isolated from P.puberulum, it is appropriate to set out here the current concepts on their formation.

The sphingolipids consist essentially of two aliphatic chains linked by a peptide bond and the subdivision of this class is governed by the nature of the substituent on the primary hydroxyl function. It seems most reasonable, therefore, to discuss their biosynthesis in terms of the known routes to the fatty acids.

In recent reviews on the biosynthesis of fatty acids[82] and lipids[47], the formation of the typical long-chain structures of the fatty acids by successive chain elongation has been described. A wealth of experimental evidence supports this pathway, which is shown in the diagram. The enzymes involved in the steps have been characterised from yeasts and pigeon liver tissue[82].

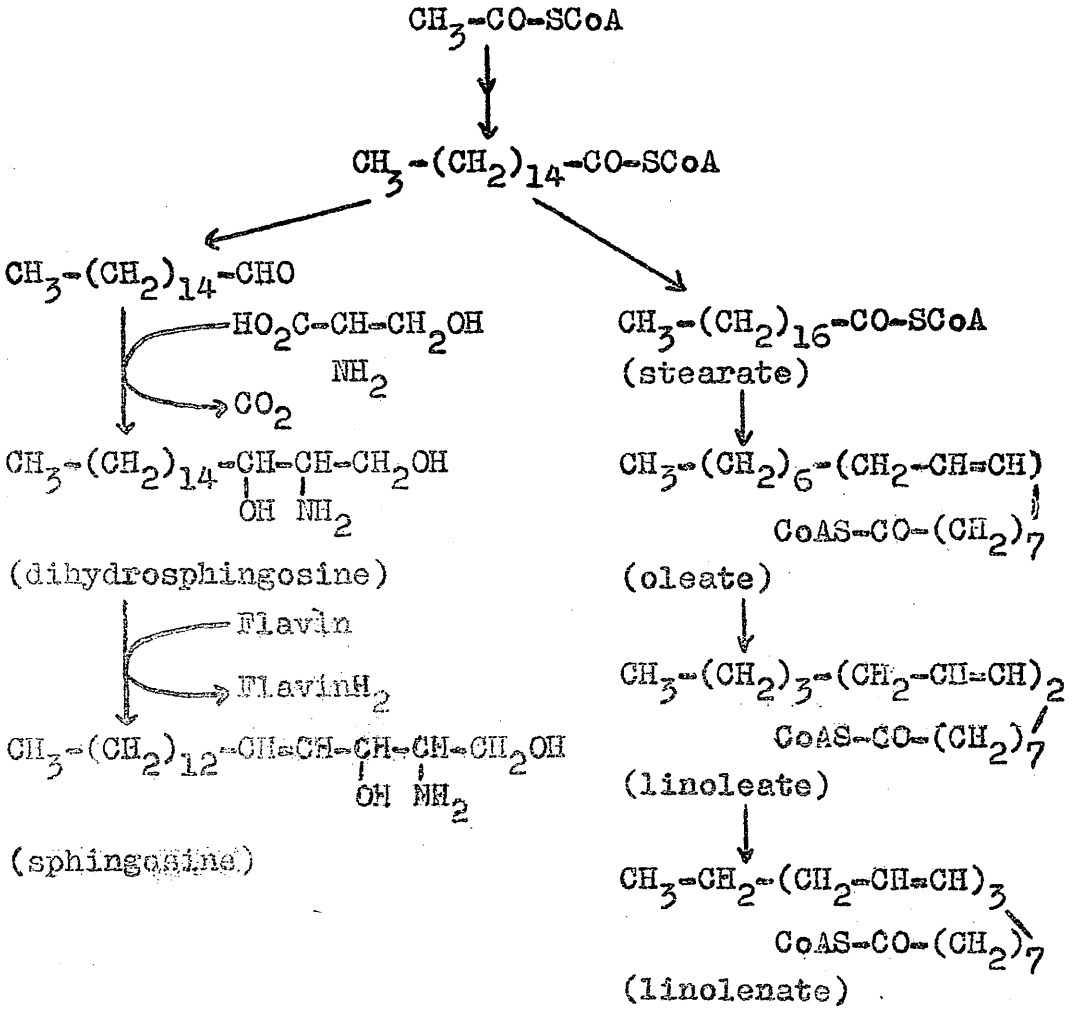
The first step is the carboxylation of acetyl -CoA by a biotin-dependent enzyme to form malonyl-CoA which then reacts with a thiol-enzyme. A

second unit of acetylCoA condenses with the malonyl-S-enzyme complex to give acetoacetyl-S-enzyme with a simultaneous loss of carbon dioxide. This decarboxylation is a driving force for the synthetic reaction and is not paralleled in the known scheme for the degradation of fatty acids. A NADPH-linked reduction of this β-dicarbonyl compound followed by elimination of water produces crotonyl-S-enzyme. A second NADPH-linked reduction step completes the formation of butyryl-S-enzyme which may then act as the starting material for a second cycle. In this manner, the chain elongation occurs by addition of two-carbon units to the terminal methyl group of the preformed chain.

Although it is acetylCoA that condenses with the malonyl-S-enzyme complex, the lack of short and intermediate chain-length acids found in the *in vitro* and *in vivo* systems supports Lynen's[83] view that these intermediate compounds in the sequence to the ubiquitous C_{16} and C_{18} acids are recycled as the acyl-S-enzyme complexes. This is an important point since, if the intermediates were recycled as the acylCoA derivatives, the enzymes capable of

hydrolysing these derivatives would be expected to act giving rise to the free short chain acids. In fact, Lynen has investigated the chain length specificity of the hydrolytic enzymes present by isotopic exchange methods and has found a marked optimum with acyl groups containing 16 or 18 carbon atoms[83]. This implies that the cycle operates until this chain length is reached and only then is free fatty acid released. This accounts in a most satisfying manner for the prevalence of palmitic (C_{16}) and stearic (C_{18}) acids. Bressler and Wakil[84] have put forward objections to some features of the scheme but not to its main outline.

At this point, the branching of the pathways leading to the two building blocks of the sphingolipids occurs. The palmitylCoA formed in the cycle shown above can add on two further carbon atoms to give a stearyl (C_{18}) derivative, which would eventually form the acyl side chain of, for example, the sphingolipids found in the mycelium of P.puberulum. Another reaction scheme, well reviewed by Strickland[47], starting from palmitylCoA is responsible for the production of the



The apparent branching point in the formation of the basic units of the sphingolipids is the elaboration of palmityl-CoA. It is of interest to note that the C_{18} compounds dihydrosphingosine and stearate are both produced by the condensation of a C_{16} CoA derivative with a C_3 acid, with the loss of CO_2 from the C_3 moiety.

sphingosine unit. In this scheme, which is supported by *in vivo* and *in vitro* radiotracer studies on brain tissue, palmitylCoA is first reduced to the aldehyde which then condenses with three carbon amino-acid serine. The decarboxylation which takes place in this reaction is a driving force for the synthetic reaction analogous to that operative in the synthesis of the fatty acids. The product of this condensation is dihydrosphingosine which is oxidised to sphingosine by a flavin-coupled enzyme. It is therefore clear that the formation of *dihydro*sphingosine precedes that of sphingosine and therefore the presence of the dihydro derivatives in the lipids of *P. puberulum* is readily explainable.

Although it has been stated[47] that 'it is probable that the latter reaction (reduction) occurs prior to any substitution on sphingosine', this is by no means certain and it is possible that the oxidation of the dihydro derivatives takes place at the ceramide (acylated) stage. Equally, the point at which the acylation occurs has not been established by biochemical experiments and

it may be of general significance that the known pathway to the cerebrosides (30) has been shown to involve acylation only as the last step, subsequent on the formation of psychosine (1-galactosylsphingosine).

In view of these ambiguities, it may be suggested that the isolation of compounds closely resembling dihydrosphingolipids as well as sphingolipids from the mycelium of P. puberulum provides circumstantial evidence that the acylation of dihydrosphingosine is a feasible process. A consideration of the fundamental differences in the acyl side chains found in the two series suggests that the oxidation of dihydroceramides to the ceramides is an unfavourable process.

Erwin and Bloch[85] have summarised the present state of knowledge on the biosynthesis of unsaturated fatty acids in micro-organisms. They conclude, on the basis of extremely sophisticated evaluations of the known pathways in a wide series of 'lower' organisms, that 'oxidative desaturation' is the most common pathway for the biosynthesis of the mono-unsaturated fatty acids. By this process

is implied the removal of hydrogen from the C_9C_{10} bond of a saturated C_{16} (palmitic) or C_{18} (stearic) acid, with the resulting formation of palmitoleic and oleic acids. This is the mechanism observed in the yeast and fungal species so far studied and is not identical with the mechanism employed by the higher plants, in which the carbon chains of laurate (C12) and myristate (C14) are elongated and transformed to oleate in some unknown manner. Palmitate and stearate are not precursors for this process.

The production of poly-unsaturated fatty acids apparently proceeds in two ways. The Δ^9 compound, e.g. oleate, may be progressively desaturated towards the terminal methyl group of the chain, giving rise to $\Delta^{9,12}$ (linoleic acid) and then $\Delta^{9,12,15}$ (α-linolenic acid). This is described as the "plant" pathway by Erwin and Bloch. In the "animal" pathway that they distinguish, linoleate is again the first desaturation product but the subsequent desaturations occur towards the carboxyl end of the chain, producing $\Delta^{6,9,12}$ - γ-linolenic acid. A recent study of the distri-

bution of the α and γ-linolenic acids in fungi[57] provides strong evidence that γ-linolenic acid is a product characteristic of the Phycomycetes, a relatively primitive fungal group. The Basidiomycetes and Ascomycetes (with which P.puberulum is classified) produce exclusively α-linolenic acid. With this in mind, it is not surprising that the degradative studies on the triene-derived acyl side chains of the sphingolipids from P.puberulum demonstrate that the possess the $\Delta^{9,12,15}$ system.

There seems little reason to doubt that epoxy-fatty acids are produced by oxidation of the olefinic precursors[86]. Since the acyl substituents of the isolated lipids are: Δ^{9}(M.W.665), 2 $\Delta^{9,12}$(M.W.647 and 679) and 6 $\Delta^{9,12,15}$(M.W.645, 659, 661, 675, 691 and693), it is most satisfying to find that the proposed structures may be accomodated into a reasonable overall scheme. In this scheme, dihydrosphingosine is produced by the condensation of palmitaldehyde and serine, and is acylated by the mono, di and trienoic epoxy - C_{18}-acids. Inherent in this pathway is the suggestion that the epoxidation, as well as the desaturation pattern of the acids is fixed before

the acylation of dihydrosphingosine. The alternative epoxidation after acylation, cannot be excluded.

The fact that the true sphingolipids found are the successive epoxidation products of a presumed linolenylsphingosine precursor permits the proposal that they are in fact so derived. The differences between the acyl groups of the sphingosines and the dihydrosphingosines constitute circumstantial evidence against the desaturation of acyldihydrosphingosines as the last step in the formation of the sphingolipids. The most likely route therefore involves desaturation of the basic C_{18} unit prior to acylation.

It must be stated at once that these are essentially speculative relationships. Proof of any of them would demand a fuller study of the chemical structures of these compounds together with detailed examination of the steps involved in their biosynthesis.

EXPERIMENTAL.

General.

Melting points were determined on a Kofler block and are uncorrected. Routine infra-red spectra were recorded on Perkin-Elmer 137B and 237 and on Unicam SP200 spectrophotometers as Nujol mulls. Reference IR spectra were obtained with a Unicam SP100 instrument on KCl discs. Ultra-violet spectra were recorded on Perkin-Elmer 137 and Unicam SP800 instruments as methanolic solutions. Nuclear Magnetic Resonance (NMR) spectra were obtained with $SiMe_4$ as an internal standard on a Perkin-Elmer R10 60 megacycle instrument.

The mass spectrum of diacetyl-'C' was recorded on an A.E.I.MS9 instrument.

Gas-liquid chromatographic analysis of certain coumarins was carried out on a Pye 'Argon' Chromatograph equipped with a ^{90}Sr detector.

Thin-layer Chromatographic analyses were carried out on glass plates coated with 0.25 mm thick layers of 'Kieselgel G'. Preparative TLC was carried out with 0.5 or 1 mm thick layers. Whatman Cellulose Powder CC41 was also used. Paper-chromatographic analysis was carried out by the ascending technique using Whatman no.I paper.

Part 1.4. Experimental.

In the following sections, the page numbers given before each description refer to the use of the data obtained in the Results and Discussion (1.2).

P.12. Extraction of the Culture Filtrate.

Accurate weights of the acetone-soluble material from the charcoal and the successive extracts from the partition are not available because of the presence of larger or smaller amounts of solvent in each.

The petroleum-ether soluble portion from the trituration of the organic phase residue weighed ca. 2.5 gm. It was taken up in 100 ml. of petroleum ether (60-80°) and reduced to 50 ml bulk. After the addition of 50 ml of ether, the solution was kept at 0°C for three weeks and two crops of colourless prismatic crystals removed. The first crop contained a trace of viridicatin and a more polar material but the second was pure 'A'. In an effort to obtain more pure 'A', the the mother liquors were chromatographed over 80 gm of Grade H alumina, deactivated with 10% w/v of 10% aqueous acetic acid. A gradient elution was carried from petroleum ether to benzene to ether and the column was then stripped with methanol. This is detailed overleaf.

P.14. Chromatogram of Petroleum-Ether Solubles.

The eluate was taken from the column in 50 ml fractions.

Fraction.	Weight (mgm)	Solvent.	
1-8	489	Pet.ether (40-60)	
9-12	187	5% benzene	
13-16	230	10% "	
17-20	167	20% "	
21-27	240	50% "	
28-33	278	100% "	
34-35	44	2% ether	
36-37	35	5% "	
38-41	36	10% "	
42-47	166	20% "	- Compound 'B'
48-49	38	50% "	
50-55	190	100% "	- Compound 'A'
56-57	22	2% methanol	
58-63	263	100% "	

Fractions 51-55 contained 147 mgm of crude 'A'. When recrystallised three times from methanol, with charcoal treatment, and combined with the recrystallised first and second crops, a total of 158 mgm of pure 'A' was available. Its melting point was 248-9°.

Recovery from column = 1.935 gm (ca.78%).

P.15. Synthesis of viridicatin (13).

This compound was synthesised by the method of Eistert and Selzer[34], a scheme which involves the reaction of phenyldiazomethane with isatin.

A. Preparation of benzil monohydrazone.

Hydrazine hydrate (11.5 gm of an 85% aqueous solution) was slowly dropped into a hot solution of benzil (40 gm) in ethanol (80 ml) with stirring. The product began to separate from solution after the addition of some three-quarters of the reagent. The solution was heated under reflux for five minutes after the addition of all the reagent and then cooled to 0°C. The hydrazone was filtered off and washed with 2 x 100 ml portions of cold ethanol. The product m.p.148-50°(lit.149-51°) was a pure white powder and weighed 43 gm, corresponding to a yield of 100%.

B. Preparation of azibenzil.

Benzil monohydrazone (43 gm) was mixed in a mortar with 86 gm of yellow mercuric oxide and anhydrous sodium sulphate (21.5 gm). The mixture was placed in a 1-litre flask and covered with dry ether (300ml). The catalyst - 5 ml of a cold, saturated solution of potassium hydroxide in ethanol - was added and the mixture shaken for 30 minutes. The orange solution

was then filtered and the residue washed with several portions of ether until the extract was only slightly coloured. The solution was diluted with more ether until the calculated concentration of azibenzil was ca. 37 gm in 850 ml of ether and this was used for the next step without isolation of azibenzil.

C. Preparation of Phenyldiazomethane.

A solution of sodium hydroxide (56 gm) in water (110 ml) and methanol (700 ml) was added to the solution of azibenzil prepared as above and allowed to stand at room temperature for 8 hours. The filtered clear-red solution of phenyldiazomethane and sodium benzoate was treated with 700 ml of a 10% aqueous sodium hydroxide and the ethereal layer separated, washed with four further portions of sodium hydroxide solution and finally dried over anhydrous sodium sulphate.

D. Preparation of Viridicatin.

Isatin (10 gm) was placed in a large conical flask and the ethereal solution of phenyldiazomethane prepared in step C added and allowed to stand for three days during which the original deep-red colour was discharged and a large amount of yellowish crystals deposited on the bottom of the flask. These were filtered off and recrystallised from methanol after

treatment with charcoal. the yield was 8.45 gm of colourless prismatic crystals m.p.267-9° (lit.[34] m.p. 268-9°)

Found: C,75.78 ; H,4.94 ; N,6.19. $C_{15}H_{11}O_2N$ requires C,75.93 ; H,4.67 ; N,5.90.

The IR and UV spectra of this compound are shown in the Results and Discussion section 1.2.1 together with its chromatographic mobility, colour reaction and structure.

Preparation of O,N-dimethylviridicatin (17).

A mixture of viridicatin (0.5 gm) and dimethyl sulphate (2 ml) was heated under reflux in 50 ml of methanol. A total of 2 gm of sodium hydroxide pellets was added over a period of 1½ hours at the end of which the solution gave no green colour with ferric chloride, indicating the absence of unreacted viridicatin. The heating was stopped and 40 ml water added. The product crystallised as colourless plates and was filtered off and recrystallised twice from methanol to give the pure product (0.525 gm, 94%), m.p.195-7°. No trace of 3-O-methylviridicatin or indeed of any impurity was observed on TLC of this compound, whose literature melting point is 197-8°[28].

Preparation of O,O-dimethylviridicatin (16).

Viridicatin (1 gm) was heated under reflux in 25 ml of methanol with methyl iodide (4 ml) and black silver oxide (4 gm) for a period of 1½ hours. The solution was filtered and the solvent removed under vacuum. The yellow solid produced was triturated with hot petroleum ether (60-80°) and the soluble material was found to be crude O,O-dimethylviridicatin (0.753 gm) which was further purified by repeated crystallisation from methanol with charcoal treatment. The final yield was 0.593 gm (53%)m.p. 82-4° (lit.m.p.86-7° [28]).

Once again, no trace of 3-O-methylviridicatin could be detected. The product insoluble in petroleum ether (0.25 gm) proved to be almost entirely the O,N-dimethylated isomer.

P.15. The structural elucidation of 'A'.

Found: C,76.10 ; H,5.33 ; N,5.72. $C_{16}H_{13}O_2N$ requires C,76.47 ; H,5.22 ; N,5.57.

The IR and UV spectra of 'A', on which this structural elucidation was mainly based, are reproduced and fully discussed in the text. Its NMR spectrum, identical with that of synthetic 3-O-methylviridicatin, is included in the data presented in 1.2.3.b.

P.19. Attempted Preparation of 3-O-methylviridicatin.

The previous methylations of viridicatin had produced only di-substituted compounds. The method of Cunningham and Freeman[27] was therefore essayed.

Viridicatin (0.3 gm) was dissolved in 5 ml of 3N aqueous sodium hydroxide and the solution warmed on the steam bath for five minutes. The sodium salt was collected from the cooled solution and dried. It was dissolved in water (10 ml) and a slight excess of silver nitrate solution added. This resulted not in a yellow but in a black powder - presumably the silver salt of viridicatin - which was suspended in a mixture of methyl iodide(2 ml) and ethanol (20 ml) and refluxed for 2 hours. The suspension was filtered and the solvent removed under vacuum to yield a solid which was triturated with petroleum ether giving the soluble material as 0.218 gm of yellow solid.

This was chromatographed over deactivated alumina (9 gm). The fraction eluted with benzene contained 127 mgm of O,N-dimethylviridicatin, identified with authentic material while elution with pure ether gave 11 mgm of a crystalline material m.p.202-4° with a positive ferric chloride test and an IR spectrum consistent with a secondary amide, which was later

shown to be identical with that of N-methylviridicatin. No trace of 3-O-methylviridicatin was observed in this preparation, which was repeated twice. The main product was uniformly the O,N-dimethyl derivative.

P.20. Preparation of 3-O-methylviridicatin.

A solution of viridicatin (1 gm) and methyl toluene-p-sulphonate (0.90 gm) in dioxan (25 ml) was heated under reflux for 4 hours. The solvent was removed at the water pump and the residue dissolved in cold aqueous sodium hydroxide (50 ml). The solution was extracted with ether and chloroform and the combined extracts dried and evaporated yielding O,N-dimethylviridicatin (0.420 gm) m.p.195-7°(lit.[28] 197-8°). The alkaline aqueous phase was acidified with dilute hydrochloric acid and extracted with ether to give a crude material which, after recrystallisation from methanol, produced 3-O-methylviridicatin(0.060 gm) m.p. 247-9° undepressed by the naturally-occurring compound 'A'. Its TLC mobility, colour reaction and IR and UV spectra were identical with those of 'A' thus completing the structural elucidation.

The remaining material in the aqueous phase was unchanged viridicatin.

Microanalysis of the synthetic compound shows -

Found: C,76.50 ; H,5.14 ; N,5.67. $C_{16}H_{13}O_2N$ requires

C,76.47 ; H,5.22 ; N,5.57.

P.21. Compound 'B'.

This was obtained from fractions 42-47 of the main column chromatogram of the culture filtrate extracts.- p.69. The crude material was crystallised twice from an ether-petroleum ether mixture to give 93 mgm of colourless needles m.p.178-80°, apparently homogeneous as judged by TLC.

Found: C,78.56 ; H,10.00 ; N,1.56,1.48.

$C_{63}H_{97}O_6N$ requires

C,78.50 ; H,10.07 ; N,1.46.

Neglecting nitrogen, however, $C_{28}H_{42}O_3$ requires

C,78.82 ; H,9.92. and $C_{30}H_{46}O_3$ requires

C,79.24 ; H,10.20.

From the NMR spectrum of this compound in CCl_4 solution, 'B' is almost certainly a steroid-type.

P.23. Chromatogram of petroleum ether-soluble material from the mycelium.

The mixture (4.63 gm) was chromatographed over 150 gm of deactivated alumina with a gradient elution from petroleum ether to chloroform to 50% methanol. The eluate was taken in 100 ml fractions. The resulting separation is detailed overleaf.

Fraction.	Weight (mgm)	Solvent.
1-4	337	Pet.ether (40-60)
5-6	10	5% $CHCl_3$
7-8	12	10% "
9-10	8	20% "
11-12	14	50% "
13-22*	2237	100% " -ergosterol
23-24	35	5% ether
25-34	850	5% methanol - 'C'
35-37	86	10% "
38-40	113	20% "
41-44	98	50% "
45-50	215	5% $AcOH_2$-methanol.

Total recovery from column = 4.021 gm (87%).

Fractions 16-19 were of 200 ml volume and in fact fractions 15-18 contained a total of 1.606 gm of ergosterol, identified with an authentic sample by the criteria mentioned.

Fractions 28-32 contained 0.685 mgm of crude 'C' purified as described to give 'C' as colourless spherical particles (280 mgm) m.p.139-41°, remelting at 144-6°.

P.24. Compound 'C'.

Found: C,73.00,72.46 ; H,11.30,11.38 ; N,2.34,2.21.

$C_{36}H_{67}O_5N$ requires C,72.94 ; H,11.30 ; N,2.36.

Acetylation of 'C'.

'C' (50 mgm) was dissolved in 3 ml of a 1:4 mixture of acetic anhydride-pyridine and heated on the steam bath for 2 hours. After the addition of 5 ml of water the resulting gel was extracted with ether. The dried ether solution was reduced to dryness and the residual gel dissolved in a small amount of methanol. Cooling resulted in a good yield (42 mgm) of fine colourless needles. These were recrystallised twice from methanol to give the pure product (31 mgm) m.p.66-7°.

Found: C,70.53 ; H,10.61 ; N,2.08. $C_{40}H_{71}O_7N$ requires C,70.90 ; H,10.49 ; N,2.07.

The change in the analytical figures when compared with those for 'C' itself corresponds well with the formation of a diacetate.

P.25. Acetylation of 'C' - forcing conditions.

'C' (39 mgm) and p-toluenesulphonic acid (50 mgm) were dissolved in 4 ml of a 1:20 mixture of acetic acid-acetic anhydride and the solution first heated on the steam bath for 2 hours and then refluxed for 1 hour. The addition of water (10 ml) and extraction as above gave 31 mgm of colourless needles identified with the diacetate of 'C' by melting point, mixed melting point and TLC. Only traces of more polar compounds were present despite these stringent conditions - they may

represent breakdown products of 'C'.

P.26. The structural elucidation of 'C'.

The IR spectra of 'C' and its diacetate are shown and fully discussed in the text, so also are their NMR spectra.

Mass Spectrum of Diacetyl-'C'.

M/e	% Abundance.	M/e	% Abundance.	M/e	% Abundance.
39	7.2	82	22.4	131	6.1
41	47	83	35.7	265	7.2
42	16.3	84	10.2	278	6.1
*43	100.0	85	16.3	308	7.2
44	36.8, 22.5	86	6.1	339	33.7
45	6.1	88	6.1	340	9.2
54	11.2	95	19.4	357	13.3
55	51	96	14.3	384	14.3
56	20.4	97	20.4	385	5.1
57	71.5	98	7.2	394	7.2
58	9.2	99	6.1	408	7.2
59	7.1	100	10.2	409	6.1
60	49	101	11.2	439	6.1
67	18.4	102	6.1	645	6.1
68	11.2	109	8.2	646	5.1
69	36.7	110	6.1	647	14.3
70	18.4	111	8.2	648	8.2
71	30.6	112	6.1	659	0.2
72	14.3	113	6.1	661	2.0
73	6.1	118	7.2	664	2.0
74	6.1	123	6.1	665	5.1
75	6.1	124	6.1	666	2.4
79	6.1	125	6.1	675	0.7
81	18.4	128	5.1	679	0.7
				693	0.2

P.37. Degradation of 'C'.

'C' (44.8 mgm) was added to a solution of 0.1 ml of conc. sulphuric acid in 2.5 ml methanol and then refluxed for five hours on the steam bath. The solution was cooled and extracted with 4 x 5 ml portions of petroleum ether (40-60) to remove the precipated fatty acids and methyl esters for further study. The acidic solution was neutralised with 4N methanolic potassium hydroxide and the precipitated potassium sulphate removed by centrifugation. The remaining liquid was made acid to litmus with glacial acetic acid and reduced to ca. 5 ml bulk on the water pump. It was then made strongly alkaline to litmus and extracted with two 10 ml portions of ether. The combined ether extracts were washed with cold water, dried over sodium sulphate and reduced to dryness finally yielding 9.2 mgm of a semi-crystalline material m.p.93-96°, which was analysed by TLC and compared with commercial sphingosine. Its IR spectrum was closely similar to that of authentic sphingosine.

P.38. Oxidation of 'C' fatty esters.

The petroleum ether-soluble fatty esters and acids were taken up in ether and extracted with sodium bicarbonate solution to remove free acids. The ether

phase was dried over anhydrous sodium sulphate and the solvent removed under vacuum to give 16.2 mgm of ester material. Its IR spectrum closely resembled those published for analogous long-chain hydroxy esters. This material was oxidised by the method of Tulloch and Craig[56] as follows: The fatty esters (16.2 mgm) were dissolved in 10 ml of pure distilled tert-butanol and added dropwise to a stirring solution of the 'stock oxidant' (20 ml), aqueous potassium carbonate (0.5%, 10 ml) and tert-butanol (50 ml). The 'stock oxidant' is an aqueous solution 0.0975 M in sodium metaperiodate and 0.0025 M in potassium permanganate. When the addition was complete (1 hour), the flask was stoppered and thoroughly shaken for 24 hours, during which time the initial purple colour was appreciably discharged. A pellet of potassium hydroxide was added and the solution reduced to 20 ml bulk under vacuum. To the resulting aqueous phase sufficient sodium arsenite solution was added to destroy the oxidant and the now colourless solution acidified with 4N sulphuric acid (10 ml). Continous ether extraction then gave the acidic breakdown products of the acyl side-chain moieties of 'C'.

These acids were methylated with excess diazo-

methane and the fatty acid methyl esters subjected to gas-liquid chromatographic analysis by the method to be described.

In order to provide a suitable standard for evaluation of the results of this important degradation 20.2 mgm of methyl linoleate (methyl octadeca-9,12-dien oate) was subjected to an identical oxidation and the esterified fragments analysed by the same method.

P.38. GLC analysis of fatty acid methyl esters.

The esters were analysed on a Pye 'Argon' instrument. The conditions were as follows:
For C_8 mono-esters and upwards, including the C_9 di-ester;
Column: 4' x ¼" glass tubing. Gas Flow: 22 ml/min.
Support: 100-120 mesh acid-washed silanised Gas-Chrom P.
Stationary Phase: 7% F-60/1% Polymer Z.
Temperature: 150° = Flash heater 200°.

Under these conditions, the retention times (Rt) of standard compounds were:

C_8 mono,	Methyl caprylate	2.0 mins.
C_{10} "	Methyl caprate	5.4 mins.
C_{12} "	Methyl laurate	14.8 mins.
C_6 di	Methyl adipate	4.4 mins.
C_7 "	Methyl pimelate	7.3 mins.

cont)

C_8 di	Methyl suberate	12.2 mins.
C_9 "	Methyl azeleate	19.8 mins.

Since C_6 mono, methyl caproate, and the C_3 di, methyl malonate, (anticipated as a breakdown product of all the polyunsaturated components of 'C') were eluted <u>together</u> from this column at a very low Rt, it was necessary to determine them separately. For this purpose, the conditions were:

<u>Column</u>: as above. <u>Gas Flow</u>: as above.

<u>Support</u>: as above.

<u>Stationary Phase</u>: 25% APL.

<u>Temperature</u>: 100° - Flash heater 190°.

Under these conditions, the Rt of methyl caproate was 11.36 minutes and that of methyl malonate was 8.7 minutes. The Rt of C_8 mono, for comparison, had increased to 46.7 minutes. Higher homologues and di-esters were not eluted from this column under these conditions.

The results of the degradation experiments with 'C' are as stated. From methyl linoleate, C_6 mono, C_3 di and C_9 di were obtained and identified with standard compounds. Evidence for a slight over-oxidation (previously reported[56]) was available in that traces of C_8 di were present. From 'C', C_9 di was at once

identified - so also was C_3 di. By a comparison of the ratio of the peak areas of C_3di and C_9 di for methyl linoleate and 'C' breakdown products, it was easily shown that 'C' gave rise to twice the amount of methyl malonate, and thus that the unsaturation pattern in 'C' has to be $\Delta^{9,12,15}$. No other arrangement would correspond with the known structures of the natural long-chain fatty acids.

In passing, the ratio of the peak areas was obtained by analysing identical quantities of the methyl esters from methyl linoleate on both columns. This gave a ratio representing the relative size of peaks resulting from a 1:1 ratio of C_3 di and C_9di in the ester mixture. Since this technique indicated that 'C' gave twice the relative amount of C_3 di, the logical inference was that two C_3 units had been excised from the middle of the 'C' side-chain moieties.

P.40. Acetylation of Base from 'C'.

The bulk of the base from 'C' (6.5 mgm), whose isolation was described on p.80, was placed in a small flask with 6 drops of dry pyridine and 6 drops of acetic anhydride and heated on the steam bath for two hours. Following the addition of water (5 ml), the product was extracted with ether. The acid-washed ether phase was dried and reduced under vacuum to give 8.4 mgm

of a semi-crystalline product which melted at 85° on the Kofler block, crystallised on cooling, and remelted at 95-8°. The literature m.p. of triacetyl-sphingosine is 101-2° [55].

P.43. The synthesis of viridicatol (20), R=H.

3'-methoxyviridicatin (20), $R=CH_3$ was prepared by Mr.C.M.Stewart by a condensation of m-methoxy-phenyldiazomethane with isatin in a manner precisely analogous to that previously described for the synthesis of viridicatin (p.70 and Ref.34). The 3,3'-dimethoxybenzil required for the first stage was prepared from m-methoxybenzaldehyde by a benzoin condensation followed by oxidation of the α-ketol with copper sulphate-pyridine. It was sublimed at 250-60° (0.2 mm Hg) to give colourless needles m.p.256-8°.

Found: C,72.01 ; H,5.02 ; N,5.34. $C_{16}H_{13}O_3N$ requires C,71.90 ; H,4.90 ; N,5.24.

Demethylation of 3'-methoxyviridicatin.

The first attempt to carry out this final step was made by boiling the compound with constant-boiling hydrobromic acid. This was ineffective and so more stringent conditions were indicated.

3'-methoxyviridicatin (30 mgm) was placed in 20 ml of 45% aqueous hydriodic acid and the solution

heated under reflux for two hours - the reflux temperature was 130-140°. From the cooled solution, continuous extraction with ether for four hours produced ca. 30 mgm of red-brown material which was sublimed (250-60°, 0.2 mm Hg) to give 18 mgm of viridicatol m.p.271-3°.

Found: C,71.09 ; H,4.23 ; N,5.49. $C_{15}H_{11}O_3N$ requires C,71.14 ; H,4.37 ; N,5.53.

This compound was found to be identical with natural and synthetic viridicatol later supplied by Dr.M.Luckner in respect of its m.p., mixed m.p., TLC mobility, IR and UV spectra and ferric chloride colour reaction.

The UV spectrum of the synthetic viridicatol showed peaks as follows; λmax(methanol) 223 (44,100), 285 (7,400), 307 (7,400), 318 (8,900),and 330 (6,800). λmax in mμ,(epsilon values).

This is to be compared with the reported UV spectrum of this compound[31]: max(methanol) 226 (28,200), 284 (8,900), 304 (9,100), 316 (11,000),and 329 (7,600). Clearly there exists a strong similarity between these UV spectra.

The synthesis of 3'-methoxyviridicatin reported since this work was completed used the same reaction

sequence and resulted in the compound m.p.257°. Demethylation with hydriodic acid gave viridicatol m.p.274°.[32]

P.44. The NMR spectra of methylated viridicatins.

In each case, the spectra of these compounds were obtained on ca. 0.25M solutions. They were recorded by Mr.J.Gall, to whom thanks are due for his technical assistance.

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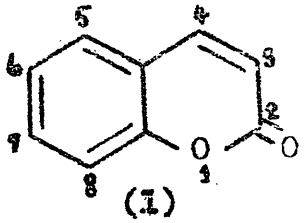
PART 2. The Biosynthesis of Plant Coumarins.

2.1. General Introduction.

The coumarins form a large and diverse group of natural products characterised by the existence of the 5,6-benz-2-pyrone (1) (coumarin) nucleus as part of their structure. They occur widely in higher plants belonging to the Umbelliferae and Rutaceae and to a lesser extent to the Leguminosae and Orchidaceae. A very few have been isolated from animals and microorganisms. For example, Karrer's compendium of natural products (1958)[1] reports the distribution of coumarins in representatives of 77 genera from 27 plant families. Of these, 18 belong to Umbelliferae and 18 to the Rutaceae, the others occurring more or less sporadically within the remaining 25 families.

At this point, it seems advisable to define the term 'coumarin' for the purposes of this research. This term comprises only those higher plant products which contain a 5,6-benz-2-pyrone nucleus derivable from a single phenylpropanoid unit, and which have only hydrogen substituents on the 3 and 4 positions of the lactone ring.

This restriction eliminates from consideration



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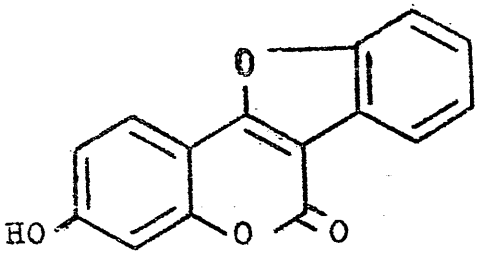

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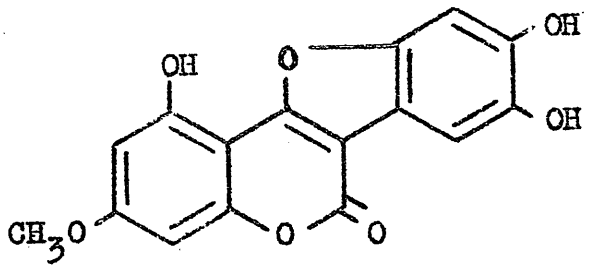

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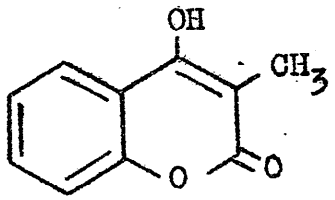

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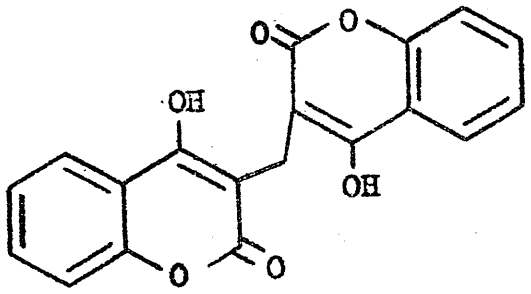

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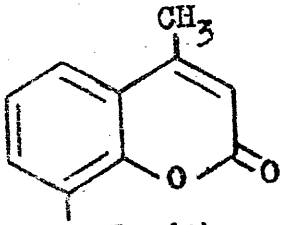

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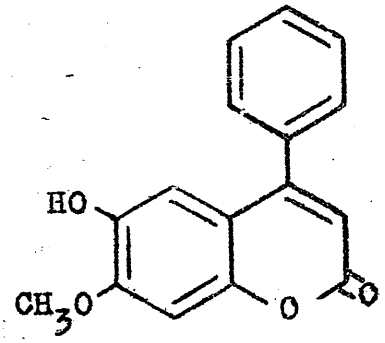

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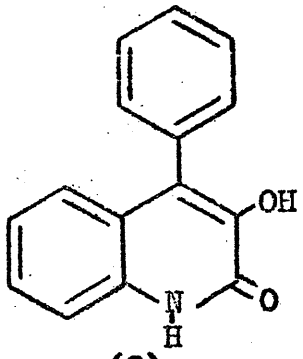

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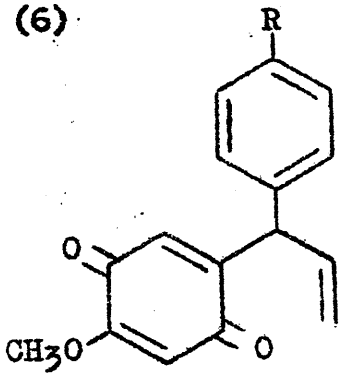

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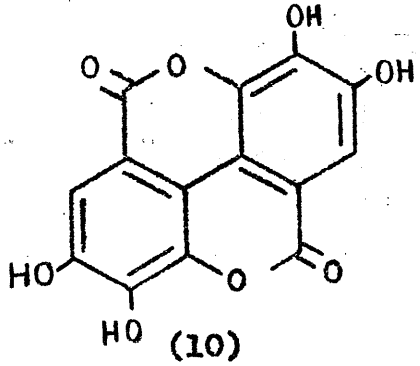
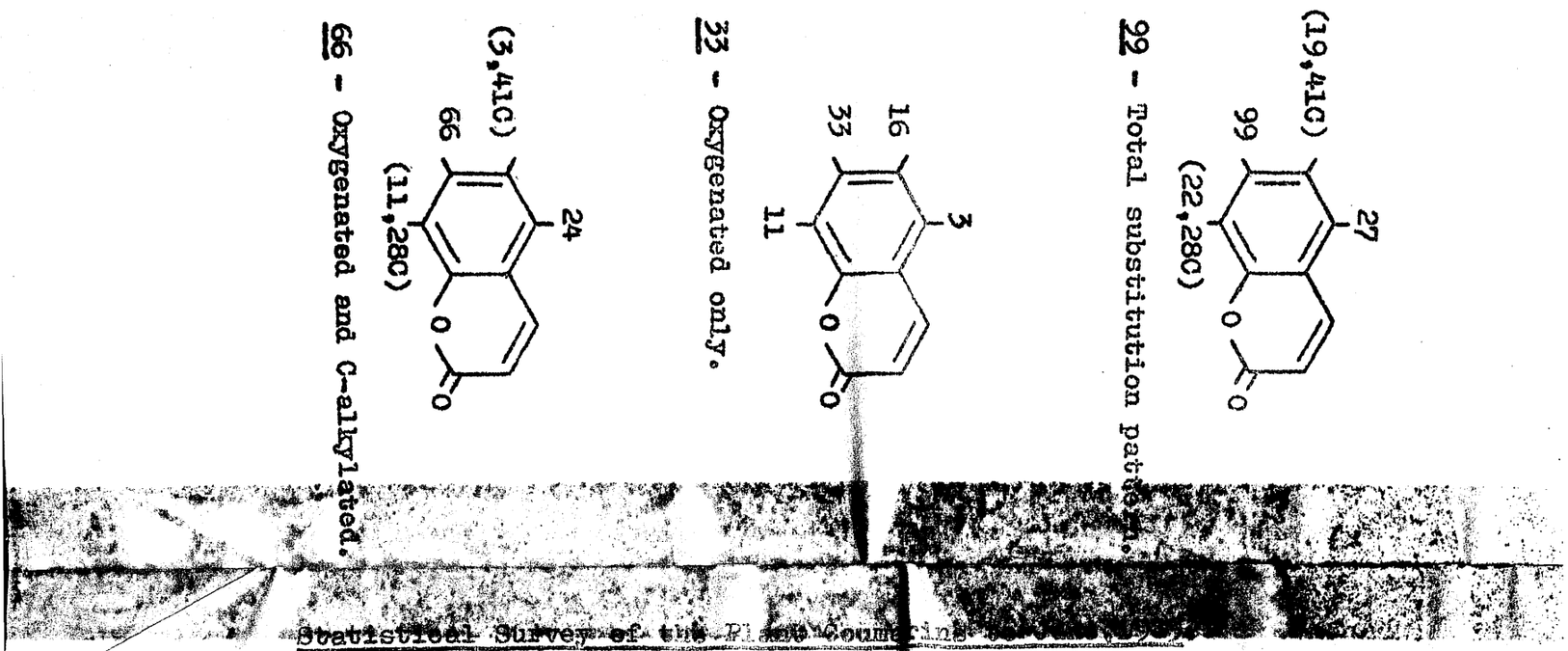


(10)

the small number of known 3-phenylcoumarins such as coumestrol(2) and wedelolactone (3). On structural grounds, they seem more closely related to the isoflavonoids and in fact radiotracer experiments on coumestrol[2] have shown this to be the case. Also eliminated are the 4-hydroxycoumarins, exemplified by 4-hydroxy-3-methylcoumarin (4)[3] and dicoumarol (5), the haemorrhagic principle in spoiled clover[4] which may be derived from coumarin in the intact plant by oxidation at the 4-position and coupling via a C_1 (formaldehyde?) unit. In addition to these small groups, the 4-alkyl and aryl coumarins e.g. (6)[5] and dalbergin (7)[6] are not classed with the 'true' coumarins since their formation probably involves a coupling between a C_n-C_3 unit and an acetate-derived C_6 precursor in a manner analogous to that proposed[7] for the formation of the isosteric viridicatins (8) discussed in the previous section. There is a strong possibility, on structural and taxonomic considerations, that the dalbergiones[8] (9), R=H,OCH_3, are closely related to dalbergin. The 3,4-benzocoumarins, e.g. ellagic acid (10), are very probably derived from gallic acid units and are not classified



Statistical Survey of the Plant Coumarins

UNSUBSTITUTED. - (2) - coumarin, dihydrocoumarin.

	6-alkyl.	8-alkyl.	6,8-dialkyl.	TOTAL.
MONO-OXYGENATED. - (43).				
7-oxygenated.				
10	12	20	1	43.
DIOXYGENATED. - (44).				
5,7-dioxygenated.				
2	15	3	2	21.
6,7-dioxygenated.				
9	- x -	2	- x -	11.
7,8-dioxygenated.				
5	7	- x -	- x -	12.
TRIOXYGENATED. - (12)				
5,6,7-trioxygenated.				
1	- x -	1	- x -	2.
6,7,8-trioxygenated.				
6	- x -	- x -	- x -	6.
5,7,8-trioxygenated.				
0	4	- x -	- x -	4.

The numbers in the first column represent those coumarins with the given oxygenation pattern which are not C-alkylated. The other three columns give the patterns for the C-alkylated coumarins e.g. 15 of the 21 known 5,7-dioxygenated coumarins are also alkylated at the 6-position. The symbol - x - indicates that alkylation at the given position is not possible.

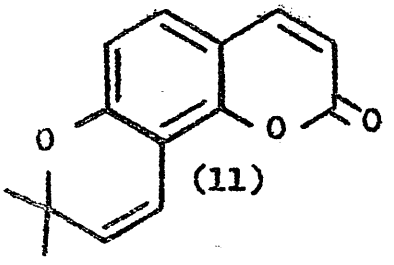
with the 'true' coumarins here.

In spite of these deletions, the remaining coumarins number over one hundred, including fourteen glucosides. The results of a statistical survey of the known structures to date[1,9] are shown in the table-more are continually being discovered. Only two completely unsubstituted coumarins, coumarin and dihydrocoumarin, are known. All the others have a free or masked oxygen function at the 7-position, a point of great biogenetic significance.

The known C-alkyl substituents are all built up from one to three C_5 isoprene units or are derivable from such a unit[10]. Di-alkylation is very rare and methylation in the benzene ring is unknown. Furthermore, alkylation of the aromatic ring at position 5 is never found in this series.

In the series of thirty-three coumarins which are oxygenated but not C-alkylated, (Column 1 and Fig.1), 5-oxygenation is rare in contrast to the findings for the 6 and 8 positions.

The remaining sixty-six of the oxygenated coumarins are also C-alkylated, (Columns 2,3,4



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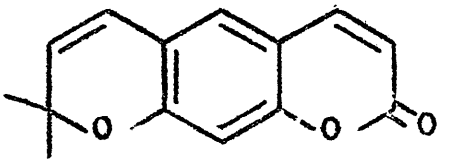

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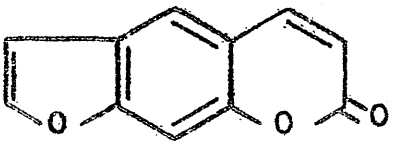

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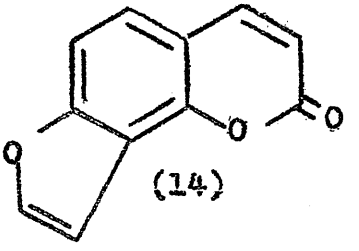

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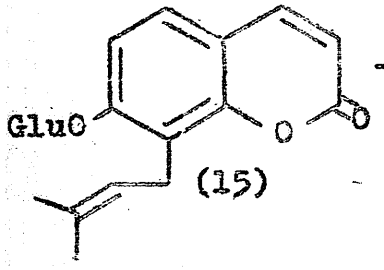

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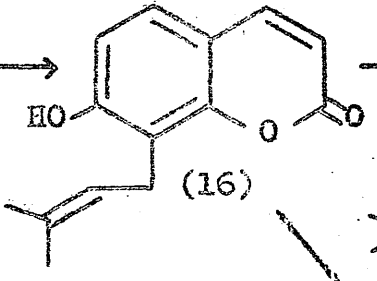

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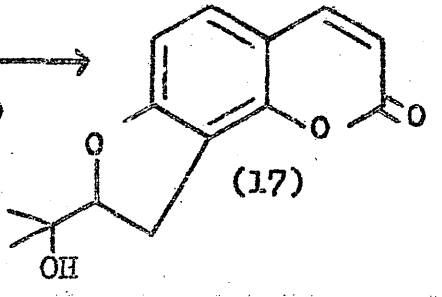

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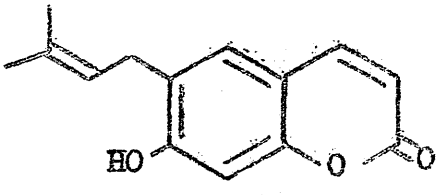

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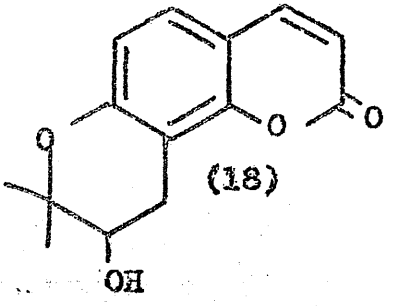


(18)

and Fig.2), and in this series 5-oxygenation is common, possibly reflecting the increased propensity for C-alkylation of a resorcinol-type aromatic system. At position 6 oxygenation is very rare while alkylation is very common. Alkylation at the 8 position is also frequent.

13 chromenes and chromans with 39 furans and dihydrofurans attest the predominance of cyclic derivatives in the naturally-occurring coumarins. It is a curious fact that in the chromene-chroman sections of the C-alkylated coumarins, 'angular' shapes such as seselin (11) predominate [11] over the 'linear' [2], e.g. xanthyletin (12), while in the furan-dihydrofuran sections, 'linear' [27] derivatives, e.g. Psoralen (13) are more frequently encountered than 'angular' [12], e.g. angelicin (14).

In general terms, the frequency of particular oxygenation patterns may be expressed as 7-mono $\gg$ 5,7-di $\gg$ 7,8-di, 6,7-di $>$ 6,7,8-tri, 5,6,7-tri. A recent survey of the coumarins[11] contains the suggestion that the 5,7-oxygenation patterns (found in 27 of 99 derivatives studied) indicates their possible formation by acetate-polyketide pathway.

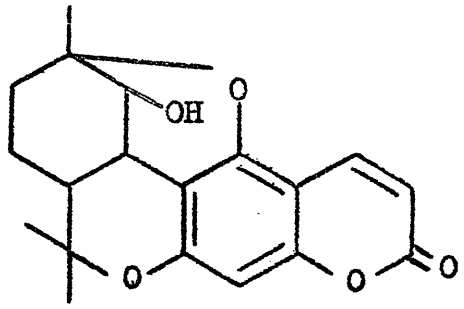
One object of this thesis is to show that

oxygenation meta to an established phenolic nucleus can in fact occur. Application of this suggestion to the problem of the 5,7-oxygenated coumarins readily accounts for their occurrence via the postulate that effective meta-hydroxylation of the aromatic ring may occur at either the cinnamic acid or the 7-oxygenated-coumarin stage.

It is of interest that 11 of the 12 known hydroxycoumarin glucosides are members of the oxygenated but not C-alkylated series since the possibility exists that the C-alkylated coumarins may be derived by interaction of an isoprenoid precursor with a preformed hydroxycoumarin glucoside. Thus, vellein (15) is the only known C-alkylated glucoside containing the coumarin nucleus. It may be readily envisaged that removal of this blocking group and cyclisation of the resulting osthenol (16) will give rise either to the angelicin series (via columbianetin (17)?) or the seselin series (via lomatin (18)?). Analogous considerations apply to the known 7-demethylsuberosin (19). However, the known co-occurrence of both simple and complex coumarins and the o-glucosides of the corresponding coumarinic acids[12]

leaves the precise timing of C-alkylation undecided. One report[13] of preliminary radiotracer studies on the furocoumarins is consistent with their derivation by 'isoprenylation' of a preformed oxygenated coumarin.

Many coumarins exhibit physiological activity in plants, animals and insects. Crude extracts of the Angelicidae, which often contain furocoumarins, have been used for centuries in China in drugs termed Tang-t'u-p'au ('Number-one Artillery'). They were enjoined as stomachic, tonic, carminative, expectorant, lenitive, rousing, stimulant and antispasmodic[14]. Excellent reviews of the extremely diverse activities of coumarins are available, describing the chemotherapeutic uses of both natural and synthetic compounds.[15,16] The chemistry of the coumarins has received close attention and their properties, together with their occurrence, have been fully documented[1,3,11,17-22]. They may be regarded as lactones of cis-o-hydroxycinnamic acids, (the coumarinic acids), which are not obtained as such due to the spontaneous lactonisation of the free acids to the coumarins. The treatment



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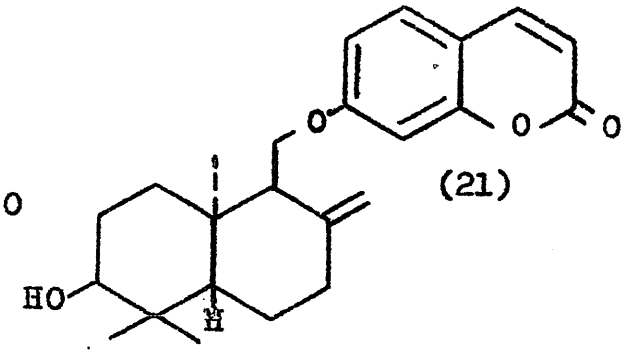

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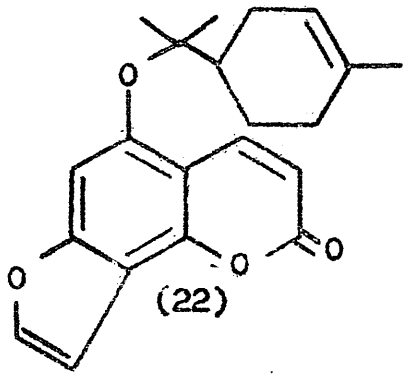

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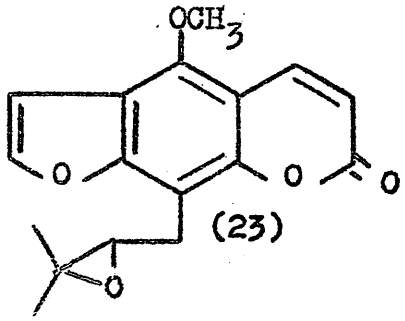

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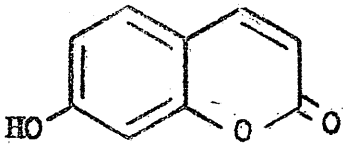

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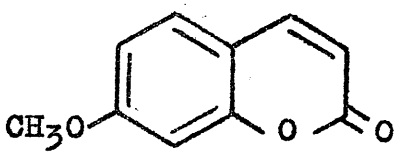

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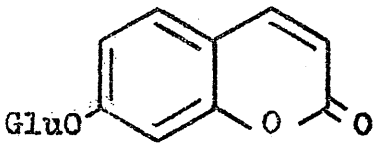

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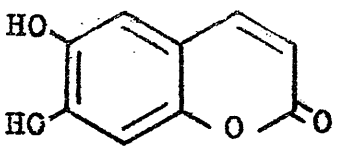

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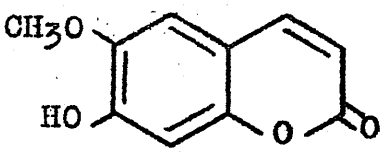


(28)

of coumarins with hot alkali or yellow mercuric oxide effects cleavage of the lactone ring and cis-trans isomerisation to give the stable trans-o-hydroxycinnamic acids (the o-coumaric acids). The reverse process, in which the o-coumaric acids are converted to coumarins, can be induced by ultra-violet irradiation of their solutions, or by heating with a trace of mineral acid.

In summary, the naturally-occurring coumarins exhibit a wide range of structural types depending on the substituents present on the benzene ring of the coumarin nucleus. Their study is not facilitated by the host of accepted trivial names, very few of which convey information on the secondary functional groups within the molecule. Some random examples of the complexity and variation achieved within the small available compass are bruceol (20), farnesiferol a (21), archangelin (22) and byakangelicol (23).

The structures of the coumarins most significant in the present study are those of coumarin (1); umbelliferone (7-hydroxycoumarin) (24), and its methyl ether, herniarin (25) and β-D-glucoside, skimmin (26); aesculetin (27) and

its 6-methyl ether scopoletin (28).

Any discussion of the naturally-occurring coumarins must include due recognition of the fundamental advances made in this field by Ernst Späth and his co-workers in Vienna during the 1930's.

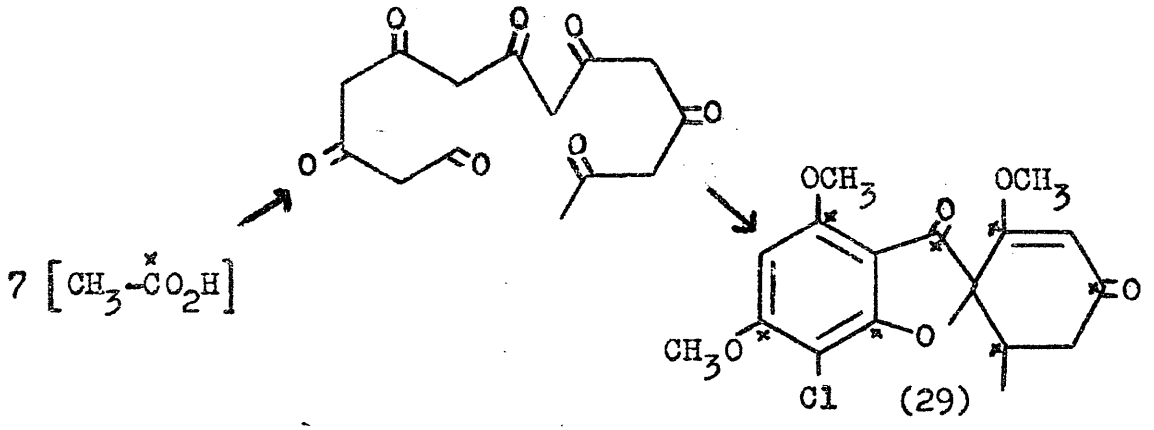
It now seems appropriate to describe the organisation of the subsequent sections. Following a general description of the biosynthesis of aromatic compounds, the theories advanced to account for the formation of coumarins are presented with a brief discussion of the relevant experiments reported before and during the present study. The main section consists of a detailed discussion of the results of the radiotracer experiments carried out here. Combining these with other work published during the last two years, the formulation of a detailed scheme for the biosynthesis of 7-oxygenated coumarins in plants is possible.

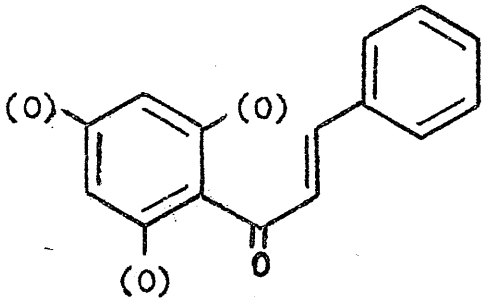
2.1.a. The Biosynthesis of Aromatic Compounds.

Any biosynthetic pathway may be regarded essentially as a sequence of chemical reactions catalysed by enzymes. The enzymes are macromolecular

proteins produced by all living cells to assist in the constant redistribution of energy which is one of the prime characteristics of life. Although many enzyme systems are capable of reactions which can hardly be reproduced in vitro, it is increasingly clear that their action is due to the induction of fundamentally 'logical' electronic rearrangements within and between favourably oriented substrates. These reactions obey the laws of thermodynamics as strictly as any performed in the laboratory.

The advent of radiotracer techniques has produced an ever-increasing flood of information on the nature of enzymes, enzyme reactions, and on the reaction sequences leading to specific compounds. Excellent reviews on the properties of enzymes are available[23,24] and the biosynthesis of aromatic compounds has been fully documented[11,25-40]. Further advances in biosynthetic studies are certain to add to our knowledge of the pathways to particular compounds but the two basic routes to the class of aromatic compounds now seem well established. These are the 'acetate' and 'shikimate' pathways. Basic to the 'acetate' pathway is the formation of





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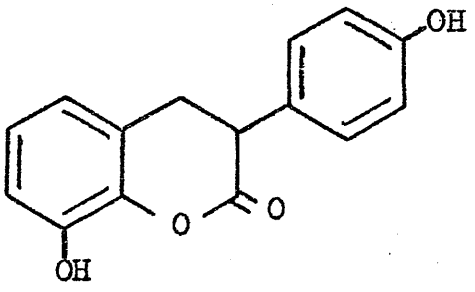

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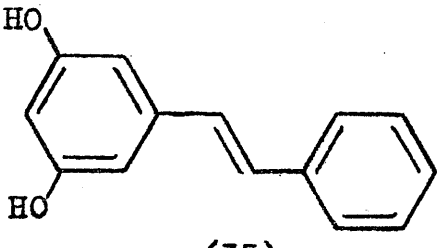
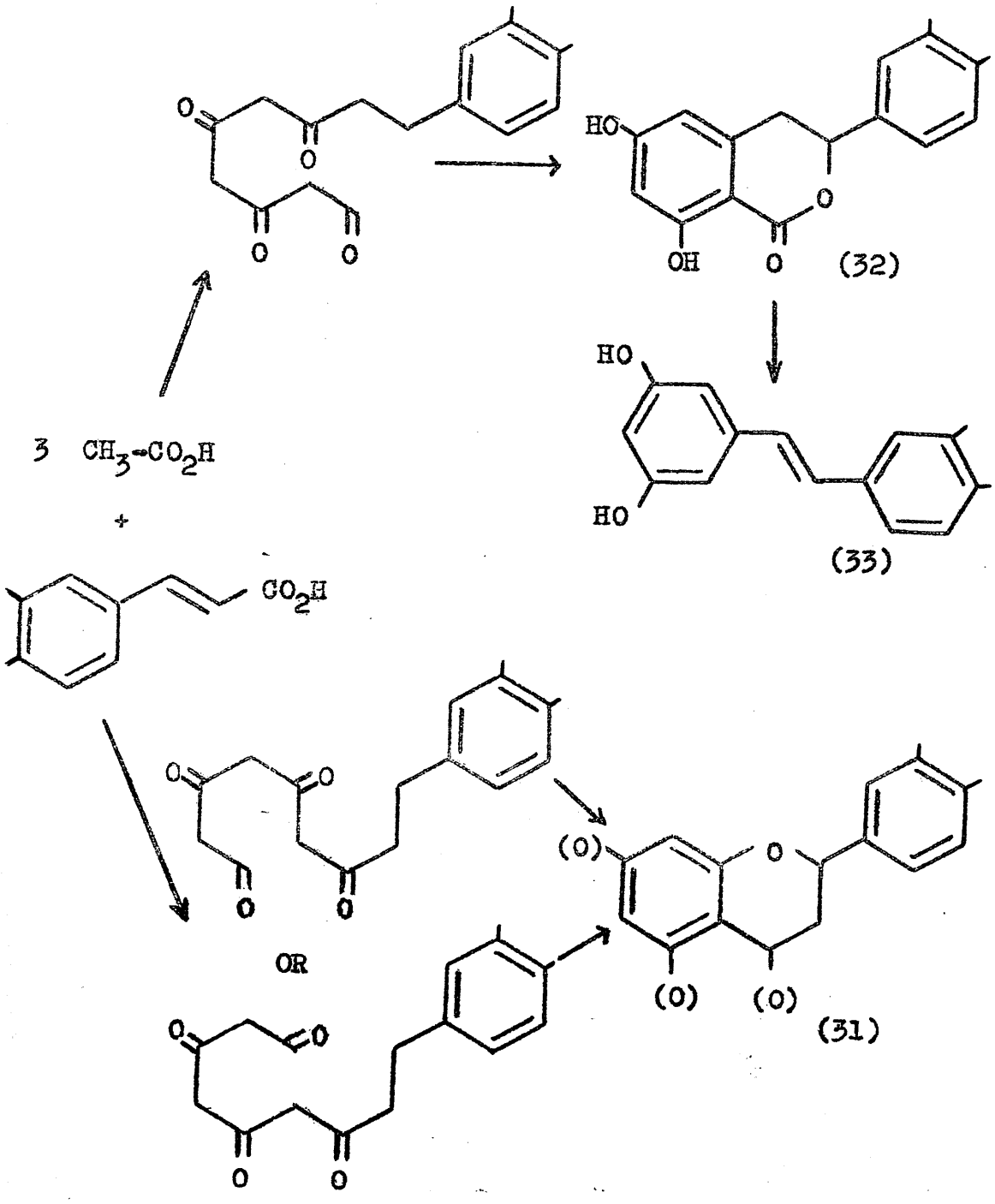


(35)

chains from effective two-carbon units. These 'poly-ketide' chains are then released from an enzyme surface and condense (spontaneously?) to yield aromatic compounds with the meta-hydroxylation pattern characteristic of the products of the overall pathway. Perhaps the most elegant manifestation of the operation of this route is seen in the formation of the mould metabolite griseofulvin (29),[41] the biosynthesis of which was studied by Birch and his co-workers in 1958. 1-^{14}C-Acetic acid was fed to the mould Penicillium griseofulvum Dierckx and after isolation the griseofulvin produced by the fungus was degraded and the activity present in each carbon atom determined. The results were fully consistent with the derivation of a 14-carbon precursor by head-to-tail condensation of two-carbon units. In fact, the current view (see[39b]) is that the methyl-terminal unit does arise from acetate but the chain elongation proceeds by addition of malonate units in a manner closely analogous to the scheme elucidated for the biosynthesis of fatty acids- (see Part 1.3.b.).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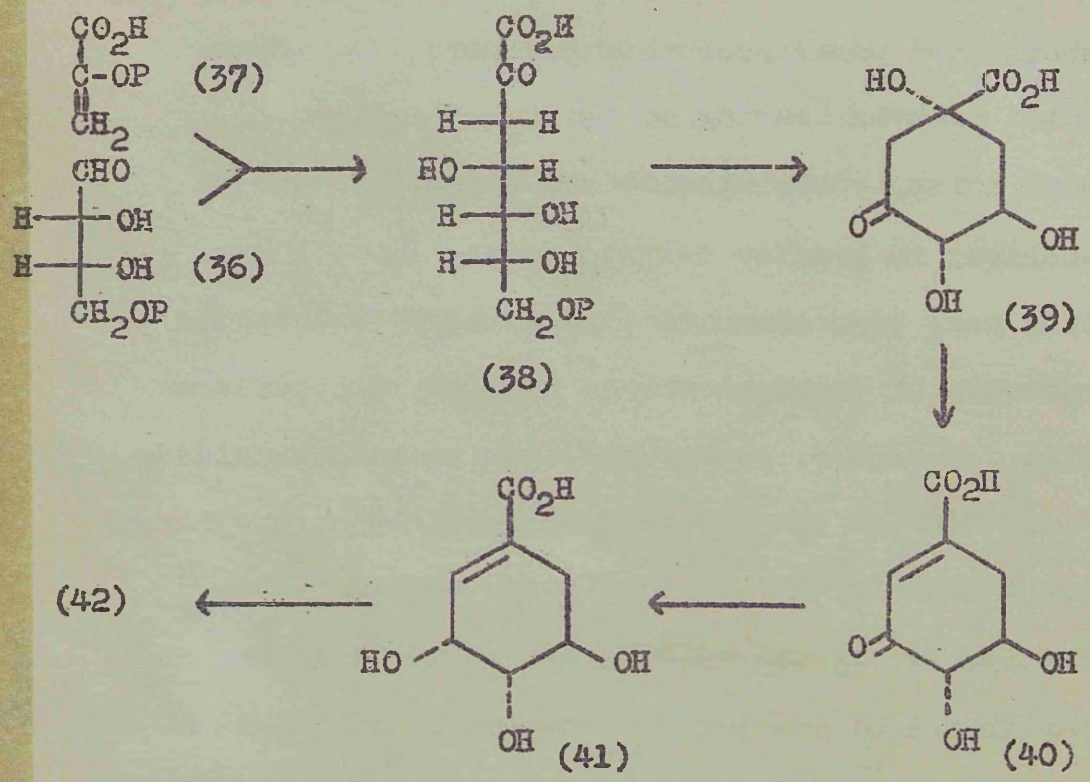
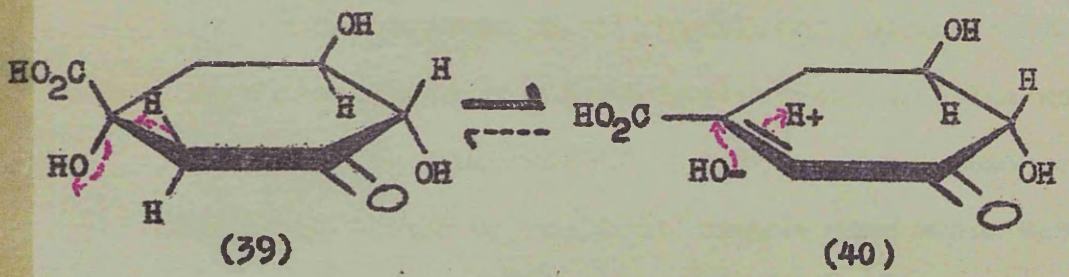
+
CO2H
OR
HO
OH
(32)
HO
HO
(33)
(O)
(O)
(O)
(31)

Since the coumarins, as defined above, are all substituted phenylpropanoid monomers, the 'shikimate' pathway leading to the C_6-C_3 aromatic amino acids, phenylalanine and tyrosine, will be described in greater detail. These two amino-acids have been shown to play a major part in the synthesis of plant phenolics such as the cinnamic acids, coumarins, flavonoids, and certain alkaloids in addition to the polymeric lignin found as a constituent of the cell walls of 'woody' plants. Furthermore, by degradation of the three-carbon side chains of the amino acids or the cinnamic acids derived from them, they may give rise to the C_6-C_2 and C_6-C_1 units which form part of a great many natural products. It is worth pointing out that definite 'hybrids' of the acetate and shikimate pathways are known, the best example being the vast group of the known flavonoid compounds[42]. These have been shown to arise by the junction of a C_6-C_3 unit with three malonylcoenzymeA molecules, yielding a postulated chalcone intermediate (30). However, such a condensation may result in the formation either of the flavonoids (31) or the


(37)
(36)
(38)
(39)
(40)
(41)
(42)

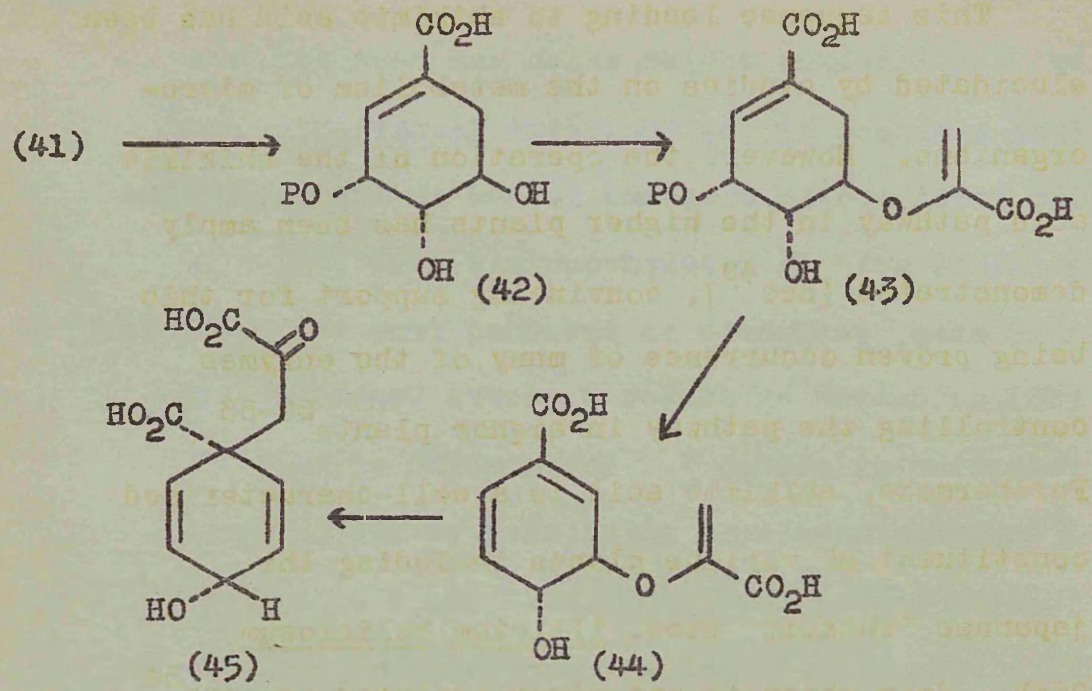

(39)
(40)

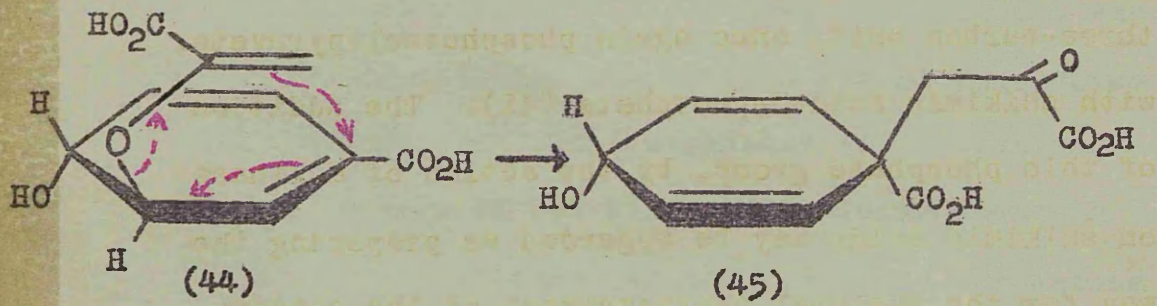
isocoumarins (32) and stilbenes (33), probably depending on the orientation of the C_{15} precursor on the active site of the cyclising enzyme. Two different folding modes could produce the flavonoid nucleus.

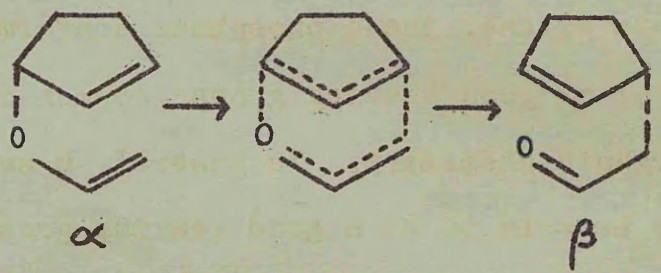
The isocoumarin hydrangenol (34) is indeed formed by the pathway shown[43,44], while studies on the stilbene pinosylvin (35) and its monomethyl ether[45,46] accord with the suggestion that they are formed by decarboxylation of an isocoumarin, stilbene carboxylic acid or of a precursor of these compounds. In passing, it should be noted that hydrangenol is a product of Hydrangea macrophylla and is a synergist for gibberellin action[47].

Intensive studies[39] make it clear that the first stage in the formation of phenylpropanoid compounds is the formation of a 'proto-aromatic' ring via condensation of erythrose-4-phosphate (36) and phosphoenolpyruvic acid (37). These compounds, which are derived from glucose by the 'pentose phosphate' and 'glycolytic' pathways respectively, yield the seven-carbon sugar sedoheptulose-7-phosphate (38). This condensation is catalysed

by a synthetase enzyme, which has been purified from extracts of the bacterium Escherichia coli.[48] The first cyclic compound formed in the pathway to shikimic acid is 5-dehydroquinic acid (39). In this step, phosphate is extruded from the precursor (38), oxidation at carbon 6 occurs together with reduction at carbon 7. Dehydration of this α-hydroxy keto-acid gives rise to the fully conjugated 5-dehydroshikimic acid and a stereospecific reduction of the ketone function produces the important intermediate shikimic acid (41). It is of interest to note that both of these reactions are demonstrably reversible. This is certainly surprising in the case of the dehydration step since, on stability considerations, the unsaturated 5-dehydroshikimic acid would not be expected to accept the elements of water readily. This may however be favoured by a mechanism such as that shown in which the polarisation of the 5-carbonyl group assists the reverse reaction. Since this reversal is stereospecific, it may well be catalysed by the same enzyme that mediates the forward reaction.


(41)
CO_2H
PO
OH
OH
(42)
CO_2H
PO
O
CO_2H
OH
(43)
HO_2C
O
HO_2C
HO
H
(45)
CO_2H
O
CO_2H
OH
(44)


HO_2C
H
O
HO
CO_2H
H
(44)
H
HO
O
CO_2H
CO_2H
(45)


O
O
O
α
β

This sequence leading to shikimic acid has been elucidated by studies on the metabolism of micro-organisms. However, the operation of the shikimic acid pathway in the higher plants has been amply demonstrated (see[49]), convincing support for this being proven occurrence of many of the enzymes controlling the pathway in higher plants[50-53]. Furthermore, shikimic acid is a well-characterised constituent of various plants including the japanese 'shikimi' tree, Illicium religiosum Sieb., from which it was first isolated in 1885[54].

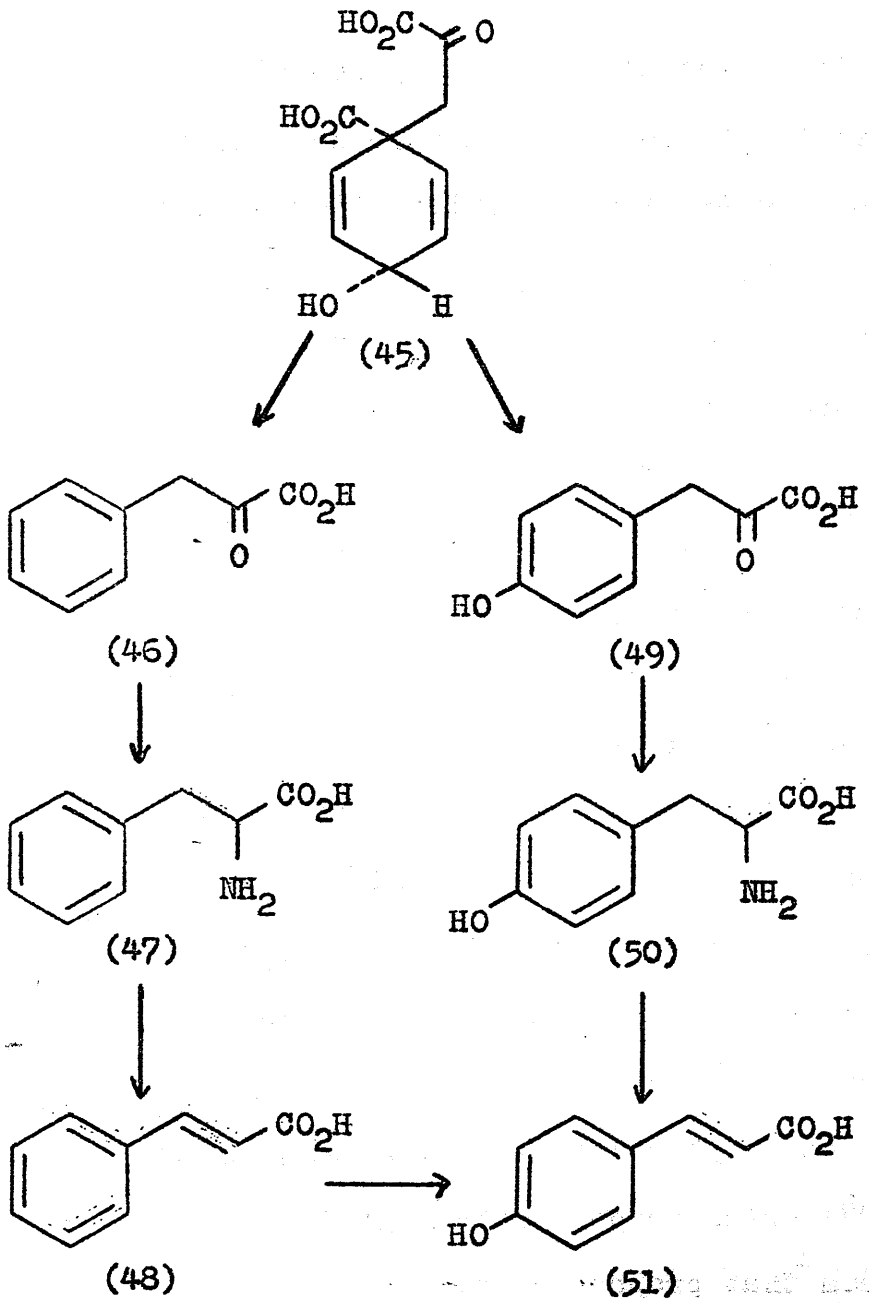
The next stage in the production of phenyl-alanine and tyrosine is the union of a further three-carbon unit, once again phosphoenol-pyruvate, with shikimic acid-5-phosphate (42). The addition of this phosphate group, by the action of a kinase on shikimic acid, may be regarded as preparing the way for the skeletal rearrangement of the conden-sation product 3-enolpyruvylshikimate-5-phosphate (43). To this extent, the 5-phosphate conforms to the concept of a 'good leaving group' common in synthetic organic chemistry. In general, a sub-stituent may be said to be a good leaving group

if its loss is a thermodynamically favoured process.

It may be significant that the non-phosphorylated analogue of (43) has long been known as a product of certain mutant bacterial strains. It could conceivably arise from the direct condensation of phosphoenolpyruvate with shikimic acid itself. Failing this, the removal of phosphate from (43) without induction of rearrangement or a stereospecific hydration of (44), (which could lead to the hydroxy-benzoic acids) must be postulated.

The elimination of the elements of phosphoric acid from 3-enolpyruvylshikimate-5-phosphate with a concomitant double bond shift yields the so-called chorismic acid (44), trans-3,4-dihydroxy cyclohexa-1,5-diene carboxylic acid 3-enolpyruvyl ether[55].

Chorismic acid is of the greatest significance in the biosynthesis of aromatic compounds since M. and F. Gibson, who discovered it in cultures of Aerobacter aerogenes mutants[56,57], have shown that it is the precursor of anthranilic acid (and thence tryptophan) as well as p-hydroxybenzoic


HO2C
O
HO2C
HO
H
(45)
CO2H
O
(46)
CO2H
O
HO
(49)
CO2H
NH2
(47)
CO2H
NH2
HO
(50)
CO2H
(48)
CO2H
HO
(51)

acid and prephenic acid (45), which is the next isolable intermediate en route to the aromatic amino acids. The structure of prephenic acid is such that its formation from chorismic acid may be visualised in terms of an orientation of the ether methylene function near the C-1 of the ring, followed by a sequence of concerted electron pair shifts through a 'no-bond' mechanism as in the rearrangement α to β [58]. Gibson and Jackman[55], however, have described the biological transformation as "presumably a stereospecific S'n$_i$ reaction" in which the leaving group decomposes during 'solvolysis' to produce nucleophilic anions that attack the carbonium ion before it can become symmetrical. Prephenic acid is the last non-aromatic intermediate in this pathway. When the enzymes prephenic aromatase[58] and prephenic dehydrogenase[59,60] react with this compound it is transformed to phenylpyruvic (46) and p-hydroxyphenylpyruvic (49) acids respectively. At first sight, it might seem that prephenic acid, rather than chorismic acid, is the true branching point in the overall sequence to the amino acids and that chorismic acid

(Greek -'separating')has been wrongly named. This is not the case, however, since the recent work of Cotton and Gibson[61] has shown that the conversions chorismic acid→prephenic acid→phenylpyruvic acid and chorismic acid→prephenic acid→p-hydroxyphenylpyruvic acid are catalysed by two <u>different</u> enzymes or enzyme complexes. Thus, chorismic acid is the true branching point in this pathway, in spite of the formal equivalence of the pools of prephenic acid involved in the biosynthesis of phenylalanine (47) and tyrosine (50).

The final step in the formation of the aromatic amine acids involves the transamination of the corresponding keto-acids. This demands the transfer of the elements of ammonia from glutamic acid to the keto-acid and the products are the α-amino acid and α-keto glutaric acid. Enzymes capable of effecting this type of transfer have been purified from micro-organisms[62], higher plants[52] and animals[63]. It is generally conceded that the formation of tyrosine by <u>para</u>-hydroxylation of phenylalanine is the major pathway in animal tissue[64,65], genetic loss of this step leading to phenolketonuria in man[66], with an accumulation of

phenylpyruvic acid. Both plants and microorganisms utilise the keto-acid route described above[39e]. However, a recent report on the properties of a phenylalanine hydroxylase system from spinach leaves[67] demonstrates that, in common with so many biochemical processes, there is no absolute division between the classes of living entities which may possess a given biochemical ability. The mere possession of a given enzyme system is no guarantee however, that its action plays a significant role in the total metabolism of a cell, or indeed that the substrates efficiently utilised in radiotracer studies are the actual in vivo substrates.

At this stage, the situation may be reviewed. Prephenic acid (45), the ultimate precursor of the phenylpropanoid compounds, is formed by the condensation of erythrose-4-phosphate (36) and two units of phosphoenolpyruvate (37). Both (36) and (37) are derived from glucose and thus, ultimately, from carbon dioxide in photosynthetic tissue. Decarboxylation of prephenic acid, with or without simultaneous dehydration, gives rise to the keto-acid precursors of phenylalanine and tyrosine respectively. The amino acids as such are formed by

transamination.

A critical feature of the sequence leading to the plant coumarins is the biosynthesis of trans-cinnamic acids. The reason for this is that it is a cinnamic acid, rather than the corresponding amino-acid, which is directly involved in the elaboration of many secondary metabolites such as the flavonoids and stilbenes discussed briefly above. This is also true for the plant coumarins studied so far.

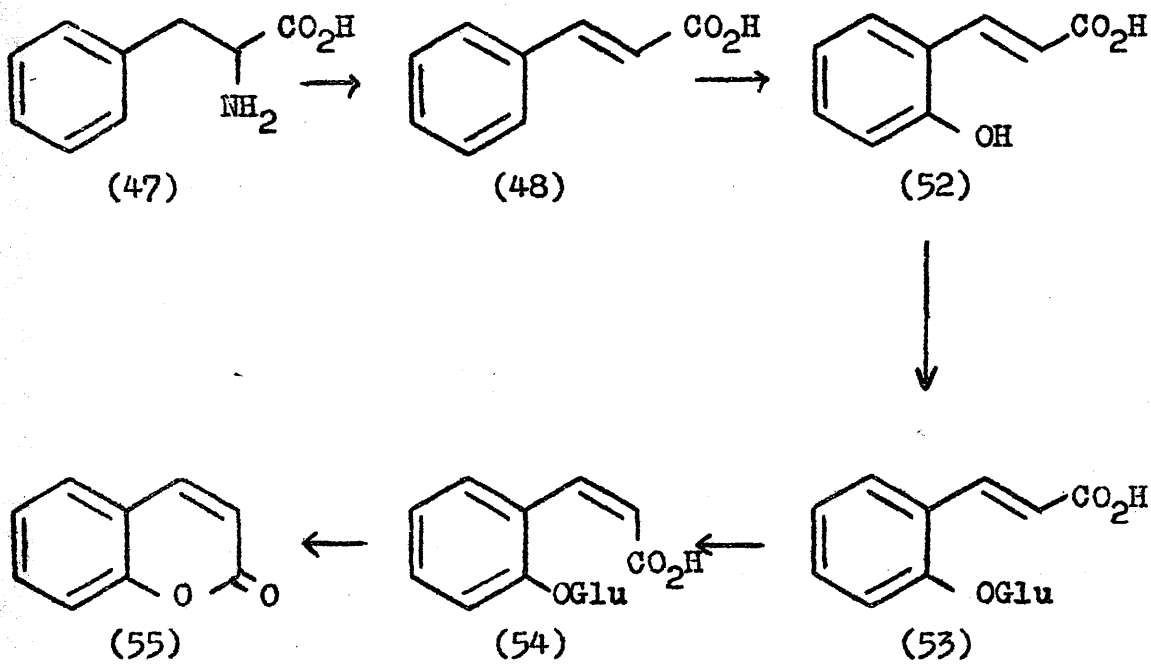
At one time it was believed that the cinnamic acid system was formed by the dehydration of a reduced phenylpyruvic acid[28]. Apparent support for this was obtained from tracer studies but it now seems clear that the good incorporations of activity achieved could have been due to the oxidation of the fed phenyllactic acids to the keto-acid precursors of phenylalanine and tyrosine.

The operation of an analogous scheme in wheat stem has been suggested[139] but the established route to the cinnamic acids has resulted from the important work of Koukol and Conn in California and Neish in Canada. They have proved[68,69] that the action of the purified enzymes phenylalanine deaminase and tyrase

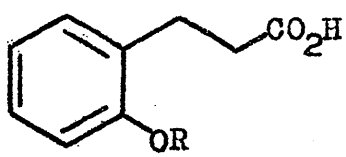
from barley results in an irreversible elimination of ammonia from the α-amino acids to give the corresponding trans-cinnamic (48) and trans-p-coumaric (51) acids as shown. Phenylalanine deaminase[68] appears to occur widely, probably indeed, in all higher plants which lignify but the distribution of tyrase[69] is much more restricted. It appears to occur only in members of the Graminae (grasses) and the substantial evidence in support of this general statement implies that the key intermediate in the further pathways to be discussed is trans-cinnamic acid (48).

2.1.b. The Biosynthesis of Coumarin

Coumarin is a fairly wide-spread natural product. Much of the interest shown in this compound stems from its well-documented action as a growth inhibitor[70] e.g. it was shown to inhibit the growth of wheat roots at a concentration of 7×10^{-6} M as early as 1907[71]. Since then, it has been implicated as a physiologically active agent in many life processes see 72,73. Perhaps the main significance of coumarin rests in the notorious haemorrhagic properties of its congenor dicoumarol in



The Biosynthesis of Coumarin.



(53a), R=H
(53b), R=Glu

spoiled sweet clover[4]. Sophisticated plant breeding studies carried out in the production of 'low coumarin' strains of this important forage crop have yielded valuable insight into the biosynthetic pathway to coumarin, which is now established, in outline at least.

The pathway to coumarin has many parallels to the sequence established for oxygenated coumarins in this and other studies. It will therefore be discussed in detail.

The first radiotracer studies on the formation of coumarin were carried out by Kosuge and Conn[74] in 1959. They showed that glucose, shikimic acid, phenylalanine and trans-cinnamic acid were effective precursors of o-coumaric acid (52) in white sweet clover (Melilotus alba Desr.) and that none of these compounds except o-coumaric acid significantly labelled coumarin, possibly due to a very rapid metabolism of coumarin to melilotic acid (53a) and melilotyl glucoside (53b).

The results obtained by Weygand and Wendt[75] for their feedings of 1-^{14}C-acetate, 1-^{14}C-glucose and U-^{14}C-phenylalanine to root tissue cultures of M. officinalis confirmed that coumarin does

not arise from acetate but rather from glucose by way of shikimic acid and phenylalanine. Degradation of the coumarin isolated from the feedings of glucose and phenylalanine proved that the distributions of activity were fully in accord with these theories.

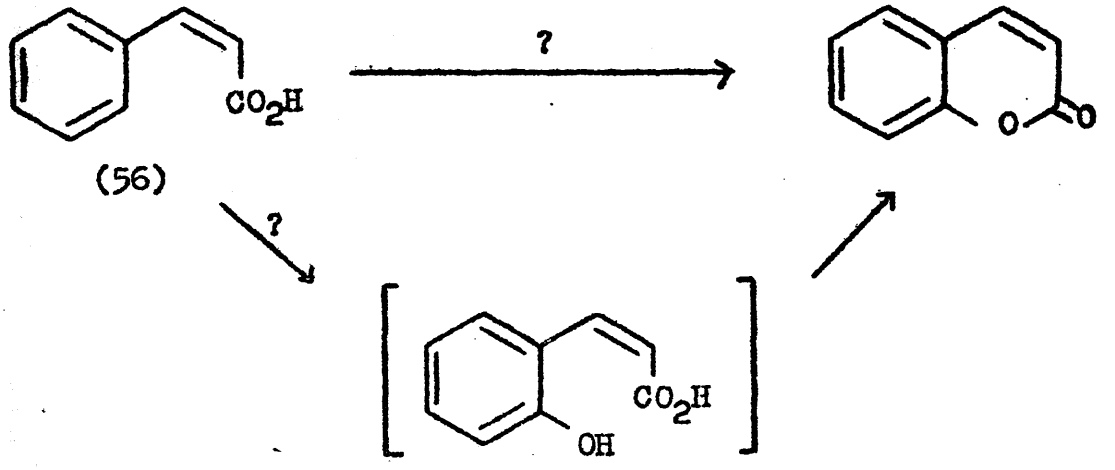
The work of Brown and his collaborators[76], using sweet grass (Heirochlöe odorata) as the subject, again showed that shikimic acid, phenylalanine and trans-cinnamic acid are good precursors for coumarin but are still more efficiently utilised in the synthesis of o-coumaric acid, which was itself readily converted to its glucoside. A critical point demonstrated here was that o-coumaryl glucoside is an efficient precursor of coumarin.

Later work by Weygand et al.[77] demonstrated that umbelliferone is not transformed to coumarin by M.officinalis. The fact that dehydroxyl'n of a para-oxygenated precursor does not play any major part in the biosynthesis of coumarin was simultaneously confirmed by Brown, whose experiments with H.odorata[78] and later with lavender[79,80] showed that tyrosine and p-coumaric acid are far less

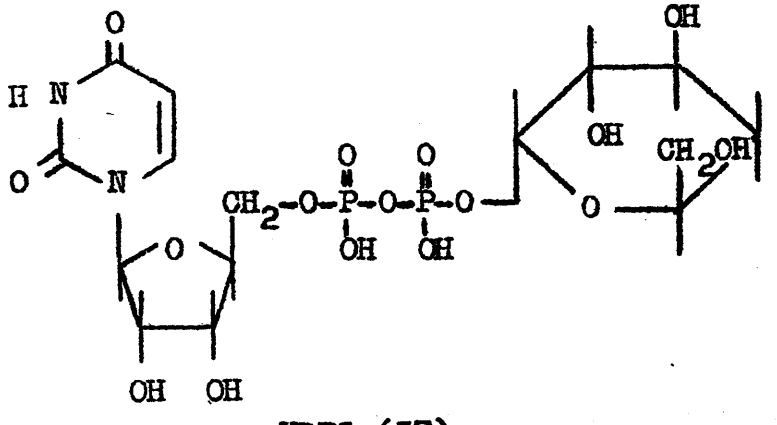
efficient precursors of coumarin than phenylalanine, cinnamic acid, o-coumaric acid and coumarinyl glucoside.

Brown suggested[79] "that, in higher plants, cinnamic acid (or an 'activated' form of it) is a common precursor of all coumarins, and that ortho- or para-hydroxylation of this compound leads subsequently to the formation of coumarin and the 7-hydroxy coumarins respectively." This hypothesis has been upheld by the later studies.

In a further contribution, Kosuge and Conn[81] showed that melilotyl glucoside (53b) and o-coumaryl glucoside (53) are metabolically active in sweet clover and that the latter is an efficient precursor of coumarin, thereby confirming the previous work of Brown et al.[76] They suggested that the incorporation of this compound probably occurs via the cis form, coumarinyl glucoside (54), and purified a β-glucosidase enzyme from the plant which was highly active against the cis but essentially inert against the trans o-glucoside. The inference drawn was that this enzyme is responsible for the final step (54)-(55) in the biosynthesis of coumarin in M. alba and a



'Direct' formation of Coumarin[87].



UDPG, (57)

similar enzyme has been shown to be active in the coumarin-rich tonka bean[82]. The specificity of this enzyme is very different from that of the commerically-available β-glucosidase (almond emulsin) which hydrolyses both the cis and trans-glucosides at the same low rate. A possible rationale of the specificity of the clover enzyme will be discussed later. in section 2.2.c.

Stoker and Bellis[87] found, in feeding ^{14}C-trans-cinnamic acid to M.alba, that the incorporations of activity into o-coumaryl glucoside, o-coumarinyl glucoside and coumarin over periods of 1, 2 and 3 days were in accord with the accepted scheme and confirmed previous results[74] indicating that melilotic acid and melilotyl glucoside are metabolites of coumarin or its precursors. They also confirmed the work of Kosuge[88], who had proved that the 'bound' form of coumarin in M.alba is indeed coumarinyl glucoside (54). The results obtained from their feedings of cis-^{14}C-cinnamic acid (56) were of great interest since they showed that the cis-acid is converted directly to coumarin, probably by ortho-hydroxylation and subsequent lactonisation without the intervention of a glucoside form. In

view of the demonstration that phenylalanine deaminase produces only trans-cinnamic acid[68] and the fact that an efficient pathway exists in sweet clover for the conversion of the trans- acid to coumarin, it is most probable that the route to coumarin via cis-cinnamic acid is not favoured and, indeed, that cis-cinnamic acid may not exist in M.alba under normal conditions. However, this possibility cannot be totally excluded.

Simultaneously with the work described above, Brown[89] presented convincing evidence, on the basis of his further studies with Heirochloe odorata, in favour of the ortho-hydroxylation scheme for coumarin biosynthesis and postulated the existence of a small but significant pool of 'free' coumarin in this plant as well as the presence of separate pools of 'bound' coumarin, coumarinyl glucoside (54).

In summary, the radio-tracer experiments on the formation of coumarin in various plants are in accord with the scheme shown, with the provisos that rapid metabolism of the coumarin can occur and that alternative routes to the lactone ring system may contribute.

The examination of the genetic control of these transformations is still in progress. It can be pointed out that the work so far has concentrated on the demonstration in M.alba of the two genes Cu, which determines whether any coumarin will be made, and B, which determines the presence of any 'free' coumarin. This has been the work of Goplen et al,[90] Rudolf and Schwarze[91] and especially Haskins and Gorz[92,93,94] and their co-workers in Nebraska. This work has been reviewed by Brown[36] and Kosuge[40], and the finding is that the Cu gene controls the ortho-hydroxylation of trans-cinnamic acid, while the B gene controls the β-glucosidase specific for coumarinyl glucoside (54). Thus, plants homozygous dominant for both genes, CuCuBB, have been shown to contain coumarinyl glucoside as well as the enzyme responsible for its hydrolysis. It is quite clear that these must be separated in the intact plant and the formation of 'free' coumarin takes place only when the plant tissue is disrupted[40] since it has been shown that no free coumarin exists in the intact plant[91,95] Plants, homozygous dominant for the Cu gene but recessive for the B, have the constitution CuCubb

and therefore, although they contain coumarinyl glucoside, they possess small glucosidase activity. The heterozygotes will of course, exhibit the activity of the dominant. The picture for Hierochloe odorata[89] is complicated in that the specific activity of 'free' coumarin was consistently higher than that of the 'bound' glucoside form. This may be explained by assuming that part of the trans-cinnamic acid precursor is converted to the cis form, and as such is 'directly' hydroxylated to give coumarin[87]. Alternatively, separate pools of coumarinyl glucoside may exist in the plant[36] and the 'free' coumarin is only formed from the small but highly active pool of coumarinyl glucoside produced after the feedings of radioactive precursor. This implies that the greater part of the 'bound' coumarin is inert within the plant. In view of the known physiological activity of coumarin against fungi and viruses[96], the presence of a latent pool of coumarin in a plant may be plausibly interpreted as a defence mechanism.

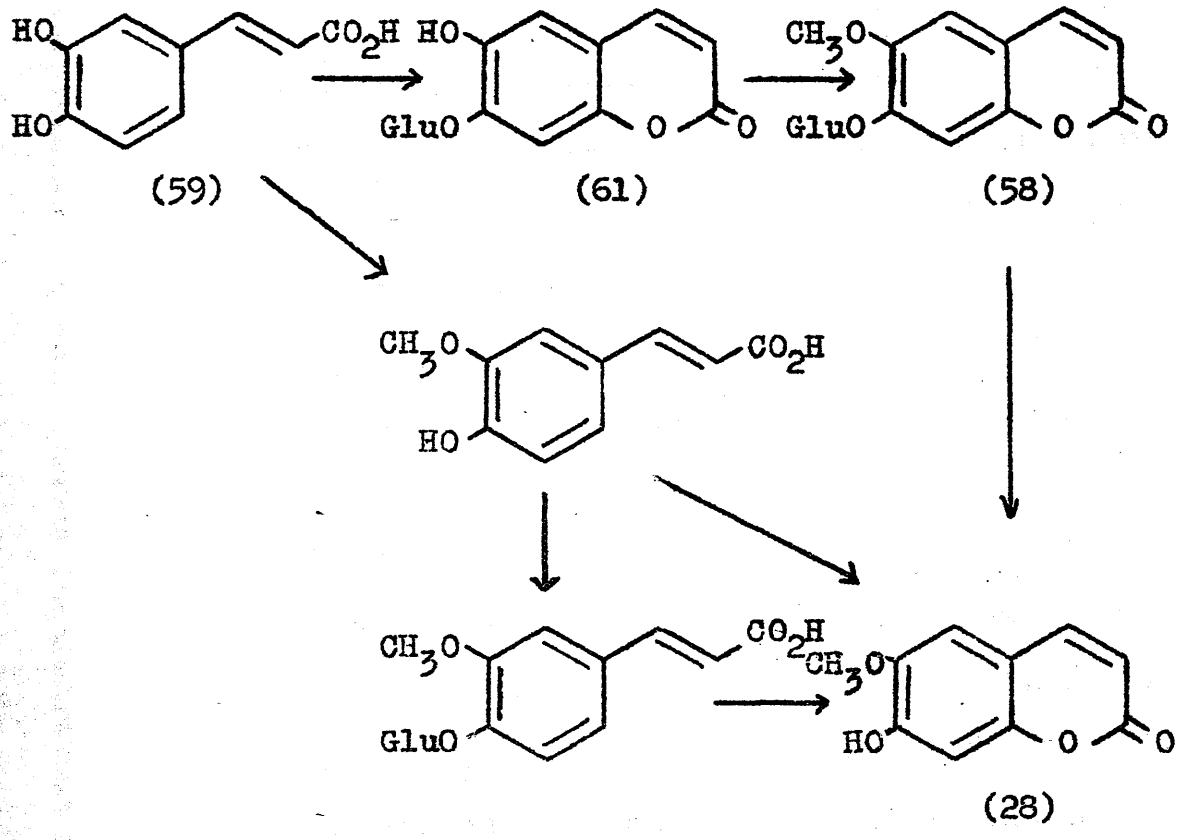
Although the ortho-hydroxylation of trans-cinnamic acid is very strongly favoured as the critical step in the sequence to coumarin, it has not

yet been demonstrated *in vitro*. However, the glucosylation of *o*-coumaric acid by an enzyme preparation from *M. alba* with added uridine diphosphate glucose (UDPG)(57), has been proved, as cited in[74]. The involvement of the α-glycosyloxy nucleotide UDPG, a frequently encountered 'High-Energy' glucose carrier in higher plants[97a], suggests a parallel with the glucoside-synthesising system first discovered by Cardini and Leloir[97].

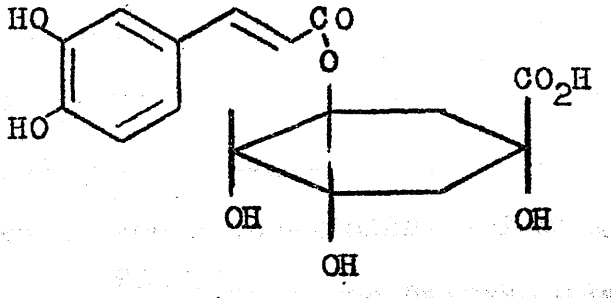
A further vexed question in the enzymology of coumarin biosynthesis is the nature of the control of the *trans-cis* isomerisation step prior to the formation of the lactone ring. As previously mentioned, the isomerisation of coumaryl glucoside is a very facile process[85], mediated by light alone *in vitro*, which in itself suggests the possibility that enzymic control is not absolutely necessary. This hypothesis has received strong support both from *in vitro* and *in vivo* studies, especially by Haskins and Gorz. Experiments carried out in the present study fully confirm their findings and demonstrate that the presence of the *o*-glucosyl group is no barrier to the isomerisation. Kahnt has adduced evidence favouring a causal connection

between the isomerisation of coumaryl glucoside and the intensity of illumination of M.alba plants[99], while this has been fully confirmed in authoritative studies of the cis-trans ratio in a wide range of species of Melilotus and Trigonella by Gorz and Haskins[100]. Their further work has yielded strong evidence in favour of a non-enzymic isomerisation step[101]. However, Stoker's report that both M.alba leaves and an enzyme preparation from them are in fact capable of inducing the trans-cis isomerisation step in the absence of light[102] is a highly significant datum contrary to this suggestion. The situation remains obscure.

Although the outline of the biosynthesis of coumarin seems clear, much remains to be done in order to establish the full details of each step in the sequence. The further metabolism of coumarin and its precursors by plants[39e,40] and microorganisms[103-105], has proved to be of great interest but will not be discussed in detail for the present purpose.



Postulated Routes to Scopoletin (28).



(60)

2.1.c. The Biosynthesis of Oxygenated Coumarins.

The striking fact that all the known plant coumarins apart from coumarin itself are oxygenated at position 7 of the benzene ring has long been interpreted as implying their derivation from para-oxygenated phenylpropanoid compounds, although an early suggestion was that they might arise from C_6-C_1 and C_2 units[138].

With the notable exception of S.A. Brown's work on herniarin[79,80,106] and umbelliferone[107], the radiotracer studies on the biosynthesis of the oxygenated coumarins have not provided detailed information on the operating mechanisms.

Reznik and Urban[108] fed β-^{14}C-ferulic acid to leaves and seedlings of Helianthus annuus, Triticum vulgare and Zea mays and were able to show by paper chromatography and autoradiography that it was incorporated into scopolin (58), caffeic acid (59) and its quinic ester chlorogenic acid (60). The first quantitative radiotracer study on oxygenated coumarins was carried out by Reid[109] using Nicotinia tabacum as the subject. He found that U-^{14}C-phenyl-alanine was a better precursor of scopoletin than 2-^{14}C-acetic acid, 1-^{14}C-phenylacetic acid and

^{14}-C-carbon dioxide.

A highly significant study by Harborne and Corner[110] of the metabolites of cinnamic acids in a wide variety of plants included the observations that glucose esters rather than glucosides were formed from phenolic acids and also that caffeic acid is converted to aesculetin (27) by radish leaves (Raphanus sativus) and to scopoletin (28) by Datura knightii leaves.

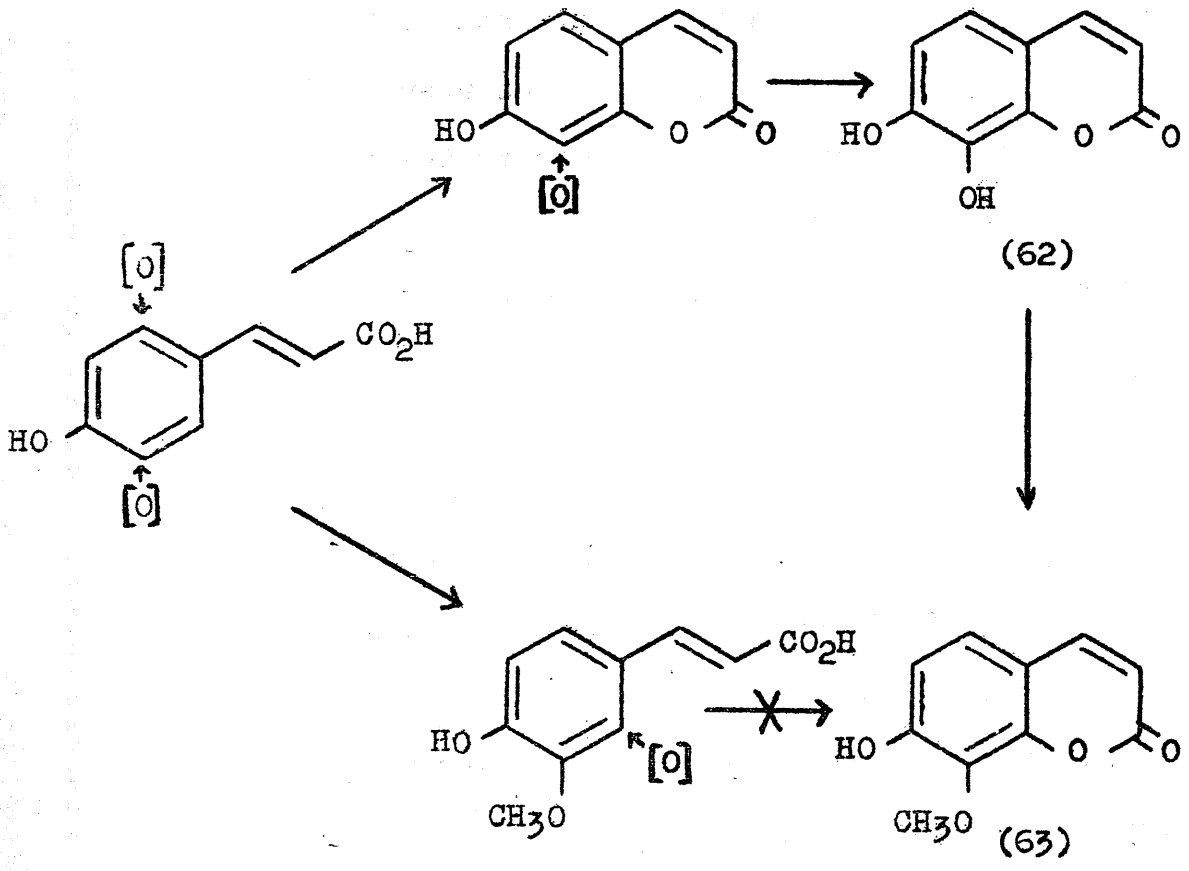
The administration of 1-^{14}C-acetic acid, U-^{14}C-glucose and 2-^{14}C-p-coumaric acid to Hydrangea macrophylla by Billek and Kindl[111,112] followed by qualitative estimation of the activity incorporated into umbelliferone (7-hydroxycoumarin) proved that only the last two were efficient precursors of this, the fundamental plant coumarin. This report led to the present radiotracer experiments on the biosynthesis of umbelliferone in hydrangea.

Brown's work on the formation of herniarin (25) in lavender and of umbelliferone (24) in hydrangea will be discussed in detail in the following section.

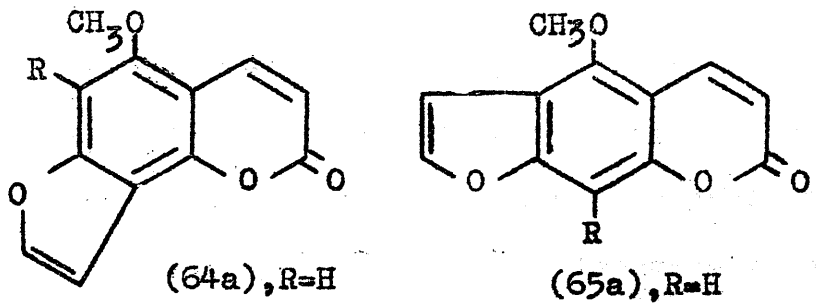
The possible pathways to scopoletin, the most frequently encountered coumarin in higher plants[113], have been investigated by Runeckles[114]. He has

concluded that the probable immediate precursor of the glucoside scopolin is cichoriin (61) (aesculetin-7-glucoside), a natural constituent of the tobacco plant.[115] The methyl group will almost certainly arise from the one-carbon metabolic pool[116]. Runeckles' prime aim was to investigate the biosynthesis of chlorogenic acid (60), but he observed that trans-2-^{14}C-cinnamic acid and 2-^{14}C-p-coumaric acid were efficient precursors of scopolin and scopoletin but not of cichoriin (61). Nevertheless, cichoriin was the most active metabolite isolated after the administration of 2-^{14}C-caffeic acid. Scopolin and scopoletin were shown to be radioactive metabolites of 2-^{14}C-ferulic acid. The possible routes of formation of scopoletin are shown in the diagram. It may be significant that aesculin, cichorin and scopolin co-occur in certain species of potato e.g. Solanum pinnastisectum[139a].

Recently, Billek and Kindl[117] have reported that the scopoletin isomer, 7-hydroxy-8-methoxy-coumarin (63) is not formed by the anticipated hydroxylation of ferulic acid ortho to the 3-methoxyl group with subsequent lactonisation.



Formation of 'Hydrangetin' (63).



(64a), R=H
(64b), R=OCH_3

(65a), R=H
(65b), R=OCH_3

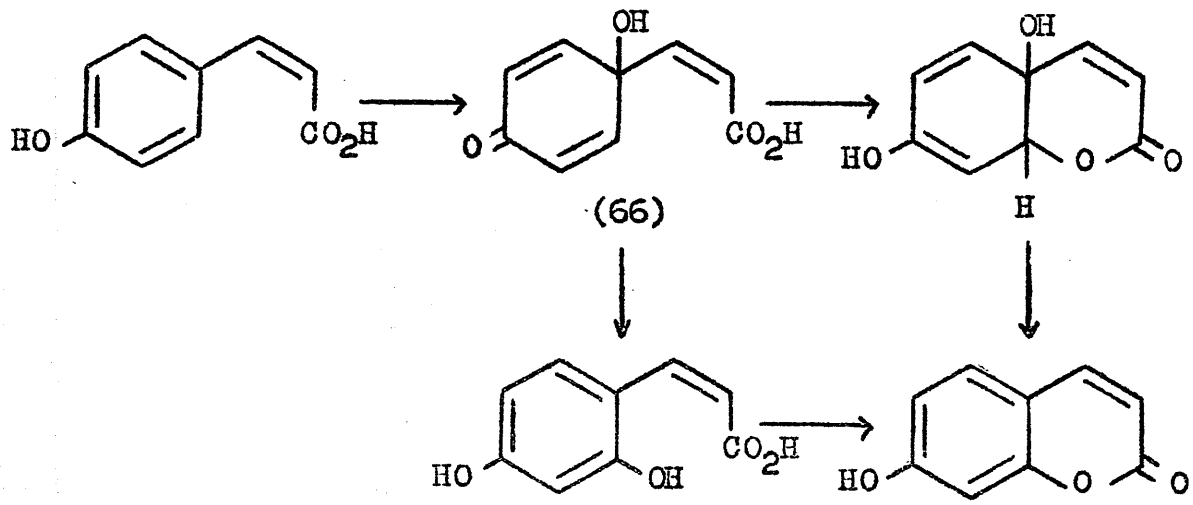
Their postulate is that it is produced rather by the 8-hydroxylation of umbelliferone to give the known daphnetin (62) which is at once methylated. This is in contrast to the previous results[108,114] which indicated that ferulic acid is the precursor of scopoletin and therefore a spatial or other requirement of the hydroxylating enzyme is implied.

The elaboration of a preformed umbelliferone nucleus is also strongly implied by the one brief communication on the formation of furanocoumarins in Pimpinella magna[13]. 2-^{14}C-Umbelliferone was a vastly superior precursor than 1-^{14}C-cinnamic acid and 2-^{14}C-coumarin for the four furanocoumarins: pimpinellin (64b), isopimpinellin (65b), bergapten (65a), and isobergapten (64a).

In spite of this general information confirming their phenylpropanoid origin, the basic problem in the biosynthesis of the oxygenated coumarins concerns the precise mode of formation of the lactone ring system. Two fundamentally different mechanisms may be envisaged. One, analogous to the scheme for coumarin biosynthesis, involves a hydroxylation ortho in trans-p-coumaric acid and thus meta to the established phenolic group. This would be followed by trans-cis isomerisation of the side-chain double

bond and lactonisation of the transitory coumarinic acid derivative. In view of the well-established ortho-para directing properties of phenolic groups, alternative schemes have been strongly favoured by organic chemists. These all involve trans-cis isomerisation of the double bond prior to an attack of the carboxyl group at the 1 or 2 positions of the ring or of a hydroxyl group at the 1 position i.e. para to the phenolic function. Appropriate rearrangements and elimination would yield coumarins.

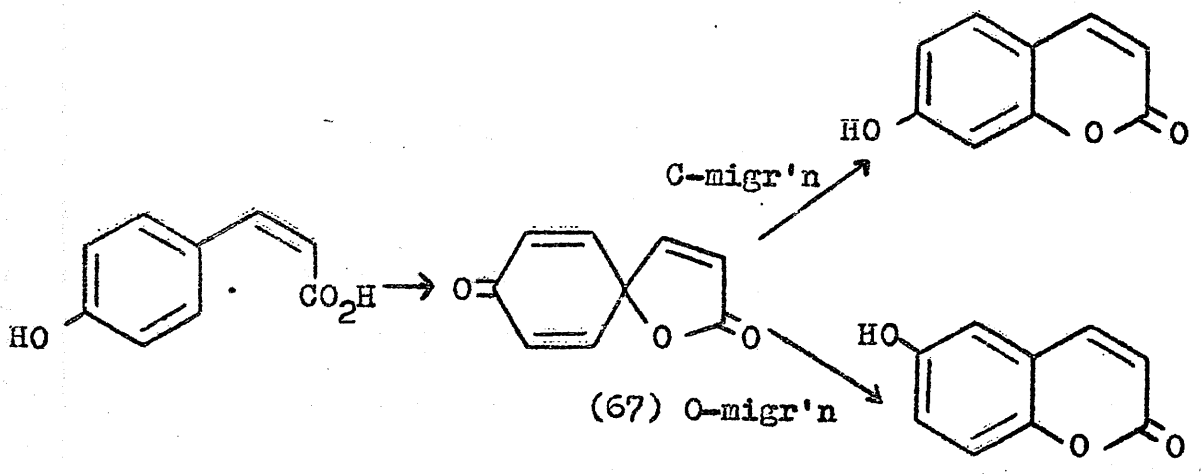
The critical difference between these schemes is that while the hydroxylation of the trans-acid cannot include the participation of the carboxyl group in the first instance, the mechanistically more attractive pathways demand that a cis-cinnamic acid derivative be the primary precursor of the oxygenated coumarins and that the carboxyl group be involved at an early stage. The latter schemes are termed the 'oxidative cyclisation routes' and one of them was proposed by Haworth as early as 1942[118]. In view of the apparent necessity of a mechanistically unfavourable hydroxylation to establish the lactone ring, he suggested an initial attack of a hydroxyl group para to the phenolic group of cis-p-coumaric acid to yield the cis-p-quinol,



(66)

(66a)

Umbelliferone formation from a cis-p-quinol (66)[118].

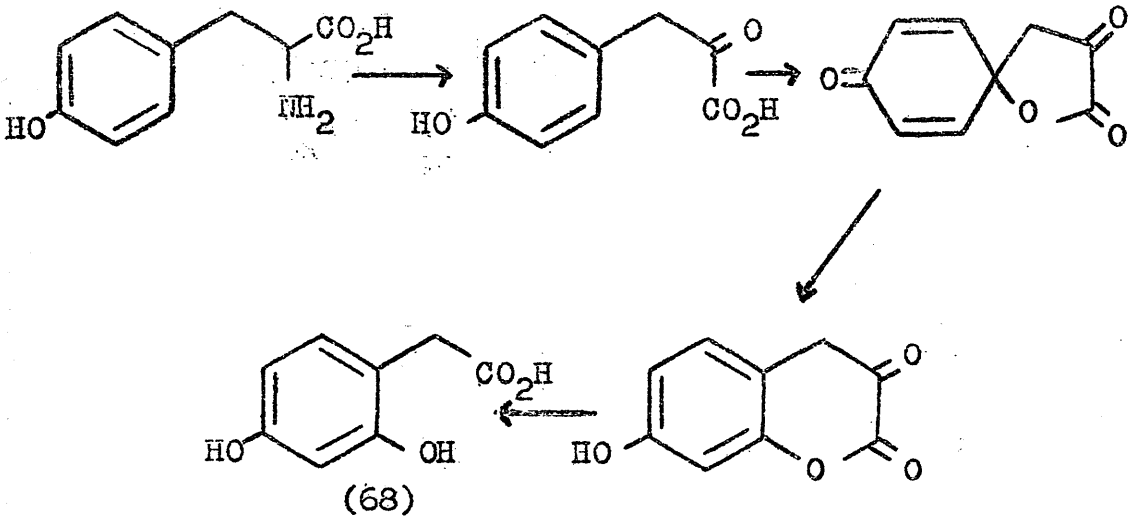


(67)

Umbelliferone formation from a spirolactone (67)[121].

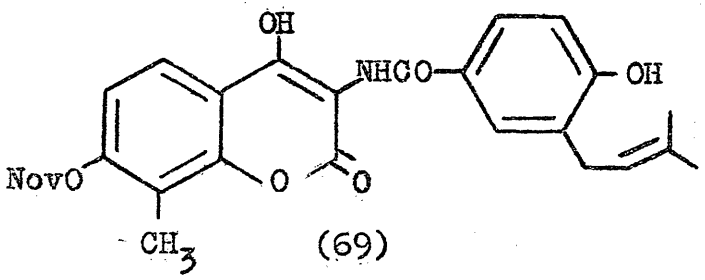
(66). His analogy was with Raper's work on the formation of melanin from dihydroxyphenylalanine[119] which had demonstrated the operation of cyclisation steps involving quinonoid intermediates. Haworth's suggestion, favoured by Birch and Smith[120], comprehended no specific cyclisation route but lactonisation onto the β-position of the dienone and subsequent dehydration of the lactone would produce the coumarin. It is clear that other rearrangements of these intermediates could result in the formation of coumarins. Thus, a 1,2-shift of the introduced hydroxyl would lead to the formation of a coumarinic acid (66a) and thence to the hydroxycoumarin.

A more recent elaboration of this general concept was proposed by Grisebach and Ollis[121] and supported by Scott[122]. It involves a direct oxidative cyclisation of cis-p-coumaric acid to give a spirolactone (67), which might rearrange by O-migration to the 7-oxygenated coumarin system. Demonstration of the feasibility of this scheme in vitro was taken as strong support for such spirolactones being intermediates in the formation of natural oxygenated coumarins[123]. However,

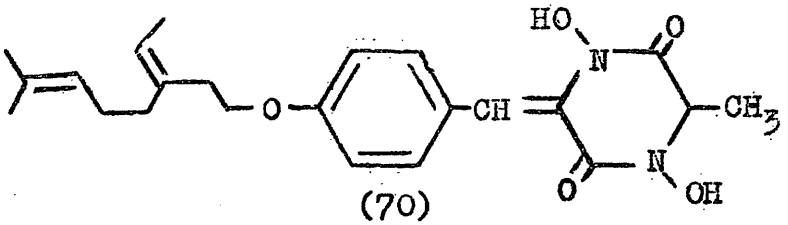


(68)

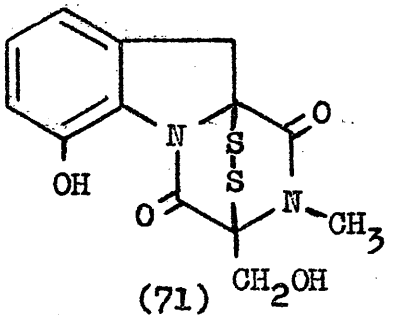
Postulated Formation of Homogentisic acid[125].



(69)



(70)



(71)

rearrangement of the spirolactone with aqueous acid produces almost entirely 6-hydroxycoumarin by C-migration [124] while treatment with base produces the related dienone hydroxy-acid and some 6-hydroxy-coumarin [124]. It is noteworthy that not one 6-mono-oxygenated coumarin is known in nature[1]. It is nevertheless true that spirolactones and spiro-dienones are well-characterised natural products and that they do participate in many biosynthetic path-ways. Thus, on the basis of model reactions, a plausible scheme (shown here) for the formation of homogentisic acid (68) from tyrosine in mammalian liver tissue has been suggested by Hassall et al.[125] A somewhat less attractive mechanism was previously postulated by Goodwin and Witkop[126].

Tracer studies on the mould metabolite (69), novo-biocin, by Kenner and his co-workers[127] have also favoured an oxidative cyclisation in the formation of the 7-oxygenated coumarin system either via a spirol-actone or direct attack by carboxyl ion or radical on the ortho position of the aromatic ring. The second mechanism does not demand the presence of a para-oxygen function but is at variance with the known pathways to coumarin. It is clear moreover

that the proposed analogy between this compound, and the plant coumarins is very strained. Thus, tyrosine and not phenylalanine is the precursor of novobiocin and deamination apparently plays no part in the sequence. In fact, it may legitimately be doubted whether any cinnamic acid derivative is involved in its biosynthesis. The structure of the compound departs significantly from the criteria laid down for the 'true' coumarins - there are two substituents on the lactone ring, and a C-methyl (unknown in the plant coumarins) at position 8. Perhaps the main objection to the attempted generalisation of the results obtained is that there is no essential correlation between the synthetic mechanisms evolved by such widely separated living systems as the fungi and higher plants. The involvement of tyrosine units as such in the production of fungal metabolites is well-exemplified by gliotoxin, (71) in which the amino-group of meta-tyrosine has apparently cyclised onto the ortho-position[128], and mycelianamide (70), in which the formation of an $\alpha\beta$-unsaturated tyrosine unit has taken place[129].

Clearly, the theories proposed to explain the formation of the 7-oxygenated coumarin system have

been designed to overcome the apparently unfavourable 'hydroxylation meta to an established phenolic group'. However, such a hydroxylation is unfavourable only in terms of the known ortho-para 'directing' influence of phenolic groups in synthetic organic chemistry and is not necessarily excluded in biological systems. Phenols are not predominantly ortho-para directing towards certain free-radical hydroxylating reagents such as the metal ion-ascorbic acid system[130,131]. Since it is recognised that enzymic aromatic hydroxylation is frequently accomplished by radical processes[132-34], it would seem that the difficulties of hydroxylation meta to the phenolic group of p-coumaric acid may well have been overestimated. In fact, Dr. Meyers has shown[124] that cis-p-coumaric acid may be exclusively meta-hydroxylated under in vitro conditions that rule out direct intervention of the carboxyl group. This was done by treatment of the cis-acid with molecular oxygen in the presence of a copper^{I,II}-ascorbic acid system in dilute aqueous solution (pH 4-6) at room temperature. Many phenol oxidases contain copper which is essential for activity and also require ascorbic acid as a co-factor[135].

For coumarins with either no oxygen substituents, or more than one, oxidative cyclisation does not have to be postulated. As already discussed, the path to coumarin involves hydroxylation ortho to the side chain while it has been shown that aesculetin is formed spontaneously in vitro on treatment of caffeic acid with manganous ions and oxygen in daylight[136,137]. In organic chemical terms, the presence of the phenolic function at the 3-position of the ring would tend to cancel the unfavourable influence of the 4-oxygen function on the required hydroxylation ortho to the side chain.

The genesis of our radiotracer experiments on the biosynthesis of oxygenated coumarins in plants was partly the result of the oxidation studies[125] briefly outlined above which cast some doubt on the necessity of the proposed oxidative cyclisation schemes. Most significant, however, was the fact that in the course of phenol oxidation studies[124], Dr. Meyers had prepared and fully characterised the spirolactone (67) one of the hypothetical intermediates of these schemes, thereby laying them open to experimental test.

2.1.d. Radiotracer Methodology.

The increased availability of radioactive organic compounds in recent years has proved of tremendous value in the analysis of the modes of action of living systems. The quantitative estimation of extremely small amounts of isolable intermediates and products following the administration of radioactive precursors has been exploited in the study of biosynthetic schemes both *in vivo* and *in vitro*.

It is important, however, to keep several cautionary facts in mind when considering the results of a radiotracer project. It may seem true that the feeding of a 'natural' precursor of some given product *should* result in a high incorporation of the precursor activity, and that an 'unnatural' precursor should not. The facts that almost every feeding amounts to an interference in the equilibrium state of the living system by a sudden flooding with exogenous precursors *and* that such interference may affect the anticipated distribution of activity must be taken into account. Furthermore, living cells possess both inherent and adaptive biological defence mechanisms quite capable of

inactivating fed material by side reactions. Thus, the compounds may not be transported to the site of synthesis but may rather be 'deposited' e.g. in the bark or heartwood. Since the feedback inhibition controls of pathways are always finely balanced, the anticipated incorporation may be blocked by the inhibition of a critical step associated with the build-up of some metabolic intermediate or product. Furthermore, the cell may dispose of the 'unnatural' precursor by using it or its breakdown products for the synthesis in question - a so-called 'aberrant synthesis'.

In view of these difficulties, it is not surprising that the general efficiency of incorporation of administered precursors is usually less than 1%, especially in tracer studies with higher plants, notorously awkward subjects. The situation is frequently otherwise in the study of mould metabolites when very high incorporation values can be obtained.

What counts in an analysis of the results of tracer studies is the coincidence of evidence to the point where the possible pathways are significantly

restricted. The words of Percival Lowell[140] are apposite here. "Proof is nothing but preponderance of probability.... Negative evidence is no evidence at all, and the possibility that a thing might be otherwise, no proof whatever that it is not so. The test of a theory is, first, that it shall not be directly contradicted by any facts, and secondly, that the probabilities in its favour shall be sufficiently great.... The odds that a thing is true from the fact that two or more witnesses agree on the same statement is not the sum of the odds that each tells the truth, but the product of these odds.... The concurrence of all (arguments) renders them not simply additively but multiplicitly effective. That different lines of induction all converge to one point proves that point to be the radiant point of the result"

Few tracer studies accord with all these stringent requirements, and few indeed can since, in the final analysis, there are so many imponderables. The best proof of a sequence at present is that its operation can be demonstrated by all available techniques. It is reassuring, but not conclusive if the individual steps can be explained rationally

in terms of known processes. Since the conclusions as to the steps in a given biosynthetic sequence rest ultimately on the measured efficiency of utilisation of a fed precursor, such efficiency must be capable of numerical expression. Although a full example of the simple mathematical treatment required will be given later, the necessary criteria may be stated as the percentage incorporation value and the dilution value for the given precursor.

The percentage incorporation value is a measure of the amount of the administered compound that has been utilised in the course of the experiment for the synthesis of the product under investigation. From the known quantities and activities of the precursor and the product the amount of the fed activity that has been incorporated into the product can be readily obtained and this is expressed as a percentage. As previously stated, the difficulties of radiotracer studies are such that an incorporation value of 1% or more is extremely high and is in itself an indication that the fed material must be closely related to the product. Of course, the problem of breakdown

and resynthesis must be taken into account, usually by analysis of the activity present in specific atoms in the product.

The dilution value is the ratio of the specific activity of the precursor to that of the product. It is an expression of the relative amounts of inactive and radioactive product isolated after the feeding. Clearly, a low dilution value suggests that the fed material is efficiently used for the synthesis of the product since the radioactive product formed during the feeding is not totally swamped by the endogenous inactive compound. Other things being equal, the higher the dilution value, the further back in a sequence is the fed precursor. In general terms, a dilution value of less than 100 suggests that the fed material is close to the 'true' pathway. Comparable values for compounds not on the pathway may range from 500 to 20,000 or more! Since the percentage incorporation value depends to a large extent on the rate of product synthesis consequent on the feeding of the precursor, and the dilution value varies with the amount of preformed product in the tissue, it is clear that to permit absolute

evaluation of a given experiment, both should be measured. For example, an efficient precursor should be well incorporated with a low dilution value. The situation may perfectly well occur that a very large amount of preformed product is present and thus a high dilution value would result. Conversely, only a tiny amount of product may be present and, although a very low incorporation of activity may be achieved, the measured dilution value may be very low, suggesting an efficient conversion. Thus the use of only one criterion could be ambiguous.

In the hydrangea experiments the counting of purified precursors and metabolites was carried out by estimation of the radioactivity of known quantities with an IDL Low Background Counter 2080 monitored with an EKCO N610B automatic scalar. This combination gave an extremely low background count (1.5 cpm) which was of critical importance in the measurement of low-activity materials from certain of the feedings. Each compound was dissolved in methanol and deposited as an 'infinitely-thin' layer on lens tissue resting in an 1" diameter aluminium

Tracer Experiment Example - VII.

A. trans-p-Coumaric acid (1.60×10^6) 2.02 mgm-1.97×10^4.

VII.	5.1%	(35)	12.50% acid	3 Days.

This method of displaying the results of radiotracer experiments will be followed in the subsequent sections.

KEY.

'A' refers to the series of feedings described.

'(1.60×10^6)' is the relative specific activity of the fed compound in cpm/mmole.

'2.02 mgm-1.97×10^4' represents the weight of compound fed and the number of cpm contained in it.

'VII' is the number of the individual experiment.

'5.1% (35)' is the percentage incorporation into the umbelliferone recovered and the calculated dilution value.

'12.50% acid' is a measure of the activity recovered after the feeding as trans-p-coumaric acid.

'3 Days' indicates the length of the metabolic period from the time of feeding to the time of extraction.

planchet. The term 'infinitely-thin' refers to the fact that below a given thickness of a dispersed radioactive compound, none of the emitted beta radiation is absorbed by the compound itself i.e. that a maximum of the beta radiation reaches the counter window. Careful trial showed that no deviation from the anticipated linear relationship between amount and activity of the radioactive compounds occurs below a planchet loading of ca. 6 mgm. All our counts were achieved with less than 5 mgm. of material, which was deposited, dried and accurately weighed.

In order to achieve sufficient accuracy in the counts, they were continued until the calculated standard error of each estimation was less than 2% and frequently still lower. Detailed treatments of the statistical problems of radiocounting are given in many textbooks[146].

To illustrate the mathematical treatment required in the evaluation of the results the best incorporation value achieved (perhaps understandably) will be described - feeding VII. Two hydrangea stems on a plant were fed with a total of 2.02 mgm. of 2-^{14}C-trans-p-coumaric acid with a specific

activity of 1.60×10^6 cpm/mmole.

This is relative activity since the efficiency of the counter used (ca.5%) was not precisely determined by comparison with an absolute ^{14}C standard. Such comparison permits the use of absolute(curie) activity terms but its lack was of no significance whatever for the present purposes since the experimental objective was comparison of different precursors.

After 5 days, the plant was cut, the stems and leaves weighed (11.0 gm) and extracted as already described to yield 3.5 mgm. of pure umbelliferone (m.wt.162.2) with an activity of 4.58×10^4 cpm/mmole. The dilution value, as previously defined, is therefore obtained by division.

$$\text{Dilution Value} = \frac{1.60 \times 40^6}{4.58 \times 10^4} = 35 \text{ (to the nearest whole number).}$$

This is a low dilution value, suggesting that the trans acid is a good precursor of umbelliferone. Real proof of this point is obtained from the percentage incorporation value calculated from a knowledge of the number of cpm recovered and the number fed.

$$\text{Cpm recovered} = \frac{3.5 \times 4.58 \times 10^4}{162.2} = 1{,}002 \text{ cpm (to four figures).}$$

The activity of the fed p-coumaric acid (m.wt. 164.2) was 1.60×10^6 cpm/mmole and its weight was 2.02mgm. Thus

$$\text{Cpm fed} = \frac{2.02 \times 1.60 \times 10^6}{164.2} = 1.97 \times 10^4 \text{ cpm.}$$

Therefore the percentage incorporation value is -

$$\% \text{ Inc.} = \frac{1.002 \times 10^3 \times 10^2}{1.97 \times 10^4} = 5.1\%$$

The reason why the dilution and incorporation values are not quoted beyond two or three figures is that the inaccuracies inherent in radiotracer counting make the last figures non-significant. The 5.1% incorporation value obtained in this particular feeding is a remarkably high value, suggesting, with low dilution value of 35, that trans-p-coumaric acid is indeed on the direct pathway to umbelliferone. The result is quoted as VII -5.1% (35).

With the efficient transport of this precursor to the site of synthesis (the leaves in hydrangea -XXXII) thus proved, it is of interest to find what proportion of the administered acid remains in a soluble free or glucose-bound form. In this case, 26.8 mgm of inactive trans-p-coumaric acid were added to the emulsin-hydrolysed basic

extract as a 'scavenger' and totally dissolved. An aliquot (4.86 mgm) of the pure sample of p-coumaric acid isolated after this addition gave 449 cpm net. Therefore justifiably assuming the weight of endogenous acid in the plant to be negligible, the number of cpm recovered can be calculated and thence the incorporation.

$$\text{Cpm recovered} = \frac{26.8 \times 449}{4.46} = 2,470 \text{ cpm.}$$

$$\% \text{ Inc.} = \frac{2,470 \times 10^3 \times 10^2}{1.97 \times 10^4} = \underline{12.5\%}$$

Since the weight of acid in the plant extract was unknown, no dilution value could be obtained for it. It would be expected to be very low, however, in view of the anticipated low quantity of endogenous p-coumaric acid before the feeding.

2.2. RESULTS AND DISCUSSION.

2.2.a. Lavender Radiotracer Studies.

The genesis of the present study was the knowledge that Brown[79] had adduced strong evidence that herniarin in *Lavandula officinalis* Chaix is formed by the glucose→shikimic acid→phenylalanine→cinnamic acid→p-coumaric acid route and that the coumarin formed simultaneously by this plant is formed from phenylalanine via o-coumaric acid and its glucoside. The co-occurrence of relatively large amounts of both coumarin and a 7-oxygenated coumarin is rare which in itself suggests that separate pools of cinnamic acid may exist in the plant which are acted on by different hydroxylating enzymes specific for the *ortho* or *para* positions. Brown demonstrated that para-oxygenated precursors were incorporated significantly only into herniarin and that the *ortho*-oxygenated compounds were utilised preferentially for the synthesis of coumarin. Since the dilution value of p-coumaric acid was quite low, it seemed clear that this acid is a direct precursor of herniarin.

The original intention, however, was to study the formation of the 'fundamental' plant coumarin

umbelliferone (7-hydroxycoumarin) and so Skimmia japonica and Daucus carota, which have been reported to contain umbelliferone[12], were analysed for this compound by extraction, hydrolysis of the glucosides present, and TLC comparison with authentic material. Neither proved to have a sufficiently high content of umbelliferone. Indeed, S.japonica exhibited at least nine fluorescent compounds. Accordingly, two varieties of lavender plants were obtained from the Glasgow Botanical Gardens by courtesy of the curator, Mr. E. Curtis, and extracted by Brown's reported method. It was found that Lavandula officinalis Chaix was not a rich source of coumarins but the cultivar described as Lavandula 'Munstead Strain' is an excellent source of the glucose-bound forms of coumarin and herniarin with smaller amounts of umbelliferone and aesculetin. This was therefore chosen as the subject for our experiments.

The radioactive precursors fed were trans-1-^{14}C-p-coumaric acid, cis-1-^{14}C-p-coumaric acid and the 2-^{14}C-spirolactone (67). A formal name for (67) is 1-oxaspiro(5,4)deca-3,6,9-triene-2,8-dione. This name has not been employed for obvious reasons.

The 1-^{14}C-trans acid was synthesised from 1-^{14}C-malonic acid and p-hydroxybenzaldehyde by an adaptation of the standard Doebner-Knoevenagel synthesis for cinnamic acids[141]. The 1-^{14}C-*cis*-acid was prepared from this by irradiation of its methanolic solution with ultraviolet light and the removal of the *trans* acid by crystallisation of the product from water (in which the *cis* form is far more soluble). Ether extraction of the solution gave the pure *cis* acid. By recycling the recovered *trans*-p-coumaric acid, it was possible to achieve conversion of over 80% of the *trans* to the *cis* form. This was used partly for feeding purposes but mainly for the synthesis of the 2-^{14}C-spirolactone (67), which was carried out by electrolysis of an aqueous solution of the *cis* acid and purification of the spirolactone from the neutral products by repeated sublimation and crystallisation[142].

Yet another point of radiotracer methodology must now be discussed, namely, the problem of introducing the precursor into the plant with maximum efficiency and with least damage. The available systems include feeding via the roots or cut petioles, by emulsion spraying onto the leaf

surfaces or by piercing the plant stem with a cotton wick and allowing the precursor solution to enter the transport system by capillary action. Since the reported technique[79] comprised root feeding, a trial was carried out. Trans-1-^{14}C-p-coumaric acid (10 mgm) with an equimolar quantity of sodium bicarbonate in 5 ml. of water was administered to the roots of a 1-year-old Lavandula 'Munstead' plant in the immediate preflowering stage. The solution (shielded from light with metal foil) was absorbed in twelve hours and a further 10 ml. of water added and absorbed in 24 hours. The plant was repotted and allowed to metabolise for six days. It was then removed from the soil, washed and weighed (7 gm.), and blended in 300 ml. of ethanol. The suspension was heated under reflux for 4 hours and the resulting green solution filtered, reduced under vacuum, and allowed to stand overnight at 0°C. The chlorophyll and fat-soluble material was removed to give a cloudy-yellow solution containing the free coumarins and their glucose-bound forms. The free coumarins were extracted with ether and were found to be negligible in quantity. The vast bulk of the coumarins were released by treatment of the solution

with almond emulsin for 3 days at room temperature and were extracted with ether as before. Repeated preparative thin-layer chromatography (TLC) of the coumarin mixture on 0.5 mm.-thick Kieselgel G with chloroform as eluting solvent resulted in a partial separation. Thus, although coumarin and herniarin ran as a single band, the umbelliferone present (0.5 mg.) was easily obtained by extraction of the intensely blue-fluorescent band corresponding to an authentic sample and fractional sublimation of the semi-crystalline eluate. The umbelliferone was found to be radioactive as a result of this trial feeding but the recovered quantity was regarded at that time as too low to permit its evaluation in the subsequent feedings.

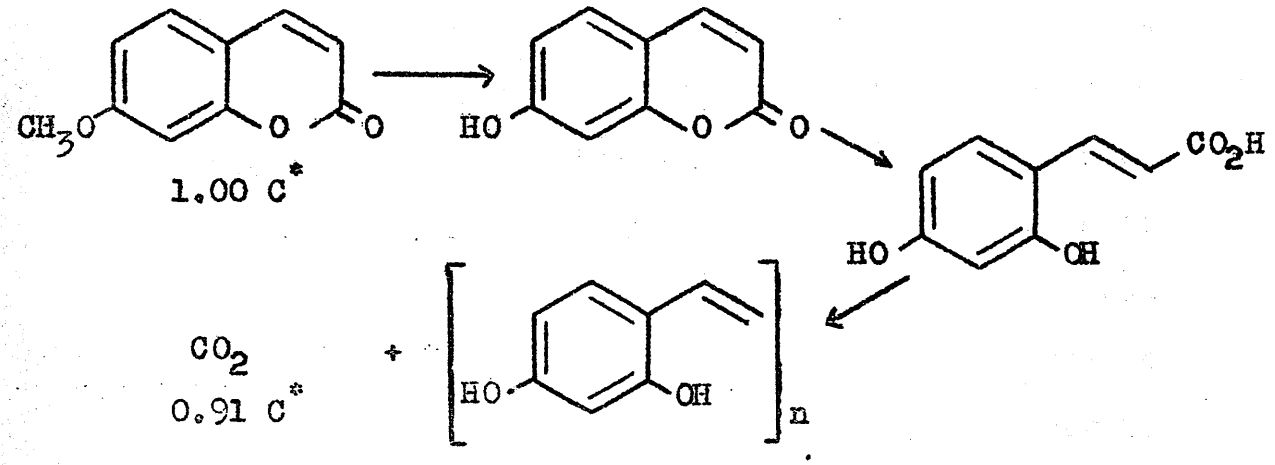
The separation and purification of the main components of the mixture, coumarin and herniarin, proved difficult because of their very similar chromatography mobility and sublimation characteristics. Accordingly, they were separated by chemical means. The binary mixture from the chromatoplate was refluxed with constant-boiling hydrobromic acid for one hour to demethylate the herniarin. The mixture of umbelliferone and coumarin thus produced was

separated and each component purified by fractional sublimation. The umbelliferone derived from herniarin was counted in a Nuclear-Chicago windowless gas-flow counter and was in fact radioactive as a result of this trial.

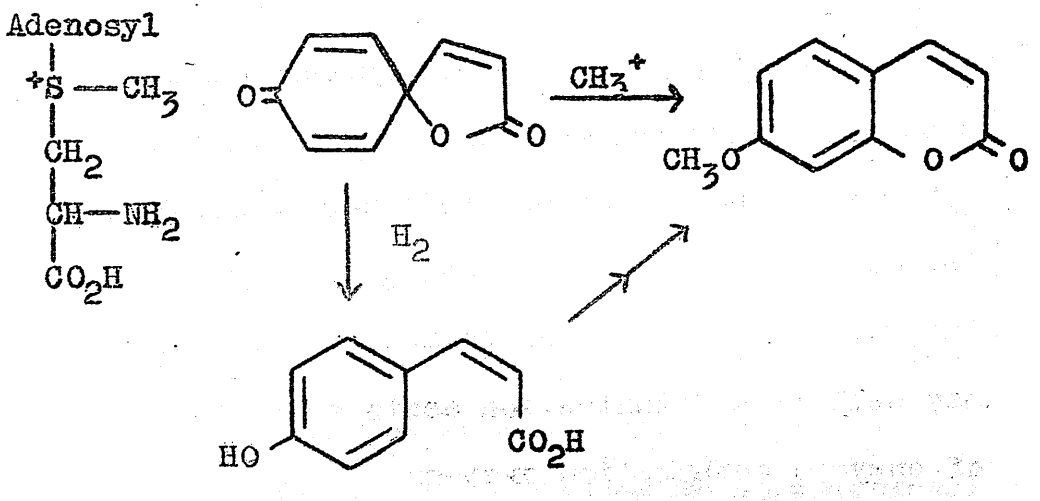
For the main lavender feedings, 30 mgm. of each precursor was synthesised and fed to three plants in equal portions as a neutral aqueous solution. The plants were repotted and allowed to metabolise for six days, the coumarin and herniarin purified as described, and their specific activities determined.

The results, expressed as the average of three feedings, show that the relative incorporations of trans-coumaric acid, cis-p-coumaric acid and the spirolactone were in the ratio were in the ratio 5:2.6:1. In contrast to Brown's findings, the absolute incorporation was very low (ca.0.02% of the fed activity) and therefore the difference in incorporation could not be accepted as definitive.

It was considered essential to prove that the incorporation of activity from the spirolactone (67) into the bound form of herniarin was not the result of breakdown and resynthesis - the so-called



Degradation of Spirolactone-derived Herniarin.



Detoxication of Spirolactone.

'scrambling' of the label. Therefore, after repeated blank trials, the umbelliferone (9.8 mgm.) purified after the spirolactone feeding was treated with alkali and yellow mercuric oxide[76] to cleave the lactone ring and isomerise the double bond. The umbellic acid produced was quantitatively decarboxylated with copper/quinoline and the carbon dioxide trapped as barium carbonate. Comparison of the activity of this compound, which represented the activity of the 1-carbon, with that of the original umbelliferone showed that over 91% of the total activity was in the 1-carbon and thus that only a small randomisation of the fed label had occurred.

A striking point in these experiments was that the plants fed the spirolactone wilted and exhibited leaf necrosis. This was decidedly not the case with the other feedings and the observed incorporation of the spirolactone into herniarin may well be a detoxication assisted by the presence of enzymic methylation systems[143]. Scott et al[123] have shown that the transitory spirolactone derived from p-methoxyphenyl-propionic acid rearranges under mild conditions to a methoxy-coumarin. An

alternative possibility is that the spirolactone may be reduced to cis-p-coumaric acid before incorporation[143].

The trend of incorporations revealed is highly significant. Contrary to the requirements of all the oxidative cyclisation theories, it is the trans-p-coumaric acid which is the most efficient precursor of the glucose-bound form of herniarin (the vastly predominant form in lavender)[80,106]. That the cis-acid and the spirolactone (67) are inferior suggests but does not prove that the proposed schemes using them as the critical intermediates cannot fully represent the situation. Without some measure of the efficiency of transport, the analysis of incorporation values must necessarily be incomplete. Thus it is clear that although the absolute incorporation values are low, they are not negligible since there is no measure available to show how much of the precursor actually reached the site of herniarin synthesis in the plant. If, for example, only 5% of the trans acid passed the physiological barrier of the roots, the observed incorporation value should be multiplied by 20 to give a true measure of the utilisation of the acid. Even

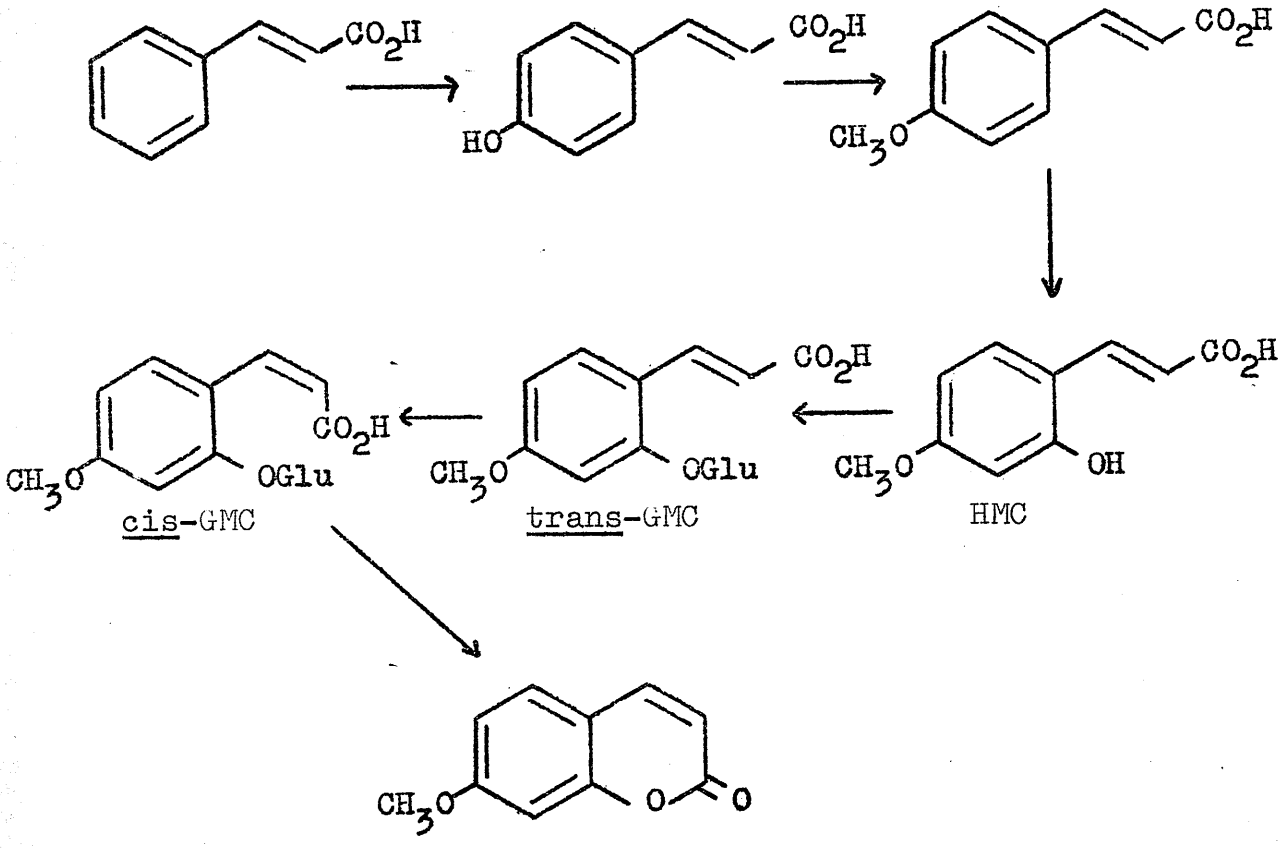
smaller incorporation values are perfectly acceptable in alkaloid biosynthetic studies.

It can be stated, nevertheless, that these results strengthen the hypothesis that the essential oxidation of the aromatic ring of oxygenated-coumarin precursors can be accomplished before the trans-cis isomerisation of the side-chain double bond i.e. that the hitherto unfavoured 'meta'-hydroxylation may well be operative.

It was abundantly clear from these results that attempts to gain meaningful information from feedings of more complex precursors via the roots would be fraught with difficulties and a search was therefore made for an improved feeding technique and for a more suitable plant subject.

In a highly significant extension of his previous studies on the biosynthesis of herniarin in lavender, Brown[80] has demonstrated that cinnamic acid is indeed a precursor of both coumarin and herniarin in lavender.

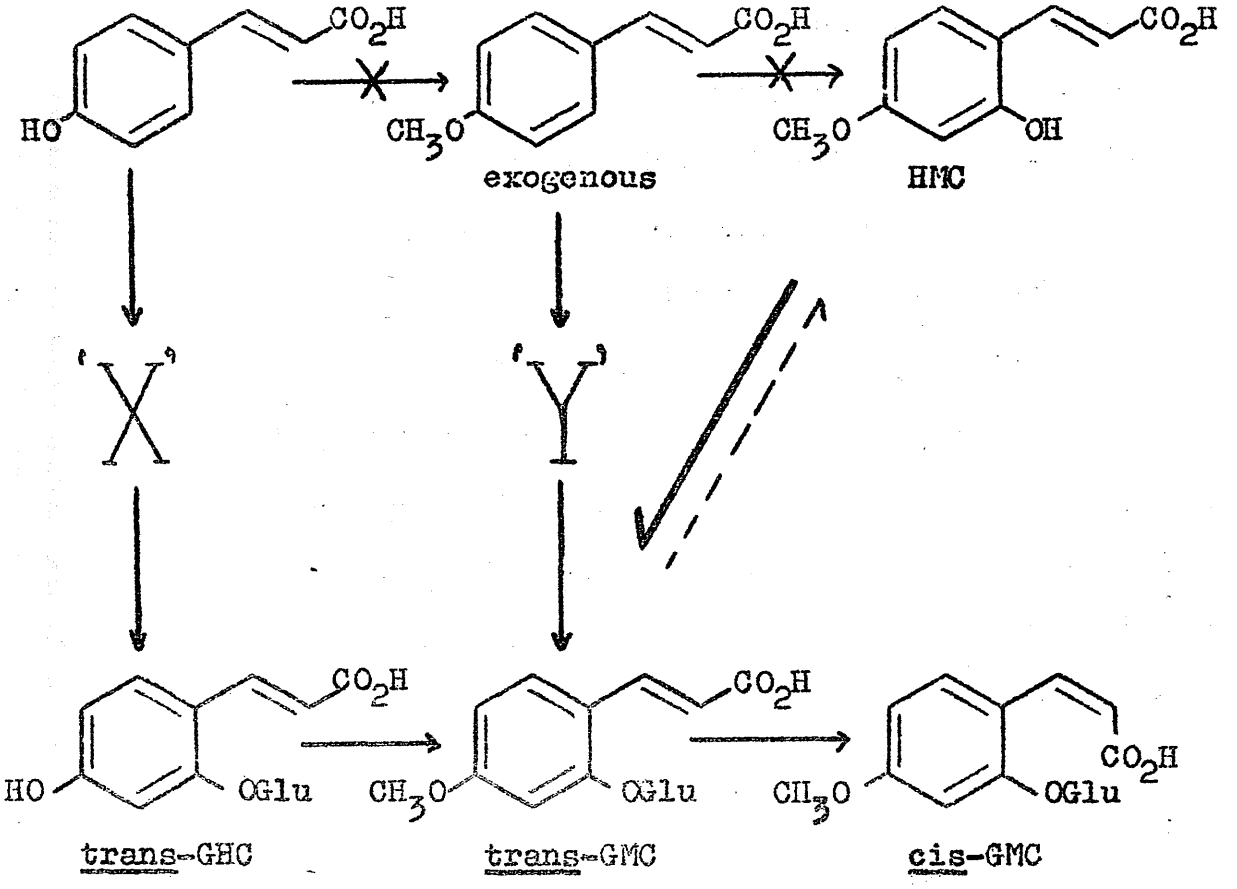
Umbellic acid and umbelliferone, while being quite well utilised for herniarin, were somewhat poorer precursors than p-coumaric acid and some 25-50 times poorer than p-methoxycinnamic acid.



S.A.Brown's scheme for the biosynthesis of herniarin[80].

By far the best precursor however was the 2-β-D-glucoside of 4-O-methylumbellic acid (HMC) referred to as trans-GMC. Since Brown also proved that the glucose-bound form of herniarin is cis-GMC and that herniarin exists predominantly in this form in lavender, it is not surprising that the trans-form is efficiently incorporated. Light- or enzyme-catalysed isomerisation would produce bound herniarin directly. Since his experiments showed that o-coumaric acid and its glucoside were selectively used for coumarin synthesis and the p-isomers were used efficiently only in the formation of the 7-oxygenated coumarin, Brown was able to propose a specific route to herniarin fully consistent with the observed dilution values of the fed precursors. This comprehends the hydroxylation of trans-p-methoxy-cinnamic acid ortho to the side chain followed by glucosylation of this introduced hydroxyl group and isomerisation of the side-chain double bond to yield the bound form of herniarin, cis-GMC. Disruption of the plant tissue and hydrolysis of this bound form (with a cis-specific glucosidase by analogy with coumarin?) will yield the lactone. Brown was careful

to point out that, although the transformation of the precursor p-coumaric acid to herniarin must involve glucoside formation after the ortho-hydroxylation step, the precise timing of the O-methylation is still uncertain. His demonstration that p-methoxycinnamic acid is a vastly more efficient precursor of herniarin than umbellic acid suggested at that time that the former compound is on the direct pathway, but he has since shown that the situation is much more complex than it might seem. In this later work, the most recent report available on the biosynthesis of 7-oxygenated coumarins, Brown[106] has adduced evidence that herniarin exists in lavender 'at least 99%' in a bound form, almost certainly cis-GMC and has identified trace amounts of a glucoside of 2-hydroxy-4-methoxycinnamic acid (HMC) in the plant, which is in fact trans-GMC. The total failure to prove the methylation of p-coumaric acid to p-methoxycinnamic acid by trapping experiments and the action of enzyme preparations is an indication that, although exogenous p-methoxycinnamic acid is very readily converted to herniarin, it may not be a 'natural' intermediate in the pathway.



S.A.Brown's extension of the scheme for herniarin[106].

This has been proposed to account for the observed greater activity of trans-GHC than of HMC after feedings of p-coumaric and p-methoxycinnamic acids. This 'anomaly' could also be explained in terms of differential solute transport. 'X' and 'Y' are intermediates of unknown structure.

Feedings of 1-^{14}C-HMC have shown that it is about one-fifth as efficient as trans-1-^{14}C-GMC, the best herniarin precursor yet found. Further trapping experiments have shown that p-coumaric and p-methoxycinnamic acids are converted to glucose-bound HMC, presumably trans-GMC, with greater efficiency than they are to free HMC. His rationale of this finding, which is contrary to earlier assumptions that free HMC should be a precursor of the bound form requires that trans-GHC (o-glucosyl -umbellic acid) should be formed from p-coumaric acid and then methylated to give trans-GMC. Exogenous p-methoxycinnamic acid may somehow be converted to trans-GMC without the intermediate formation of HMC and thus will be efficiently incorporated. In this connection, it would be of great interest to know whether the methyl group of fed p-methoxycinnamic acid is the same as the methyl group of the final product of the sequence, cis-GMC.

2.2.b. Hydrangea Radiotracer Studies (1).

Our experiments with lavender provided some evidence against the proposed oxidative cyclisation schemes. Brown's compelling evidence in favour of the mechanistically unfavourable meta-hydroxylation process prompted more detailed study

of the operative oxidation mechanisms in the formation of 7-oxygenated coumarins.

Billek and Kindl[44] had carried out qualitative studies on the biosynthesis of umbelliferone in Hydrangea macrophylla Ser. which showed that, while acetate is not a direct precursor, both glucose and trans-p-coumaric acid are efficiently incorporated.

The first object was therefore to find a suitable species of hydrangea. Accordingly, fresh stems and leaves of H.sargentiana, H. macrophylla, H. hortensia and H.arborescens-grandiflora were obtained by various means. The plant material was extracted as before and submitted to acid hydrolysis followed by ether extraction of the aqueous solution. Fractional sublimation of the product gave a high-temperature portion which was weighed and analysed by TLC. Of these four, H.macrophylla was clearly the best source of umbelliferone since it gave the greatest quantity least contaminated by other compounds. Later analysis of H.villosa, H.paniculata-grandiflora and H.petiolaris reinforced this finding.

Three cultivars of H.macrophylla were obtained

commercially and evaluated for the production of umbelliferone. The cultivars 'Bouquet Rose' and 'Blue Bird' were inferior to 'Blue Wave' which consistently produces some 0.03% of umbelliferone from fresh plant stems and leaves. The subject for the present research was Hydrangea macrophylla Ser. variety 'Blue Wave', one of the many familiar ornamental species.

In passing, it may be noted that hydrangea is a good example of the delightfully inconsequent applications of plants by man. Thus, aqueous extracts of the roots and rhizomes of H. arborescens (a rich source of umbelliferone) were used by the Cherokee Indians as a specific against urinary calculi and cystitis[144]. Hydrangea is also used in the Japanese ceremony of 'Hanamatsuri' (Flower Festival Time) which is carried out to this day in honour of the birth of the Buddha. 'Amacha', a tea-like decoction of the dried leaves of H. macrophylla Ser. variety Thunbergii Makino, is served as part of the religous ritual. Since this particular variety is a good source of the sweet-tasting isocoumarins phyllodulcin and hydrangenol,[145] it was not surprising that equivalent extracts of

Growing Box - Feedings II-VII in progress.

Wick-feeding Technique - Experiment No.VII.

our own plants were distasteful in the extreme. To provide a controlled environment, a growing box with 'daylight'-type fluorescent lighting and electrical heating was built under the supervision of Mr. A. Hislop to our design. When in use, the temperature varied from 17° to 22° and the humidity was almost constant at 50%. The 1-year old plants exposed to a 16-hour 'day', were grown in a siliceous earth 'Peralite' and top-watered regularly with a modified Hoaglands nutrient solution. In a room with large window area, the plants flourished under these conditions and were suitable for feeding experiments after some two months. A comparison of the results of the feedings at the inception and conclusion of the overall tracer experiments suggests that no significant change in their umbelliferone metabolism has occurred during this period (but see[139]). The growing box was so successful that tracer experiments were possible even through a Scottish winter.

Since root-feeding had proved inefficient, a technique of wick feeding was adapted from that reported by Leete for his alkaloid studies. The (usually) woody stem was pierced with a single

cotton thread introduced into a small sample tube containing a neutral aqueous solution of a known amount (usually 6.1 μmole in 0.1 ml) of the radioactive precursor. Capillary action resulted in the uptake of this liquid within a few hours, after which a 'chaser' 0.1 ml. of water was added to the tube and absorbed. Two stems were fed for each plant and trials showed that essentially all the active material was taken up by the plant. This was undoubtedly due in part to the efficient liquid transport system of hydrangea which has proved to be an excellent subject for these experiments.

This new feeding method constituted a tremendous advance in technique and permitted detailed investigation of the operating pathways.

The incorporation of precursor activity into umbelliferone was measured after the purification procedure now described. At the end of the appropriate metabolic period, the plant stems were cut below the site of feeding. The stems and leaves were blended in boiling 80% ethanol (500 ml.) to inactivate endogenous glucosidases as far as possible, and the suspension heated under reflux

for two hours. After filtration, the ethanol was removed in the reduction of the extract to 50 ml. bulk. Almond emulsin (0.1% w/v) was then added in solution to hydrolyse the β-glucosides present. After incubation at 30° for two days, sodium bicarbonate (0.6 gm.) was added to retain free acids and the solution continuously extracted with ether. The ether-soluble residue was applied as a band on 0.5 mm.-thick Kieselgel G chromatoplates and eluted with ethyl acetate-chloroform (1:4) to produce a sharp umbelliferone band intensely blue fluorescent in ultraviolet light (350 mμ). This band was excised and placed in methanol overnight. Filtration and evaporation gave a residue which was carefully sublimed under vacuum (120-160°; 0.02 mm Hg). The sublimate, which was completely pure umbelliferone, was weighed and, if necessary, a known weight of non-radioactive umbelliferone sufficient to yield a total of 3-5 mgm added. Twice repeated crystallisation from water provided umbelliferone as needles, m.p. 228-232°. After drying at 110° under vacuum, the umbelliferone was counted. Stringent trials have shown that consistently over 90% of the plant umbelliferone is recovered by the procedure

described and that the contamination of the compound by the fed radioactive precursor is essentially nil ($< 0.02\%$).

In many of the experiments it was desired to measure the activity incorporated into both free and glucose-bound trans-p-coumaric acid to provide valuable information on the uptake of precursor activity into this important intermediate. This was done by a so-called 'inverse dilution analysis'[146]. Addition of sodium bicarbonate to the emulsin-hydrolysed plant extract before removal of the umbelliferone was followed by a 'scavenging' of any active p-coumaric acid present, with a large (ca. 30 mgm) known quantity of inactive compound. Acidification of the solution followed by ether-extraction and repeated crystallisation of the diluted radioactive acid produced a quantity of pure material which was counted after drying under vacuum. Its purity was rigorously proved by GLC analysis of the methyl ester. Feeding 1 was a trial of the overall feeding, extraction and purification systems. The incorporation of activity from trans 2-^{14}C-p-coumaric was 1.3% after 3 days, some 65 times the value achieved in the lavender studies.

FEEDINGS.

A. trans-p-Coumaric acid (1.825×10^6) 2.24 mgm-2.49×10^4.

I.	1.305%	(286)	4.21% acid	3 Days.

A. trans-p-Coumaric acid (1.60×10^6) 2.02 mgm-1.97×10^4.

II.	4.02%	(53)	8.10% acid	1 Day.
III.	2.6%	(49)	18.90% acid	
IV.	4.2%	(57)	-	3 Days.
V.	2.7%	(41)	6.90% acid	
VI.	4.9%	(51)	12.40% acid	5 Days.
VII.	5.1%	(35)	12.50% acid	

B. cis-p-Coumaric acid (1.60×10^6) 2.00 mgm-1.95×10^4

VIII.	0.49%	(319)	8.90% acid	1 Day.
IX.	-		12.90% acid	
X.	0.33%	(112)	15.80% acid	3 Days.
XI.	0.26%	(778)	13.20% acid	
XII.	0.65%	(217)	19.20% acid	5 Days.
XIII.	1.16%	(72)	22.40% acid	

C. trans-Cinnamic acid (1.82×10^6) 2.00 mgm-2.35×10^4.

XIV.	0.24%	(119)	4.40% acid	1 Day.
XV.	-		6.22% acid	3 Days.
XVI.	0.33%	(118)	9.35% acid	5 Days.

E. Spirolactone (1.19×10^6) 1.90 mgm-1.40×10^4.

XVIII.	0.062%	(1140)	6.70% acid	1 Day.
XIX.	0.094%	(2430)	3.60% acid	3 Days.
XX.	0.146%	(1470)	5.90% acid	5 Days.

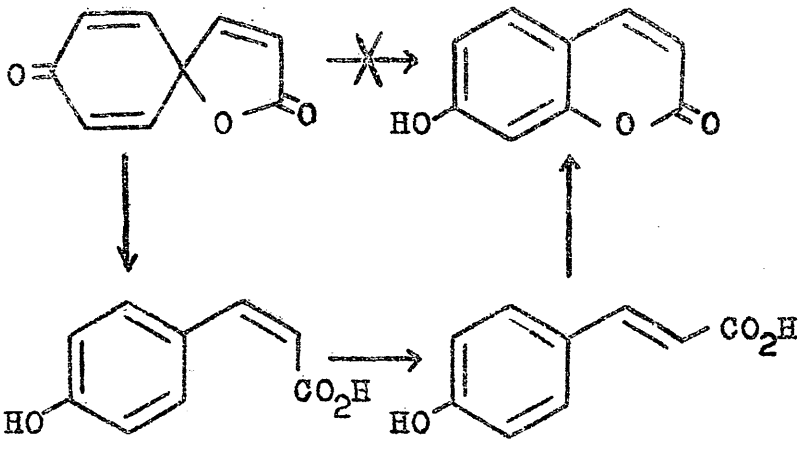
All of the compounds fed to hydrangea were labelled with ^{14}C in the 2-position of the side-chain. They were administered as neutral aqueous solutions (shielded from light) by cotton wick into the stem. After the given period the stems and leaves were extracted as detailed in the discussion.

The main experiments consisted of the feeding of equimolar quantities of the 2-^{14}C-precursors synthesised from 2-^{14}C-malonic acid to randomly selected plants for periods of 1,3 and 5 days. This was to permit an analysis of the time-course of incorporation, frequently significant for detailed mechanistic studies.

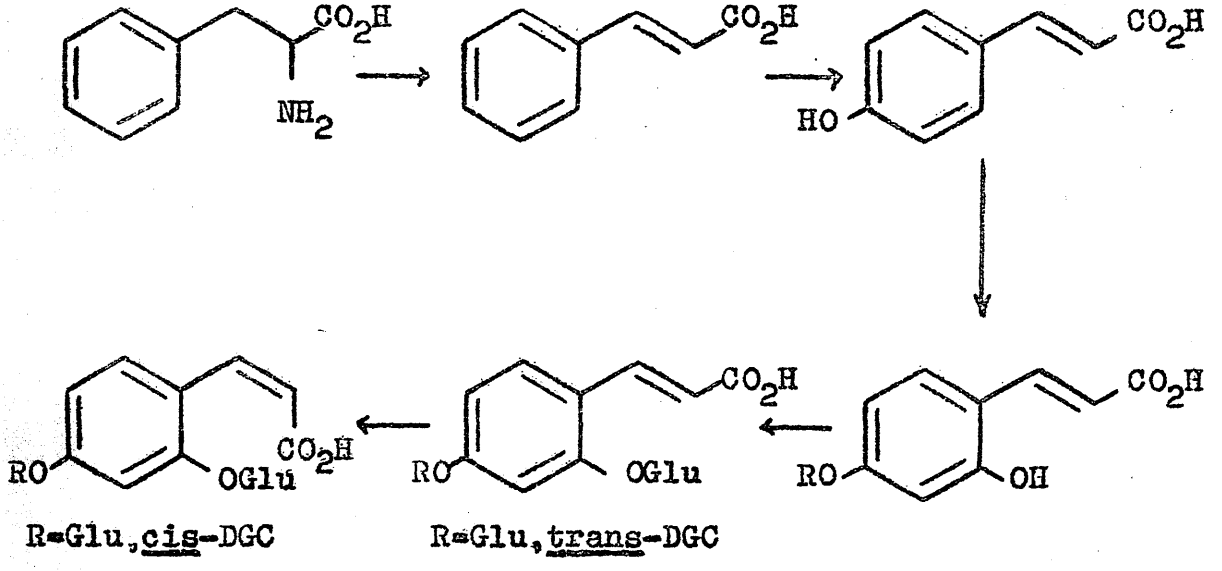
The results (II - XVI, XVIII - XX) demonstrate in a gratifying clear-cut manner that trans-p-coumaric acid proved to be an excellent precursor of umbelliferone in hydrangea whereas cis-p-coumaric acid was only about one-seventh as efficient. This latter incorporation may in fact be the result of isomerisation (light-induced or otherwise) to the trans-acid, probably in the leaves. This is entirely consistent with the observed low recovery of the trans acid, which would be expected from its known metabolic activity as a lignin[39d] or, indeed, carbohydrate[147] precursor. The consistently higher recovery of trans-acid formed by isomerisation of the administered cis form may be readily explained. When fed, the acids are bound predominantly as insoluble esters[148], but also as soluble glucose esters[110] or glucosides[84] by the enzymic

glucosylating systems present. While trans-p-coumaric acid and/or its conjugates readily enter the biosynthetic pathways leading to lignin and to other products including umbelliferone, the soluble cis compounds, as 'unnatural' precursors, may be transported to the leaves where the normal light-induced equilibration of cinnamic acids takes place. This equilibration will give rise to the trans forms which may then enter into the pathway to umbelliferone. It is however frequently true that the steps in a biosynthetic sequence are separated not merely in time but also in space. It is probable therefore that the cis acid conjugates may not be transported to the same cell organelles as the 'natural' trans forms, and thus the cis-derived trans acid conjugates would require further transport to the site of umbelliferone synthesis. The results of feeding XXV favour this general hypothesis and will be discussed in due course.

The spirolactone (67), a theoretical intermediate in certain of the proposed oxidative cyclisation routes, was still less utilised in the formation of umbelliferone than the other, cis-p-coumaric acid. In the event, it was less than one-fortieth as efficient as trans-p-coumaric acid.

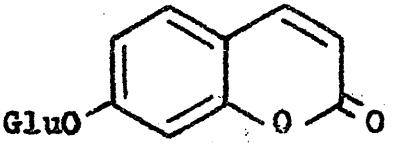


Rationale of spirolactone incorporation, XVIII-XX.



R=Glu, cis-DGC R=Glu, trans-DGC

First proposed scheme for umbelliferone biosynthesis[150].



Skimmin

The strong possibility exists that even this small incorporation was not achieved by direct rearrangement to umbelliferone or skimmin since the trans-p-coumaric acid recovered after the spirolactone feedings was appreciably radioactive. This may be explained in terms of a hydrogenolysis of the spirolactone yielding cis-p-coumaric acid, a fraction of which may isomerise to the trans form.

The excellent incorporation of trans-cinnamic acid itself into p-coumaric acid and thence into umbelliferone is in accord with the previously discussed fact that phenylalanine, rather than tyrosine, is the precursor of p-coumaric acid in plants other than grasses[69, 149]. The combined evidence negates the basic premise of the oxidative cyclisation theories that a cis-p-oxygenated cinnamic acid derivative must be the primary precursor of the 7-oxygenated coumarin system. Since these results so strikingly excluded oxidative cyclisation as the major pathway for the biosynthesis of umbelliferone, a generalisation of Brown's scheme for herniarin[80] was proposed instead[150].

The fore-going analysis of these early experiments includes concepts which were not envisaged

at the time, such as the possible formation of soluble and insoluble ester conjugates. The present state of knowledge demands their present discussion.

It was clear from preliminary paper and thin-layer chromatographic evidence that the major part of the umbelliferone within hydrangea is not present as such but is rapidly released on emulsin hydrolysis of some glucose conjugate. Before the adoption of the technique of TLC on cellulose powder, it was not possible to identify skimmin (umbelliferone β-D-glucoside) in plant extracts due to the presence of interfering fluorescent compounds and we proposed[150] that the bound form of umbelliferone might be cis-2,4-di-β-D-glucosyloxycinnamic acid (cis-DGC) formed by light-induced isomerisation of the trans form. Later, and vastly improved, TLC techniques have permitted the assignment of skimmin as by far the main bound form of umbelliferone with cis-DGC as a minor component. Therefore, unless otherwise stated, the incorporation data recorded for these experiments refer principally to the activity found in skimmin.

Feeding XXI was primarily designed to produce a measure of the proportion of the glucose-bound form

FEEDINGS.

F. trans-p-Coumaric acid (1.11×10^6) 4.9 mgm-3.32×10^4.

*XXI.	Free.	0.11%	(53)	10.4%acid 1 Day.
	Bound.	0.81%	(41)	

H. cis-p-Coumaric acid (2.3×10^6) 3.0 mgm-4.20×10^4.

XXV.	Free.	0.033%	(368)	1 Day.
	Bound.	0.33%	(381)	

G. trans-p-Glucosyloxycinnamic acid (2.17×10^6) 4.11mgm-3.32×10^4.

XXII.	2.5%	(85)	7.40% acid	1 Day.
XXIII.	4.1%	(41)	1.30% acid	3 Days.
XXIV.	3.2%	(29)	11.20% acid	5 Days.

*10.4% acid value in XXI is an estimate from feedings I-III.

present. A large amount of trans-p-coumaric acid was fed to hydrangea for 1 day and the plant extracted as before. However, the cloudy-yellow aqueous solution containing both free and glucose-bound umbelliferone was extracted with ether to remove the free phenol before the addition of emulsin. A second ether extraction removed the hitherto bound umbelliferone. The relative weights and activities of the free and bound umbelliferone demonstrated that over 85% is in fact present in the bound form and that the activity of this bound material is slightly higher than that of the free. This strongly suggests that the formation of bound umbelliferone must precede that of the free. Furthermore, this result is in direct contradiction to the postulated oxidative cyclisation pathways which all comprehend the formation of free umbelliferone before the bound.

Anticipating the true order of feedings somewhat, XXV is of interest in this connection. Stoker and Bellis[87] had shown that cis-cinnamic acid is incorporated, presumably via the unstable coumarinic acid, directly into coumarin without the prior formation of a bound form. The possibility existed, contrary to the previous interpretation of

the results of VIII-XIII, that cis-p-coumaric acid might be similarly incorporated into umbelliferone in hydrangea. Therefore, cis-2-^{14}C-p-coumaric acid was fed to a hydrangea plant and the incorporations into free and bound umbelliferone determined after a one-day metabolic period.

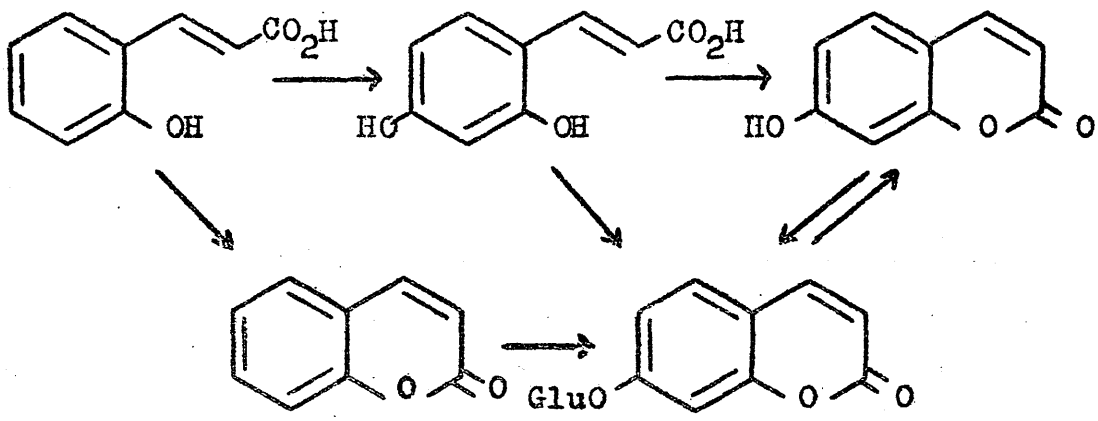
The results show unequivocally that the situation in *M. alba* is not paralleled in hydrangea since the free umbelliferone (9% by weight) is not significantly more active than the bound fraction (91%), a finding which lends strong support to the hypothesis that the exogenous cis-acid is necessarily isomerised to the trans form before its activity can be incorporated into umbelliferone. Yet again this result militates against the oxidative cyclisation theories.

It is well known that phenolic acids can be converted into glucose esters and glucosides when fed to higher plants[110,151,152]. Since the proposed scheme could include the ortho-hydroxylation of trans-p-β-D-glucosyloxycinnamic acid as a critical step in the formation of skimmin, the decision was made to evaluate the precursor efficiency of the p-glucoside by direct experiment. The 2-^{14}C-labelled glucoside was prepared by the technique of

methyl esterification of the free acid and reaction of this phenolic ester with acetobromo-glucose[153]. Selective removal of the blocking methyl and acetyl groups was accomplished by treatment with barium hydroxide solution. The required glucoside was then purified by repeated crystallisation from methanol. Paper-chromatographic analysis showed no significant contaminant.

The results of feedings XXII-XXIV show that the glucoside of p-coumaric acid is a very good precursor of skimmin in hydrangea and, indeed, is apparently as efficient as the aglycone. An interesting point from these results is that the dilution values into skimmin decreases significantly with time, suggesting that the p-glucoside may be less readily metabolised to lignin etc. within the plant than its aglycone and thus that a proportionately greater amount of activity should be found in skimmin. It must be emphasised that, although this p-glucoside is efficiently incorporated into skimmin, no further evidence has been found in the course of this work either confirming or denying its status as a 'natural' precursor.

Thus far, it has been shown that <u>trans-para-</u>



Pathways for incorporation of o-coumaric acid.

FEEDINGS.

I. o-Glucosyloxycinnamic acid (8.26×10^6) 3.84 mgm-9.75×10^4.

XXVI.	0.025% (3850)	1 Day.
XXVII.	0.025% (4900)	3 Days.
XXVIII.	0.024% (5000)	5 Days.

J. o-Coumaric acid (1.08×10^6) 2.1 mgm-1.39×10^4.

XXIX.	0.10% (850)	1 Day.
XXX.	0.12% (802)	3 Days.
XXXI.	0.14% (804)	5 Days.

oxygenated cinnamic acids are good precursors of 7-monooxygenated coumarins. By analogy with Brown's work on herniarin, ortho-oxygenated precursors should be decidely inefficient for the biosynthesis of umbelliferone.

The synthesis of 2-C^{14}-o-coumaryl glucoside was achieved by the condensation of helicin (salicyl-aldehyde glucoside) with 2-^{14}C-malonic acid[80] and o-coumaric acid was obtained from its glucoside by emulsin hydrolysis.

The anticipated low utilisation of these precursors is convincingly demonstrated by the experimental facts. (XXVI-XXVIII; XXIX-XXXI). These results leave little doubt regarding the order of the hydroxylation steps operating in the formation of the necessary 2,4-dioxygenated-trans-cinnamoyl precursor of skimmin and thus, ultimately, of umbelliferone. Ortho-hydroxylation must follow para and so the critical step in the biosynthetic scheme is hydroxylation ortho to the side-chain of a para-oxygenated-trans-cinnamic acid derivative.

As will become apparent, the gross effect of this mechanistically unfavoured process could be achieved by indirect and mechanistically feasible means.

The observed incorporation of activity from 2-^{14}C-o-coumaryl glucoside was so low as to approach the zero level of the counting procedure employed. The significantly higher uptake of labelled o-coumaric acid is worth discussing since it is of the same order as that found for trans-cinnamic acid and could arise in one of two interesting ways. Clearly the o-coumaric acid has not been O-glucosylated before its incorporation since the values for the glucoside are 4-6 times lower. The o-coumaric acid may therefore be hydroxylated para to the side chain, i.e. meta to the established phenolic hydroxyl, yielding umbellic acid for transformation to umbelliferone. Alternatively, o-coumaric acid may be isomerised by light or otherwise to form coumarin via the unstable coumarinic acid. Hydroxylation of coumarin at the 7-position (as in certain bacterial cultures) would produce umbelliferone without the necessary intervention of a glucose conjugate.

Previous work had established that free coumarin is rapidly metabolised within the sweet clover plant[74,81] and feeding XVII was intended to show whether rapid breakdown of the bound form of umbelliferone occurs in hydrangea. A relatively large amount

FEEDINGS.

D. trans-p-Coumaric acid (1.60×10^6) 5.1 mgm-4.98×10^4.

XVII.	2.85%	(36)	2.11% acid	21 Days.

K. trans-p-Coumaric acid feeding to a single-stemmed plant.

XXXII. (2.3×10^6) 2.0 mgm-2.80×10^4.

Leaves.	0.58%	(36)		0.41 mgm.
Stem.	0.66%	(146)	Total 1.29%	2.07 mgm.
Roots.	0.05%	(18,300)		16.78 mgm.

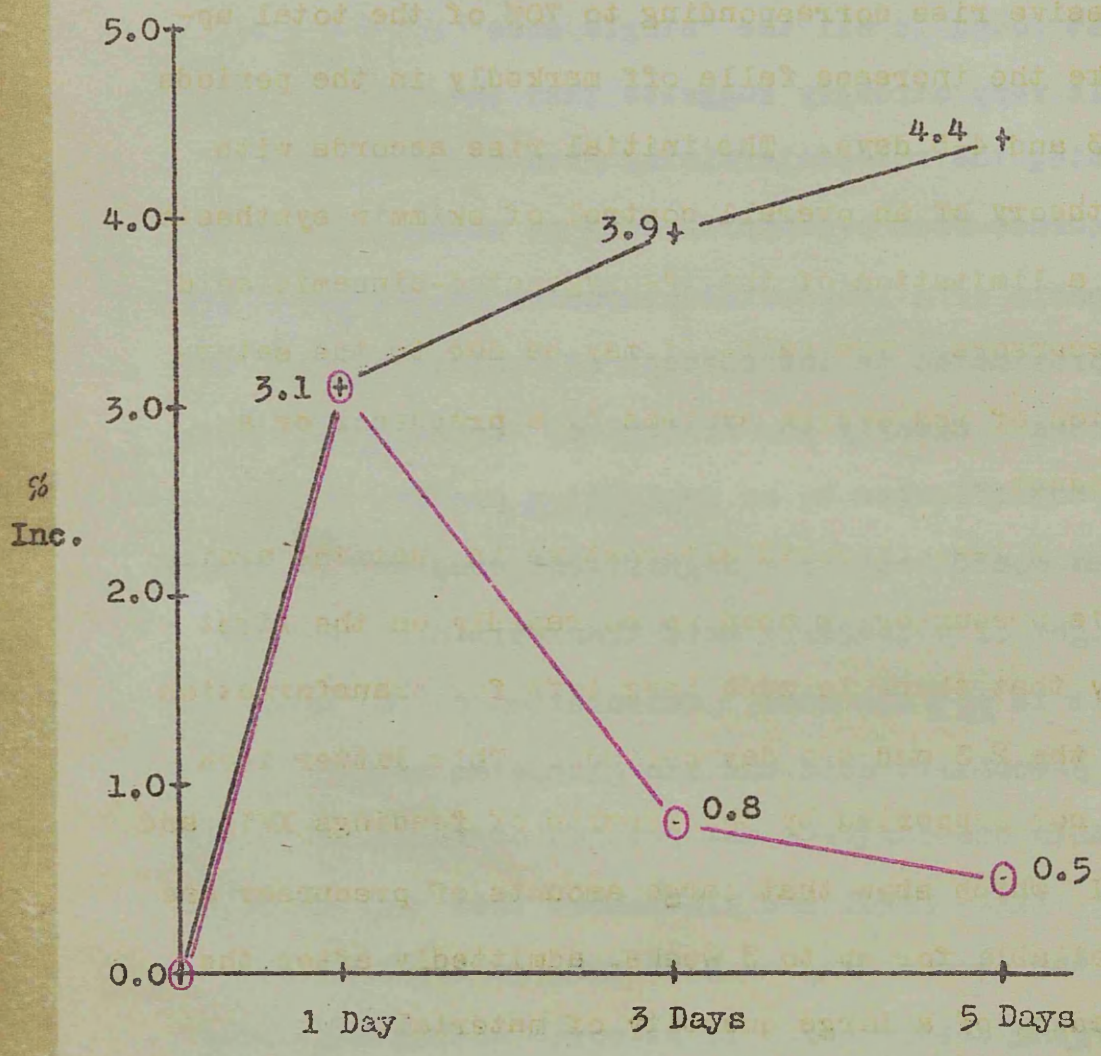
of labelled p-coumaric acid was fed to two stems of a hydrangea plant and allowed to metabolise for 21 days. The result obtained can be directly compared only with that of feeding XXl because of the high dose rate of precursor. Such comparison suggests that there is an inverse relationship between the incorporation into bound umbelliferone and the amount of recoverable free and glucose-bound p-coumaric acid with time. This is entirely reasonable in terms of the hypothesis that the metabolic pools of p-coumaric acid and its conjugates are indeed the precursors of bound umbelliferone. As the metabolic period after feeding increases, so these pools would be expected to diminish.

The three-fold increase in incorporation over three weeks indicates that skimmin, once formed, is resistant towards further metabolism since, if the destruction of the newly-formed bound umbelliferone were to proceed at a rate comparable to that of its synthesis, no significant increase would be possible. Furthermore, if account is taken of the fact that translocation of skimmin from the leaves to the roots is a continual process, the true incorporation increase should be still higher.

This significant point concerning the fate of skimmin was demonstrated by feeding XXXII, for which a single-stemmed hydrangea was fed the usual total amount of radioactive p-coumaric acid (12 μmole) for one day. The leaves, stems, and roots were separately extracted and the weight and specific activity of the umbelliferone recovered from each determined. The results strongly suggest that the leaves are, on a percentage for weight basis, the most active site of synthesis. It can be readily envisaged that rapid translocation of the skimmin accounts for the observed activity in the stems and roots. It is furthermore clear that while the roots contain the highest concentration of umbelliferone, the woody stem also has a fairly high proportion of the compound, possibly deposited in the lignified cells. It has been shown in later work (to be described) that over half of the umbelliferone in the roots is in the form of skimmin. It is reasonable to assume therefore that, after synthesis in the leaves, the skimmin is transported to the roots where it accumulates.

In this feeding, XXXII, the plant was exposed to a relative 'double dose' of precursor. In view

of this, it is striking that the total incorporation of 1.29% in the stems and leaves is close to half that found in all the 'single dose' experiments. This very strongly suggests that the enzymes mediating the rate-determining step or steps in the sequence from p-coumaric acid to skimmin are only capable of a low, defined, turnover of precursor and approximates to the concept of 'saturation' of the system. Clearly the control of the substrate flow is not effected by an inhibition process since that would lead to a significant drop in the actual weight of p-coumaric acid transformed to skimmin. This is not the case. Calculation of the quantity of p-coumaric acid and its glucoside transformed within one day (from the results of feedings II-III, XXI, XXII, XXXII and XXXV) shows that they are all of the same order, ca. 0.05 mgm. in spite of the varying dose rates. It is worth noting that this apparently small turnover of precursor represents the synthetic abilities of hydrangea when it is operating at maximum rate immediately after flooding with precursor. This is shown by a graph of the average incorporation of 2-^{14}C-p-coumaric acid and its glucoside occurring within each metabolic



Chain-label incorporation from trans-p-coumaric acid and its glucoside. (average of II-VII, XXII-XXIV and XXXV-XXXVII).

——+—— = total incorporation to end of period.

——⊙—— = increment over previous percentage.

period, reasonably regarding the incorporation of activity as an additive phenomenon. After an initial massive rise corresponding to 70% of the total uptake the increase falls off markedly in the periods 2-3 and 4-5 days. The initial rise accords with a theory of an overall control of skimmin synthesis by a limitation of the p-oxygenated-cinnamic acid precursors. The fall-off may be due to the saturation of the enzyme systems by a precursor or a product.

A less favoured alternative is that the available precursor is used up so rapidly on the first day that there is much less left for transformation in the 2-3 and 4-5 day periods. This latter idea is not supported by the results of feedings XVII and XXI, which show that large amounts of precursor are available for up to 3 weeks, admittedly after the feeding of a large quantity of material.

There seems little reason to doubt that the formation of skimmin in hydrangea is under a strict control and that the normal rate of synthesis is much less than that induced by the feeding of precursors.

	2% HAc on paper	5% HAc-TLC cellulose	AAW-TLC cellulose	*
Umbellic acid	0.22	0.29	0.81	a
Umbelliferone	0.45	0.59	0.90	b
o-Glucosyloxy-cinnamic acid	0.65	0.76	0.47	c
p-Glucosyloxy-cinnamic acid	0.64	0.80	0.35	d
Skimmin	0.74	0.82	0.28	a
7-cellobiosyl-oxy-coumarin	0.68	0.79	0.05	e
trans-DGC+	0.70	0.81	0.035	f
cis-DGC+	0.70	0.78	0.035	f

Rf values of oxygenated cinnamic acid derivatives.

+2,4-di-glucosyloxycinnamic acid.

*This column refers to the fluorescence of these compounds in light at 350 mu.

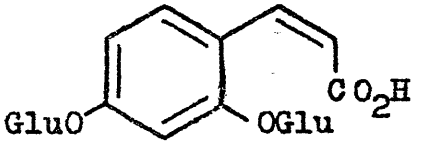
a - purple, b - blue-purple, e - weak purple.

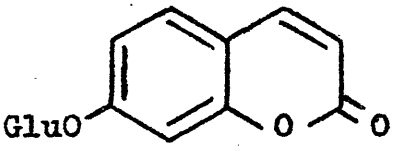
c,d and f all exhibit quenching in light at 254 mu, but after hydrolysis c is yellow and f is blue-purple, d is purple after hydrolysis and exposure to ammonia vapour.

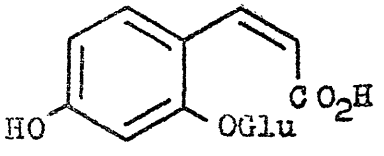
2.2.c. Chromatographic Analysis of Hydrangea and Postulated Enzymology.

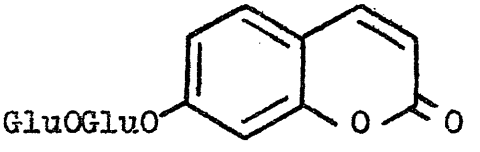
In the fore-going discussion, repeated reference has been made to skimmin (umbelliferone β-glucoside) as the major form in which the hydroxycoumarin occurs in hydrangea. This was shown to be the case as the result of paper and thin-layer chromatographic analysis of hydrangea leaf extracts and comparison with synthetic standards.

The preliminary work, using paper chromatographic techniques, did not permit an identification of the bound form of umbelliferone in hydrangea due to the presence of interfering fluorescent substances. An improved separation was obtained by TLC of the leaf extracts on unbound cellulose powder (Whatman CC41) with 5% aqueous acetic acid as eluent. This system visualised one spot (Rf 0.82) yielding umbelliferone (Rf 0.59) on emulsin hydrolsis. The presence of bound umbelliferone conjugated otherwise than with glucose has been eliminated. In spite of the result, chromatography of a series of standard cinnamic glucosides in this system showed that all of these compounds have similar Rf values, thus prohibiting the unequivocal identification of the









Possible 'Bound' Forms of Umbelliferone.

true bound form. After due experimentation, the ideal solvent for the required separation was found to be the upper phase of amyl alcohol: acetic acid: water (4:1:5), referred to as AAW. The use of this partition system of a buffered organic eluent and cellulose powder affords excellent separation of diglucosides, monoglucosides and phenolic aglycones, and could be generally useful.

The rationale for the formation of umbelliferone in hydrangea is that cis-DGC is the precursor of skimmin, in its turn the precursor of free umbelliferone. However, if the pathway to umbelliferone is indeed analogous to those for coumarin and herniarin, any one of all of the four glucosides shown may be present as the bound forms. It is noteworthy that a crystalline diglucose conjugate of umbelliferone has been isolated from H.paniculata[154] and H.macrophylla var.Hortensia[155]. In spite of repeated attempts, no such compound could be isolated from the variety of H.macrophylla used in the present study. For direct comparison with the naturally-occurring compounds, Dr. Meyers synthesised 7-cellobiosyloxycoumarin and trans-DGC[12].

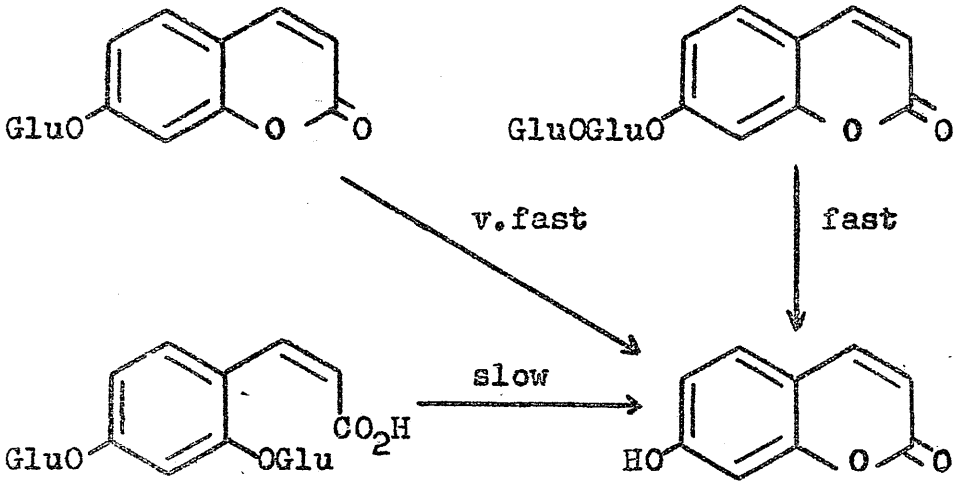
When a hydrangea leaf extract was chromatographed

on cellulose powder thin layers using the AAW solvent, two umbelliferone producing spots (Rf values 0.02 and 0.24)were observed, as a result of emulsin hydrolysis on the plate and elution in the second dimension with 5% acetic acid. The material with Rf 0.24 was by far the major component (ca. 95%), had purple fluorescence in light at 350 mμ and was positively identified with skimmin by its Rf values on paper and cellulose powder chromatograms, very rapid hydrolysis with emulsin, characteristic yellow fluorescence with aqueous sodium hydroxide, and by comparison of its ultraviolet spectrum with that of authentic skimmin. The minor component with Rf 0.02 was only very slowly hydrolysed by emulsin and, because of its position on the chromatogram, it was believed to be a di-glucose conjugate. It was clearly not the 7-cellobioside since this has a weak but visible purple fluorescence (anticipated for all coumarin glucosides) in light at 350 mμ and is rapidly hydrolysed by emulsin, though less dramatically than skimmin, which undergoes almost instantaneous hydrolysis.

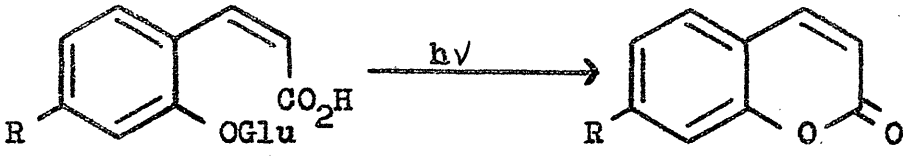
The synthetic trans-DGC, on chromatography with 5% acetic acid on cellulose powder gave

essentially one spot (Rf 0.81) which did not show significant fluorescence in light at 350 mμ, and exhibited quenching at 254 mμ. Hydrolysis of this material with emulsin for 2 hours and elution in the second dimension with the same solvent showed two spots (Rf 0.26 and 0.58) with blue-purple fluorescence in light at 350 mμ. The material of Rf 0.26 was by far the major component of the mixture and corresponded in all respects to umbellic acid. That with Rf 0.58 was identified as umbelliferone. This showed that the synthetic diglucoside was predominantly trans-DGC with a small amount of the cis isomer present. Its analysis with the AAW solvent showed that no skimmin was present.

In an attempt to obtain pure cis-DGC for comparison with the natural diglucoside, a methanolic solution of trans-DGC was irradiated with ultraviolet light from a mercury vapour lamp for 1 hour. TLC of the product with AAW gave rise to two spots (Rf 0.04 and 0.27). Both yielded umbelliferone on hydrolysis but the spot with Rf 0.04 produced a trace amount of umbellic acid, proving it to be predominantly cis-DGC. The component with Rf 0.27 exhibited a purple fluorescence in light at 350 mμ and was conclusively identified with skimmin.



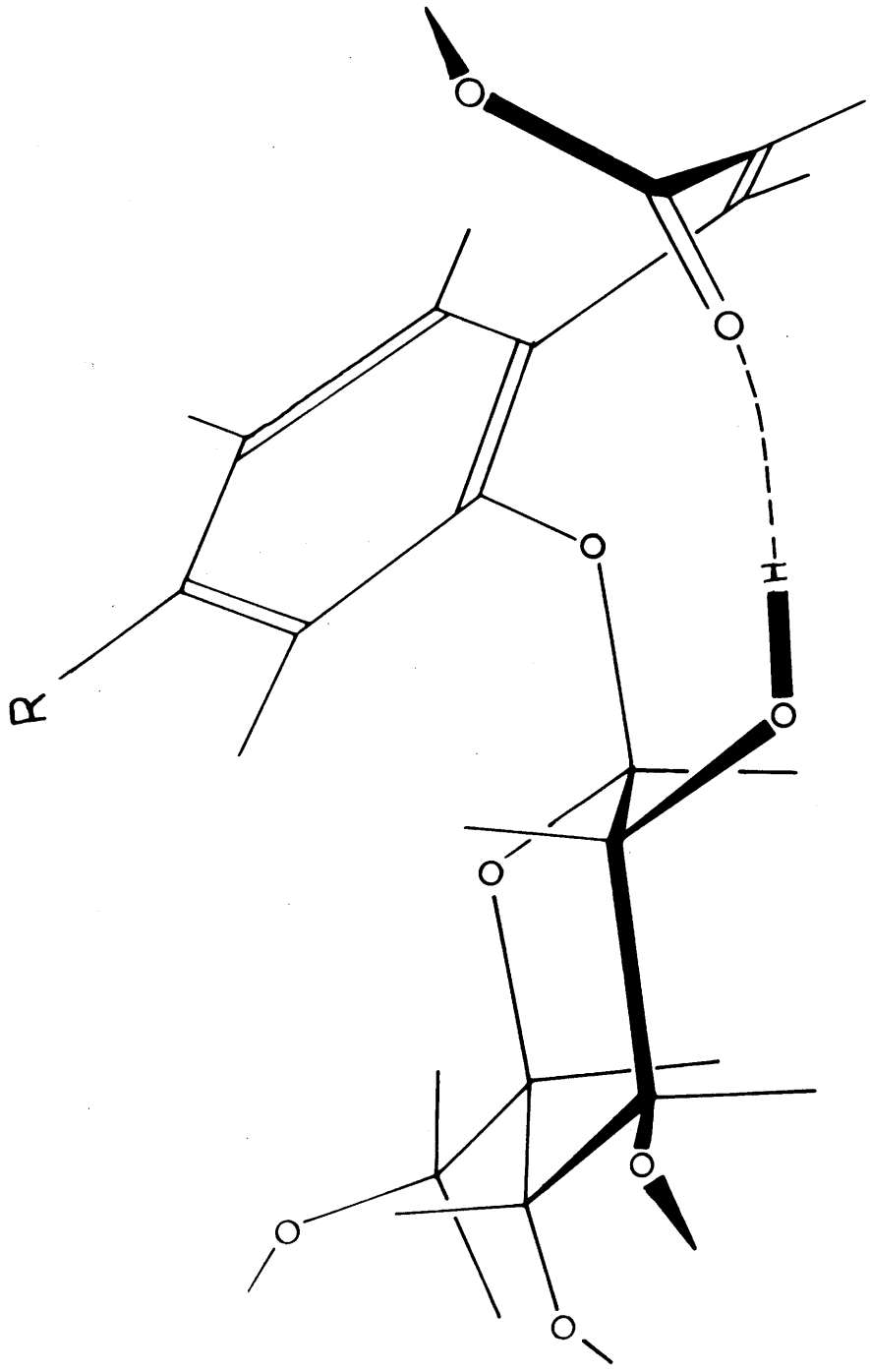
Relative rates of emulsin hydrolysis.



UV-induced lactonisation of coumarinic acid glucosides, R=H, OGlu, OCH_3, etc.

Lutzmann has reported an analogous lactonisation of coumarinyl glucoside to coumarin as a result of irradiation[85] and this has been fully confirmed in our own experiments. This lactonisation is so striking that the naturally-occurring di-glucose conjugate of umbelliferone was removed as a band from a preparative cellulose-powder TLC plate (AAW solvent) and irradiated for 1 hour in the same way as the synthetic trans-DGC. Evaporation of the methanol solvent and analysis of the residue as above showed two umbelliferone-producing spots (Rf 0.01 and 0.29). The predominant, faster-migrating material was identified as skimmin by the usual tests. From this evidence, coupled with its slow emulsin hydrolysis, the structure of this minor bound form of umbelliferone in hydrangea appears to be cis-DGC, since irradiation of 7-cellobiosyloxycoumarin produced no skimmin. This latter observation should apply equally to other umbelliferone disaccharides while skimmin itself did not break down under this ultraviolet irradiation.

At no time during the work described so far has free or glucose-bound umbellic acid been detected in hydrangea extracts. In view of the small amount of cis-DGC present this is not surprising,


O
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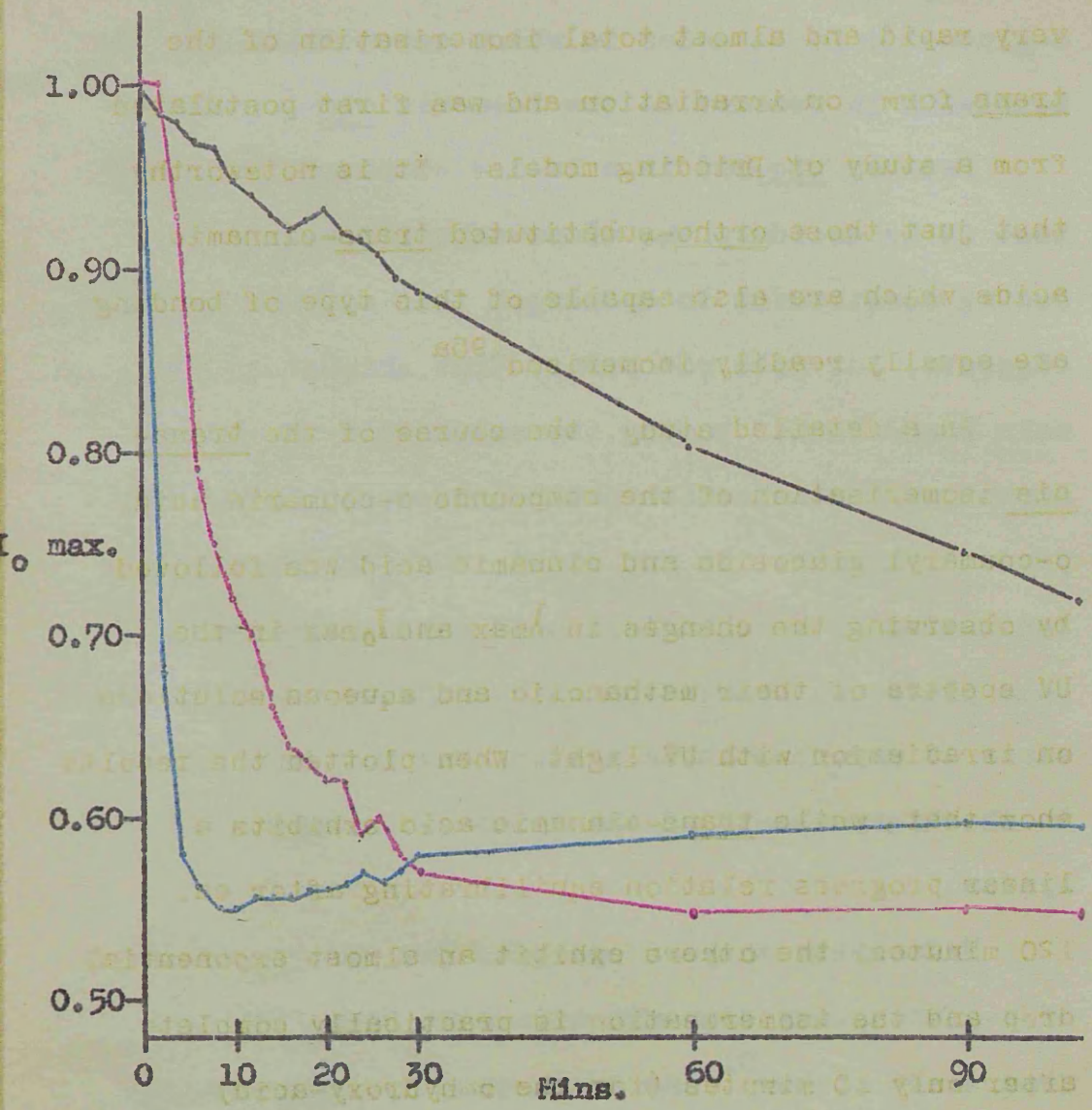
since Brown has observed only a trace of a trans-o-glucoside in lavender in which essentially all the herniarin is present as the isomeric cis-o-glucoside[3,106].

Because cis-DGC is a labile compound, the fact that it is decidely the minor umbelliferone-producing constituent in hydrangea leaf extract strongly suggests, but does not prove, that skimmin is the main bound form. During the extraction, a certain amount of lactonisation could conceivably occur to produce skimmin. In any event, the fact that lactonisation of o-coumarinic acid glucosides does take place on irradiation in vitro must be discussed since it may constitute a model for the final stage in the biosynthesis of the coumarin lactone ring system.

Hydrolytic enzymes specific for the cis form of o-glucosyloxy-cinnamic acid have been isolated from sweet clover[81] and tonka bean[82]. They possess unusual properties which may be discussed here.

The basic assumption is that strong intramolecular hydrogen bonding can occur only in the cis forms of o-glucosyloxycinnamic acids and then only between the carbonyl group and the 2-hydroxyl of the glucose (diagram). Such an interaction



UV Isomerisation Studies on Cinnamic Acid Derivatives.

trans-cinnamic acid =

o-coumaric acid =

o-coumaryl glucoside =

provides an excellent rationale for the observed very rapid and almost total isomerisation of the trans form on irradiation and was first postulated from a study of Drieding models. It is noteworthy that just those ortho-substituted trans-cinnamic acids which are also capable of this type of bonding are equally readily isomerised[195a].

In a detailed study, the course of the trans-cis isomerisation of the compounds o-coumaric acid, o-coumaryl glucoside and cinnamic acid was followed by observing the changes in λmax and I_0max in the UV spectra of their methanolic and aqueous solutions on irradiation with UV light. When plotted the results show that, while trans-cinnamic acid exhibits a linear progress relation equilibrating after ca. 120 minutes, the others exhibit an almost exponential drop and the isomerisation is practically complete after only 10 minutes (for the o-hydroxy-acid) and 30 minutes (for the o-glucoside). With longer irradiation of the trans-o-glucoside the cis isomer initially produced is increasingly transformed to coumarin. Now the o-hydroxy acid may be said to have a maximal thermodynamic driving force (lactonisation to an inert product) favouring the

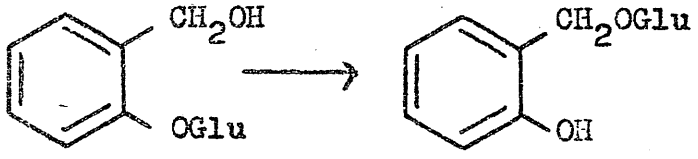
isomerisation. The fact that the o-glucoside so closely follows its pattern militates in favour of an equivalent driving force.

It is known[83, 84, 23(pp.224-5)] that glucosidase enzymes are highly specific for the sugar moiety in their substrates. Thus, epimerisation about a single carbon in the sugar ring is sufficient to prevent the action of the corresponding enzyme. Furthermore, substitution on the hydroxyl groups of the sugar usually has a profound effect; with β-glucosidase any substitution on the 2,3 or 4 hydroxyl functions completely prevents hydrolysis. The aglycone specificity is significant but less demanding. In the cis form of the o-glucoside, which is the known precursor of coumarin in M.alba, the postulated hydrogen bonding would be expected to lower the rate of emulsin hydrolysis of the cis-o-glucoside, compared to that of the trans isomer since it is effectively an interference with the normal substitution of the 2-hydroxyl. Kosuge and Conn have shown[81] that the rate of emulsin hydrolysis of the cis-glucoside is in fact less than of that of the trans-glucoside, as shown in the table.

Hydrolysis is merely a particular case, in which

Substrate.	Relative Rate of Hydrolysis.	
	Sweet Clover enzyme.	Emulsin.
β-glucosides of:		
Coumarinic acid	100	6.1
o-Coumaric acid	0.9	7.5
Melilotic acid	50	8.3
o-Hydroxyphenylacetic acid	0.6	0.4
Salicylic acid	61	9
Phenol	0.4	1.2
Salicyl alcohol	11	15.7
Salicylaldehyde	54	100

Demonstration of sweet clover and emulsin activities.
(Data from Kosuge and Conn[81])



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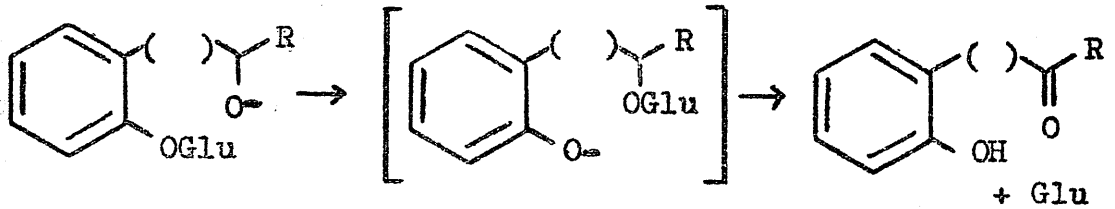
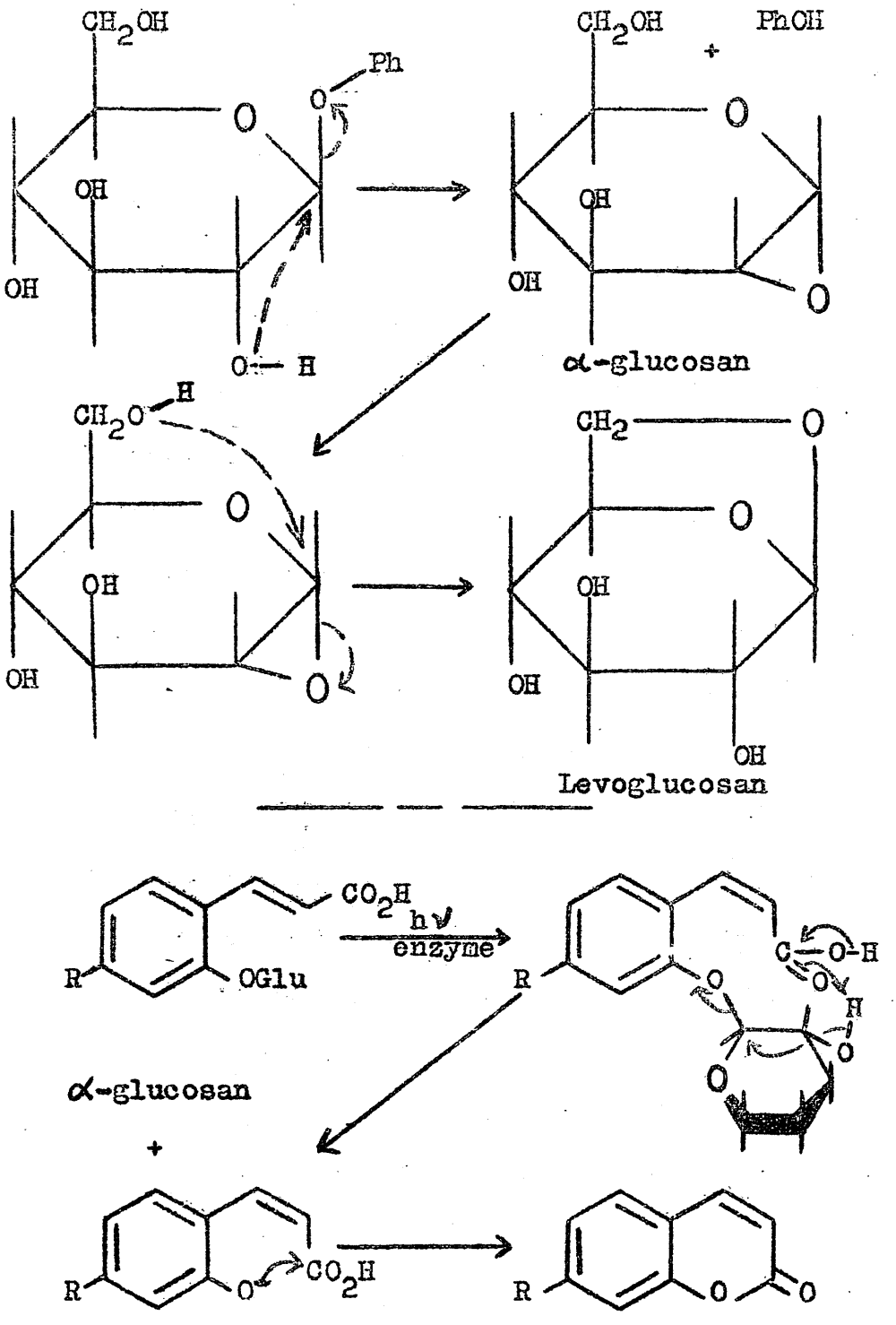


Postulate 1.- Rationale of Sweet Clover enzyme action.

the acceptor happens to be water, of the more general transfer properties of enzymes. A well-known example of intramolecular glucose transfer was first described by Rabaté[86] who showed that a glucosidase from Salix purpurea can transfer glucose from the phenolic hydroxyl group of saligenin (72) to the primary hydroxyl group with retention of the β-configuration of the glycosidic linkage. It is now proposed by analogy that the rate-limiting step in the action of the sweet clover glucosidase is basically a transfer of glucose from the o-oxygen function the ionised side-chain carboxyl or other carbonyl function which is held near the 2-hydroxyl of the glucose by hydrogen bonding and that a linked action is the rapid elimination of glucose from the initial product. This accounts well for for the observed specificity of the sweet clover enzyme (table), thus those substrates with suitably oriented carbonyl groups in the ortho-position are rapidly attacked whereas those which cannot readily form hydrogen bonds to the 2-hydroxyl of the glucose are only slowly hydrolysed. The glucoside of o-hydroxyphenylacetic acid is very slowly hydrolysed by both emulsin and the clover enzyme. This may reflect



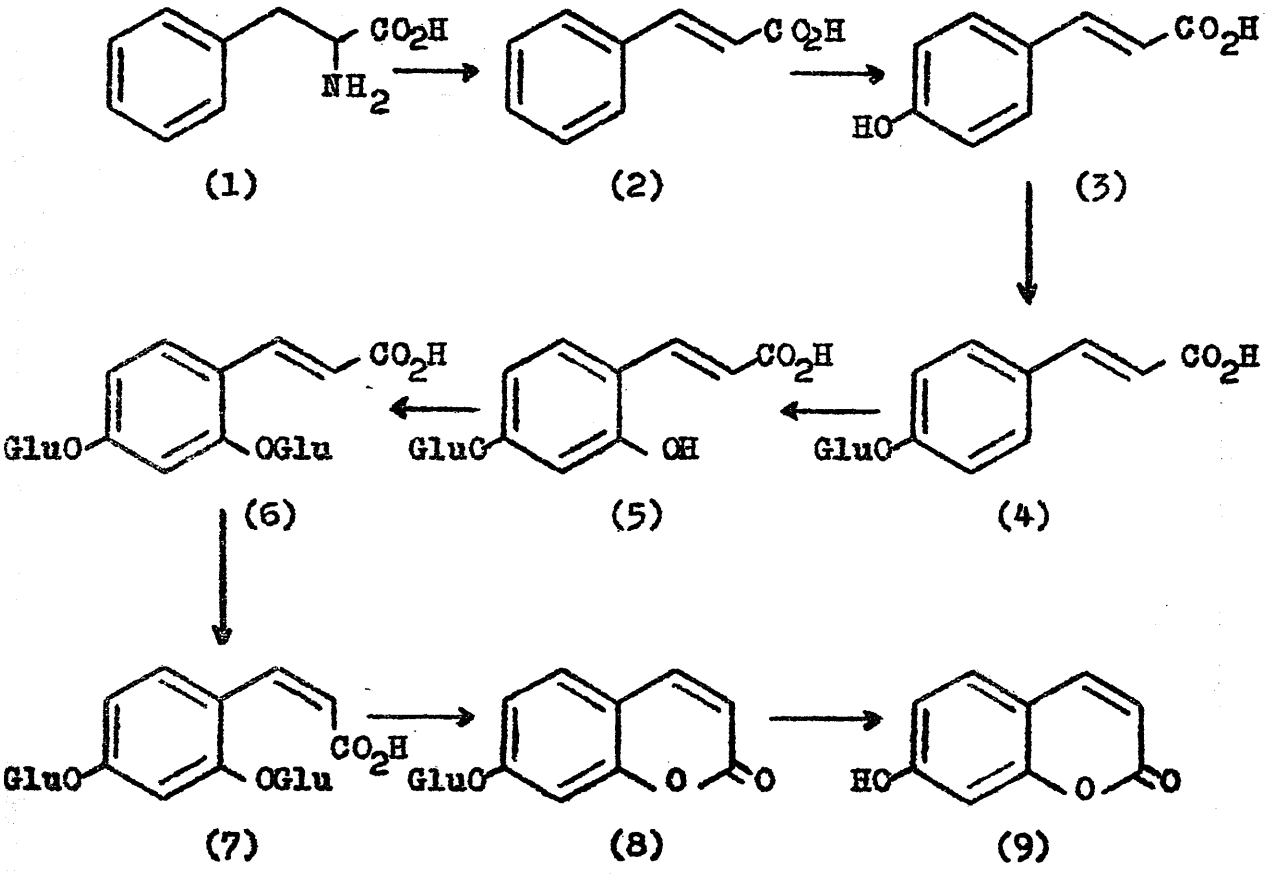
Postulate 2. Rationale of UV- and enzyme-induced lactonisation of coumarinic glucosides.

the possibility, visualised by inspection of models, that in this case the hydrogen bonding may be so favoured as to preclude the rapid action of either hydrolytic enzyme.

The glucose transfer just described is an alternative to feasible mechanisms involving effective base-catalysed cleavage of the glycosidic bond but they are by no means mutually exclusive. The familiar generalisation that the glucosidic linkage is stable to alkali is in fact quite wrong[156], since the resistant properties of 'normal' acetals are frequently not shared by glycosides. A large body of data indicates that the most general scheme for the alkaline hydrolysis of phenyl β-glucosides comprehends 'double inversion'[150]. An attack of the ionised 2-hydroxyl function at the anomeric 1-carbon with expulsion of the phenoxide ion gives rise to 1,2-anhydro-β-D-glucose, (α-glucosan), which has been prepared from β-D-glucose by thermal dehydration in vacuo[157,158]. The formation of this quasi-epoxide has been proposed, on good evidence, to be the slow, rate-determining step of the cleavage reaction while its subsequent transformation into 1,6-anhydro-β-D-glucose is rapid. The production

of the stable 1,6-anhydro form (levoglucosan), which is often obtained in near quantitative yield, is visualised as an attack of the 6-hydroxyl function at the anomeric carbon. Either α-glucosan or levoglucosan could be taken into the pool of the 'activated' glucosylating reagent, uridine diphosphate glucose (UDPG).

The possible relevance of this *in vitro* scheme to the enzymic hydrolysis of cis-o-coumaryl glucosides may now be demonstrated. The postulate of hydrogen bonding between the carboxyl group and the 2-hydroxyl of the sugar moiety provides a good explanation for the facile and almost complete *trans-cis* isomerisation of these compounds on irradiation. The same postulate may account for the observed specificity of sweet clover β-glucosidase. Now, if the carboxyl group in the *cis*-form is indeed held near the 2-hydroxyl, it can be readily seen that its presence would *accelerate* the formal ionisation of this group and thus would accelerate the formation of the intermediate 1,2-anhydro-β-D-glucose and the free coumarinic acid. This scheme explains not only the observed *in vitro* lactonisation of *cis*-DGC and *cis*-o-glucosyloxycinnamic



Scheme for Umbelliferone Biosynthesis consistent with the results of feedings I-XXXII.[12]

acid on irradiation but also may represent the reaction catalysed by the sweet clover and tonka bean enzymes. The concept of hydrogen bonding is therefore of critical significance in this preliminary rationale of the final stages in the biosynthesis of the coumarin lactone ring. In a recent report, Diedrich[159] has considered hydrogen bonding within dihydrochalcone glucoside derivatives in a study of their inhibitory action on the renal reabsorption of glucose.

The scheme shown, consistent with the results of the tracer experiments so far is proposed as a working hypothesis to account for the formation of umbelliferone in hydrangea[12]. The steps 6-7-8 in this overall scheme are paralleled by an observed *in vitro* transformation. The simultaneous identification of cis-DGC (7) and skimmin (8) in hydrangea leaf extracts greatly enhances the plausibility of this scheme, when combined with the results of our tracer experiments which rule out oxidative cyclisation and prove the order of the hydroxylation steps.

2.2.d. Recent Work on Umbelliferone Biosynthesis.

The work of Dr. S.A. Brown of Saskatoon has

been of the greatest importance in establishing the possible pathways to the coumarins and he has also made significant contributions on the problems of lignin biosynthesis. Following our preliminary report on the formation of umbelliferone in hydrangea,[150] we were most grateful to receive from him a copy of his (then) unpublished work on the same subject carried out in conjunction with Drs. G.H.N. Towers and D. Chen of Montreal[107]. By good fortune, the experiments carried out by the Canadian workers complement rather than reproduce our own, thereby aiding a fuller analysis of the situation. It should be stated that our tracer studies were planned prior to our knowledge of their work.

As part of a previous study by Bohm, Ibrahim and Towers[160] on the coumarin constituents of H. macrophylla, umbelliferone and a 7,8-hydroxymethoxycoumarin were isolated and shown to become radioactive as a result of feedings of U-^{14}C-shikimic acid, 1-, 3-, and U-^{14}C-phenylalanine and trans-2-^{14}C-cinnamic acid. It was implied that the hydroxymethoxycoumarin, identified as the 7-hydroxy-8-methoxy isomer (collinol[161] or hydrangetin[107]) became radioactive as a result of feeding with labelled umbelliferone. 'Hydrangetin' has been

isolated from a few of the experiments carried out here but it was not possible to study its precise formation although Kindl and Billek[117] have done so.

The feeding techniques adopted by Brown, Towers and Chen differed significantly from our own. Whereas we had fed intact plants by cotton wick, they allowed the uptake of the precursor solution through the cut ends of plant stems. This necessitated much shorter metabolic periods and no time-course studies were carried out. In their later 'trapping' experiments, with unlabelled umbellic acid and active coumaric acids , detached hydrangea leaves were fed the mixture of compounds via the cut ends of the petioles. The object of the 'trapping' technique is to permit an evaluation of the conversion of the fed radioactive material into the recoverable portion of the unlabelled compound, which is usually a suspected intermediate in a sequence. The activity of the final product of the particular sequence may also be determined thus permitting an evaluation of the status of the putative intermediate.

In their qualitative preliminary studies, the trapping technique was employed. In five

successive experiments, 1.33 μmoles of 3-^{14}C-cinnamic acid were fed to cut hydrangea shoots together with 5 μmoles of p-coumaric, o-coumaric, ferulic and caffeic acids and coumarin respectively. The plants were kept in light for 30 hours and then extracted. After each feeding, various phenolic acids as well as umbelliferone and 'hydrangetin' were isolated on paper chromatograms and their activity evaluated by radioautography. Of these, only p-coumaric acid and the two coumarins were ever found to be appreciably radioactive. Significantly, o-coumaric acid was detected only from those plants specifically fed the compound and even then it was not radioactive. This conversion of cinnamic acid to oxygenated coumarins while o-coumaric acid remained unlabelled was in accord with the results of Brown's studies with lavender[80].

In their quantitative feeding experiments, the efficiency of utilisation of each compound was judged by its dilution value only. The results obtained after the feeding of amounts ranging from 33 to 59 μmoles of each precursor for one day showed that trans-2-^{14}C-p-coumaric acid is more efficiently utilised than 3-^{14}C-cinnamic acid for the

synthesis of both umbelliferone and 'hydrangetin'. There was a measurable incorporation of activity from 2-^{14}C-coumarin, but the dilution was almost 60 times that of p-coumaric acid so that the direct 7-hydroxylation of coumarin does not appear to be a major pathway in the formation of umbelliferone or 'hydrangetin' in hydrangea. This accords with Brown's proof that the ortho-oxygenated-cinnamic acid precursors of coumarin are poorly incorporated into herniarin in lavender[80]. The corollary, that para-oxygenated precursors (including umbelliferone) are not significantly transformed to coumarin, has been demonstrated by the studies of Weygand[77] and Brown[78-80].

In their second series of tracer experiments, 1-^{14}C-o-coumaric and 2-^{14}C-p-coumaric acids were compared with 1-^{14}C-umbellic acid as precursors of umbelliferone during a metabolic period of one day. As predicted by analogy with the previous work on herniarin, umbellic acid was an excellent precursor and is in fact by far the best yet tested, with dilution values from 1 to 3. In passing, these very low values indicate that the fed umbellic acid must have swamped the endogenous unlabelled 2,4-dioxygenated cinnamic acid pool. This is further proof that the normal rate of

umbelliferone formation is much less than the potential, as previously inferred from our present results.

This direct evidence strongly favours our suggested involvement of a trans-2, 4-dioxygenated cinnamic acid in the biosynthesis of umbelliferone[150] and confirms and extends Brown's previous work on herniarin. It constitutes yet more evidence contrary to the requirements of the oxidative cyclisation theories.

Thus far, the results gained by the Canadian workers were in agreement with Brown's general postulate[78] that, in the biosynthesis of 7-oxygenated coumarins, para-oxygenation of the fundamental trans-cinnamic acid precedes ortho-hydroxylation. The feedings of o-coumaric and p-coumaric acid in their second series showed, however, that in direct conflict with this postulate and the evidence on which it was based, these acids were almost precisely equivalent precursors of umbelliferone! This contrasts with the results of Brown's investigations on herniarin biosynthesis and indeed to the qualitative preliminary results obtained in this particular study. Since our

experiments[142] on the utilisation of ortho-oxygenated cinnamic acid derivatives in the biosynthesis of umbelliferone in the same species of hydrangea were in complete accord with the postulate and the collateral evidence in its favour, the results obtained by Brown, Towers and Chen are at first sight inexplicable. One solution other than direct conversion is that a breakdown of the radioactive o-coumaric acid has occurred with a rapid incorporation of the fragments into skimmin. However, this 'aberrant synthesis' had not taken place as shown by their degradation of the umbelliferone recovered after the feeding of 1-^{14}C-o-coumaric acid. Because the specific activity of the carbon dioxide evolved (equivalent to the lactone carbonyl) was identical to that of the original hydroxycoumarin, the direct conversion of o-coumaric acid to umbelliferone without randomisation was established.

Ibrahim[162], working at Montreal, failed to find o-coumaric acid but did find p-coumaric acid in Hydrangea macrophylla (almost certainly the same variety as that used by the other Canadian workers). The best explanation of the apparent ambiguity would thus seem to be that their variety, unlike our own,

does possess enzyme systems capable of converting exogenous o-coumaric acid to umbelliferone, probably via umbellic acid, although it cannot convert exogenous cinnamic acid to o-coumaric acid. This may well be due to a one-gene difference (here suggested for the first time) between the genetic constitution of these hydrangea varieties since the Canadian hydrangea variety displays efficient enzymic 'meta'-hydroxylation of o-coumaric acid controlled by a gene either absent or masked in our own. An alternative is that the feed-back inhibition[163-166] of this step is operative in our cultivar.

Feedback inhibition may be defined as that control of a biosynthetic sequence which involves the inhibition of one of the steps by one of the products or intermediates when in excess of a given level. The exciting prospect of 'mutant' studies with higher plants would appear to exist since very strong evidence favouring at least the phenotypic enzyme difference was obtained by Brown, Towers and Chen. In trapping experiments, either o-coumaric acid or p-coumaric acid, labelled with ^{14}C in the carboxyl (1) carbon, was fed with an equimolar amount of unlabelled umbellic acid to detached

hydrangea leaves via the petioles. The total metabolic period was only two hours but both coumaric acids were efficiently transformed to umbellic acid. A close study of their reported dilution values of the radioactive acids into the free and glucose-bound forms of umbellic acid shows that the bound umbellic acid was uniformly more radioactive than the free, suggesting either that, (1) coumaric acids are glucose-bound before hydroxylation to give the umbellic acid conjugate directly or (2) that the umbellic acid formed from the free coumaric acids is not in contact with the large pool of exogenous unlabelled umbellic acid. In both cases, even limited hydrolysis of the conjugate during the extraction procedure would result in appreciable but lower radioactivity in the recovered free umbellic acid. Hypothesis (1) implies that free umbellic acid is not directly on the pathway to umbelliferone while hypothesis (2) indicates that the coumaric acids may be more rapidly transported to the site of synthesis than umbellic acid during the short metabolic period employed.

Such hypotheses may also explain the remarkable fact that, in three of the eight activity

determinations in their umbellic acid trapping experiments, Brown et al. found that the activity of recovered umbelliferone was significantly higher than the recovered umbellic acid, in seeming contradiction to their previous evidence that umbellic acid is in fact a precursor of umbelliferone! Since they unfortunately employed only the dilution value criterion in the evaluation of their tracer studies, an unambiguous explanation of this anomaly is impossible. However, they postulated that either differential transport of precursors as described above (hypothesis 2) or 'a second major pathway not involving umbellic acid' could account for it. It is clear that the formation of a glucose-bound form of umbellic acid from coumaric acid without the liberation of the free dioxygenated compound is precisely equivalent to their 'second major pathway'.

A further result of their trapping experiments was that in two of the four determinations of the relative dilutions of activity into free and glucose-bound umbelliferone, the free compound was apparently more active than the bound, in contrast to our own findings and contrary to the postulate that bound umbelliferone is the precursor of the free. However this can again be explained by assuming the existence

of larger or smaller pools of unlabelled bound umbelliferone within the leaves, or by proposing a limited hydrolysis of the highly radioactive bound form during extraction.

In summary, this significant study establishes the existence of a pathway: cinnamic acid → p-coumaric acid → umbellic acid (free or bound) → umbelliferone (free or bound) in hydrangea but the normal participation of o-coumaric acid has not been proved. These suggestions are confirmed by our own tracer experiments[142] and TLC analyses of hydrangea[12], which in themselves provided sufficient evidence to permit the advancement of the biosynthetic scheme as a working hypothesis.[150]

2.2.e. Hydrangea Radiotracer Studies (2).

Reverting now to the tracer experiments carried out in the present study, the fact that fed trans-2-^{14}C-p-glucosyloxycinnamic acid is used as efficiently as its aglycone in the formation of skimmin had already been established by feedings XXII-XXIV. Therefore the precise nature of the p-oxygenated substrate of the ortho-hydroxylating enzyme was in doubt. In general, if only one of the two possible precursors is in fact hydroxylated, two hypotheses

can account for the almost equal precursor status of glucoside and aglycone. 1. The p-glucoside may be rapidly hydrolysed by an endogenous glucosidase or 2. an equally fast glycosylation of p-coumaric acid may occur. Another possibility is that 3.the ortho-hydroxylation enzyme is not sensitive to the presence of a glycosyl group at the para-position. On balance, the results of feedings XL and XLI suggest that glucosylation of fed precursors is a favoured process while the fact that skimmin is the main bound form of umbelliferone in Hydrangea macrophylla (also in Skimmia japonica[167], Skimmia laureola[168] and Hieracium pilosella)[169] could best accord with the second proposal.

The further work now to be described, however, supports the concept of deglucosylation before hydroxylation i.e. hypothesis 1.

In a stimulating discussion of mutual problems with Dr. S.A. Brown, he suggested that feeding experiments with p-glucosyloxycinnamic acid labelled with ^{14}C in both the phenylpropanoid and the sugar moities might permit a closer definition of the substrate of the ortho-hydroxylating enzyme. Accordingly, following Dr. Meyers departure to Belfast,

these studies were carried out (XXXV-XXXVII) together with several experiments designed to test p-coumaric acid and its glucoside competitively as precursors of skimmin (XXXIII-XXXIV, XXXVIII-XXXIX). The last two feedings made as part of this project were intended to throw some light on the glucosylation and hydrolysis of fed precursors (XL-XLI).

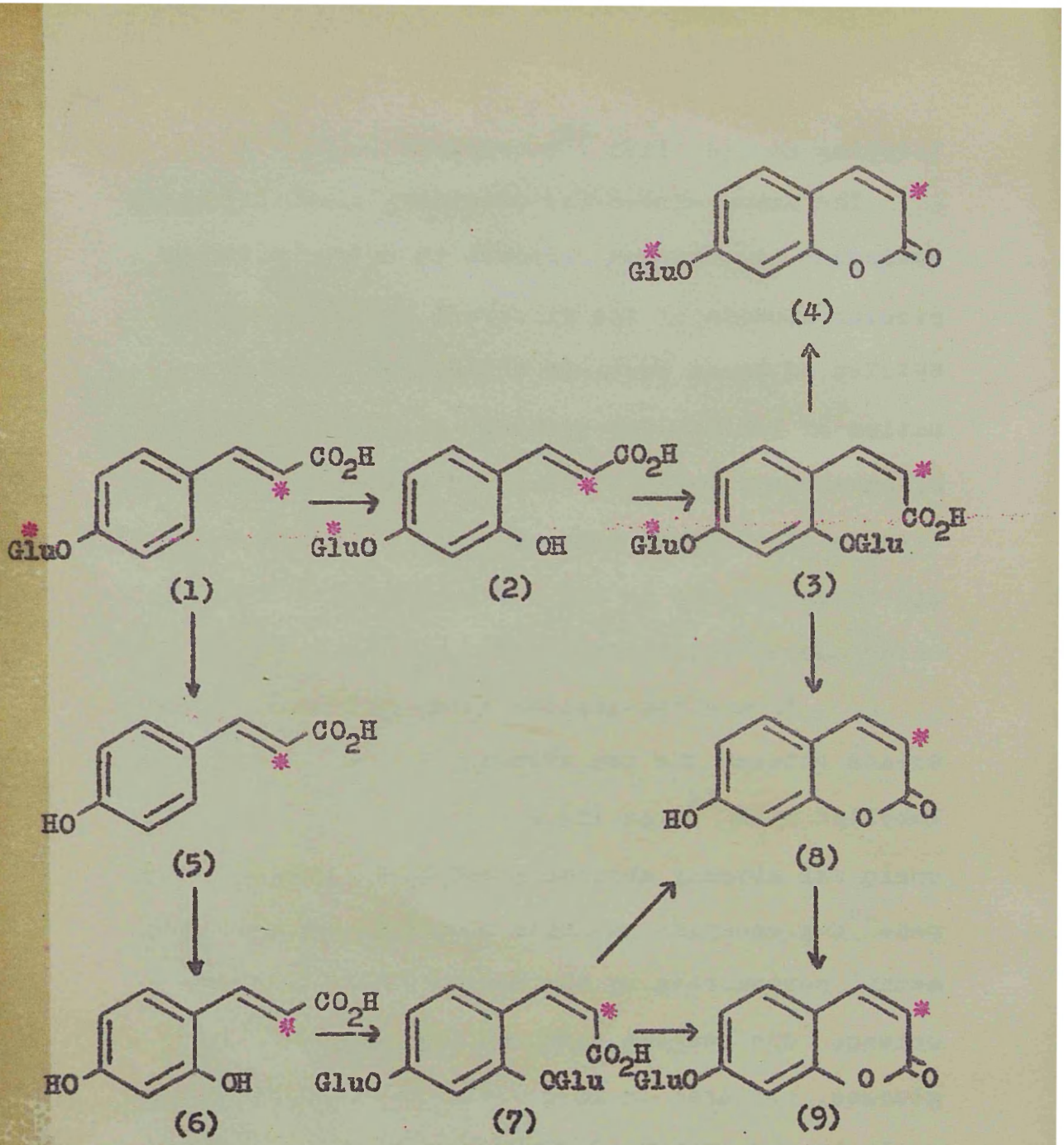
Multiple-label experimentation involves the administration of molecules labelled with the same or different isotopic species in a known proportion at various sites to biological or abiological systems. Subsequent on some given transformation, an evaluation of the relative proportions of the isotopic species remaining in each position frequently affords a greater insight on the mechanism of the transformation than may be obtained by direct feedings of singly-labelled compounds.

In this case the problem was to decide whether the p-glucoside is in fact a direct precursor of skimmin. A hydroxylation of this glucoside would at once give rise to a glucose-bound form of umbellic acid. This step is postulated for its ready explanation of the observation that bound umbellic acid was more radioactive than the free in Brown's

trapping studies with ^{14}C-coumaric acids[109].

The usual method for obtaining doubly-labelled compounds for feeding purposes is to mix together precise amounts of the different singly-labelled species of known specific activities. The alternative of making, for example, a labelled derivative of an already labelled compound yields a product with no significant proportion of actual doubly-labelled molecules due to the low abundance of the labelled atoms even in moderately enriched preparations.

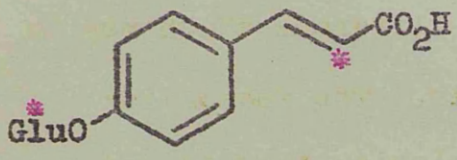
From a statistical viewpoint, no difference exists between the two techniques. The p-glucoside, labelled with ^{14}C at the 2-position of the side-chain was already available and for the present study p-U-^{14}C-glucosyloxycinnamic acid was prepared from methyl p-coumarate by the method previously described. The reagent used was acetobromo-U-^{14}C-glucose, prepared in good yield on a semi-micro scale by the method of Fischer[170] after trials of several more recently reported techniques had failed. Removal of the blocking groups from the intermediate tetracetyl methyl ester was accomplished by selective hydrolysis with barium hydroxide and the product rigorously purified by preparative



Alternative pathways for utilisation of doubly-labelled precursor (1).

TLC on cellulose powder and repeated crystallisation from methanol. Its specific activity was determined and each of three randomly-chosen hydrangea plants was fed a neutral aqueous solution containing known amounts of both radioactive species (i.e. Glu-labelled and Chain-labelled). For comparison purposes, the total quantity fed to each plant was 12 µmole, as with most of the previous feedings.

The essential feature of these experiments was that, if the ratio of the activities present in the side-chain and the glucose of the skimmin recovered after the metabolic period were identical to that of the precursor p-glucoside, this would provide very strong evidence for the en bloc incorporation of the p-glucoside (1-2-3-4) and thus indicate that the hydroxylation substrate is p-glucosyloxycinnamic acid. The alternative, that little or no activity should be found in the glucose label compared to the side-chain incorporation, would suggest either that the para-glucosyl group had been lost before the hydroxylation (1-5-6-7-9) or (1-5-6-7-8-9) (and thus that the substrate is p-coumaric acid itself) or that the p-glucose could be lost after hydroxylation and replaced by inactive glucose from the endogenous



HYDRANGEA.

80% EtOH extract.

40 ml. aqueous solution. — Ether extraction → 'FREE UMB.' (chain label)

1/4 — emulsin, ether extraction → 'BOUND UMB.' (chain label)

3/4

1. +10 mgm skimmin.
2. 3x TLC.
3. +40 mgm skimmin.
4. 4-5x cryst.

→ SKIMMIN. = 'BOUND UMB.' (chain plus glucose label)

Flow-sheet for double-label experiments.

UDPG pool (1-2-3-8-9). It should be noted that steps 3-4 and 7-9 represent a selective hydrolysis of cis-DGC while 3-8 and 7-8 may be described as a total hydrolysis giving rise to umbelliferone which may be glucosylated 8-9 giving skimmin. The reason for postulating such a total hydrolysis is that the formation of umbelliferone differs from those of coumarin and herniarin since the final product is in fact a coumarin while the others are coumarinic acid glucosides. Because a selective hydrolysis of the coumarinic glucoside form has not occurred with the others, it may be argued that it is possibly unlikely to take place in the biosynthesis of umbelliferone.

The purification of pure skimmin from hydrangea threatened to be extremely difficult, repeated attempts to obtain a crystalline glucoside from this plant having failed. Accordingly a new technique was devised which has not only permitted the accurate measurement of the side-chain incorporation into free umbelliferone and into skimmin but has also made possible the determination of the glucose incorporation into the recovered skimmin (flow sheet). After each metabolic period, the two hydrangea stems were cut below the point of feeding as before

and refluxed in boiling 80% ethanol for 30 minutes to inactivate endogenous glucosidases as far as possible. The stems and leaves were then blended and subjected to further reflux in the same 80% ethanol for two hours. Reduction of the solution under vacuum to ca. 40 ml. volume removed all the ethanol. As described for the determination of the ratio of free and bound umbelliferone in hydrangea (XXI, XXV), ether extraction of the cloudy-yellow solution at this stage gave a small quantity of free umbelliferone which was purified, weighed and counted by the established techniques. In order to obtain a measure of the amount of skimmin present in the plant extract, the solution freed from umbelliferone was diluted to exactly 50 ml. volume and a 12.5 ml. aliquot removed. This aliquot was treated with almond emulsin (0.1% w/v) and incubated at 30° for two days. The umbelliferone thus released from skimmin (by far the predominant bound form) was extracted with ether and purified. Clearly its weight may be equated to one-quarter of the total weight of skimmin present while its specific activity corresponds to one-quarter of the total side-chain incorporation from the doubly-labelled precursor

into skimmin. From this data, the side-chain incorporation into skimmin and the weight of skimmin in the remaining 37.5 ml. of extract were determined.

The remaining problem was to find the total specific activity of the skimmin present in each case. This was accomplished by dissolving a known quantity of unlabelled skimmin (ca. 10 mgm) in the plant extract and reducing it to dryness. The residue was then subjected to twice-repeated preparative chromatography on 0.5 mm-thick layers of cellulose powder. The solvents used were 5% acetic acid, 5% acetic acid and AAW, in that order. In each case the purple-fluorescent band corresponding to skimmin was eluted from the support and its purity examined on analytical cellulose-powder thin-layers by the established two-dimensional technique with emulsin hydrolysis between the successive runs. The skimmin from the third (AAW) plate was almost completely pure and its ultra-violet spectrum was identical with that of an authentic specimen. From the epsilon value of the authentic material at 318 mμ, the amount of skimmin produced from the chromatographic separations was calculated. A further known quantity (ca. 40 mgm) of the unlabelled skimmin was added to this methanol

solution and the task of crystallising the skimmin to constant activity was commenced. The best solvent for the purpose was found to be water from which the glucoside crystallises as colourless needles melting at 216-8° on the Kofler block after drying at 110° under vacuum (lit.m.p. 219-21° in sealed tube)[167]. The mother liquors from the first crystallisation of each sample were shown to contain a trace of cis-DGC in addition to a very small amount of a monoglucose-umbellic acid conjugate of uncertain structure which was not detected in unfed plants.

Five crystallisations of the XXXV (1 day) skimmin produced a material of constant activity and complete purity as judged by chromatographic and spectroscopic comparison with authentic skimmin. For feedings XXXVI and XXXVII, only four crystallisations were necessary. The change in total specific activity over the last purification was uniformly less than 2.5%.

Since the specific activity due to the chain label was already known, simple subtraction of this value from the total specific activity of the skimmin yielded an accurate measure of the activity

DOUBLE-LABEL FEEDINGS.

The compound fed was p-glucosyloxycinnamic acid labelled with ^{14}C at the 2-position and uniformly in the glucose moiety. The chain-label activity was 1.07×10^6 cpm/mmole and that of the glucose moiety was 1.04×10^6 cpm/mmole.

Cpm fed as chain label = 1.352×10^4.

Cpm fed as glucose label = 1.312×10^4.

M. trans-p-Glucosyloxycinnamic acid 4.12 mgm.

XXXV.	Free Umb (chain)	0.125%	(78)	
	Bound Umb (chain)	3.050%	(45)	1 Day.
	Bound Umb (glucose)	0.203%	(674)	
XXXVI.	Free Umb (chain)	0.288%	(64)	
	Bound Umb (chain)	4.116%	(36)	3 Days.
	Bound Umb (glucose)	2.509%	(58)	
XXXVII.	Free Umb (chain)	0.150%	(52)	
	Bound Umb (chain)	4.276%	(24)	5 Days.
	Bound Umb (glucose)	3.944%	(26)	

The relative incorporation of the glucose label as compared with that of the chain label may be expressed:

XXXV. - 6.65%.

XXXVI. - 60.98%.

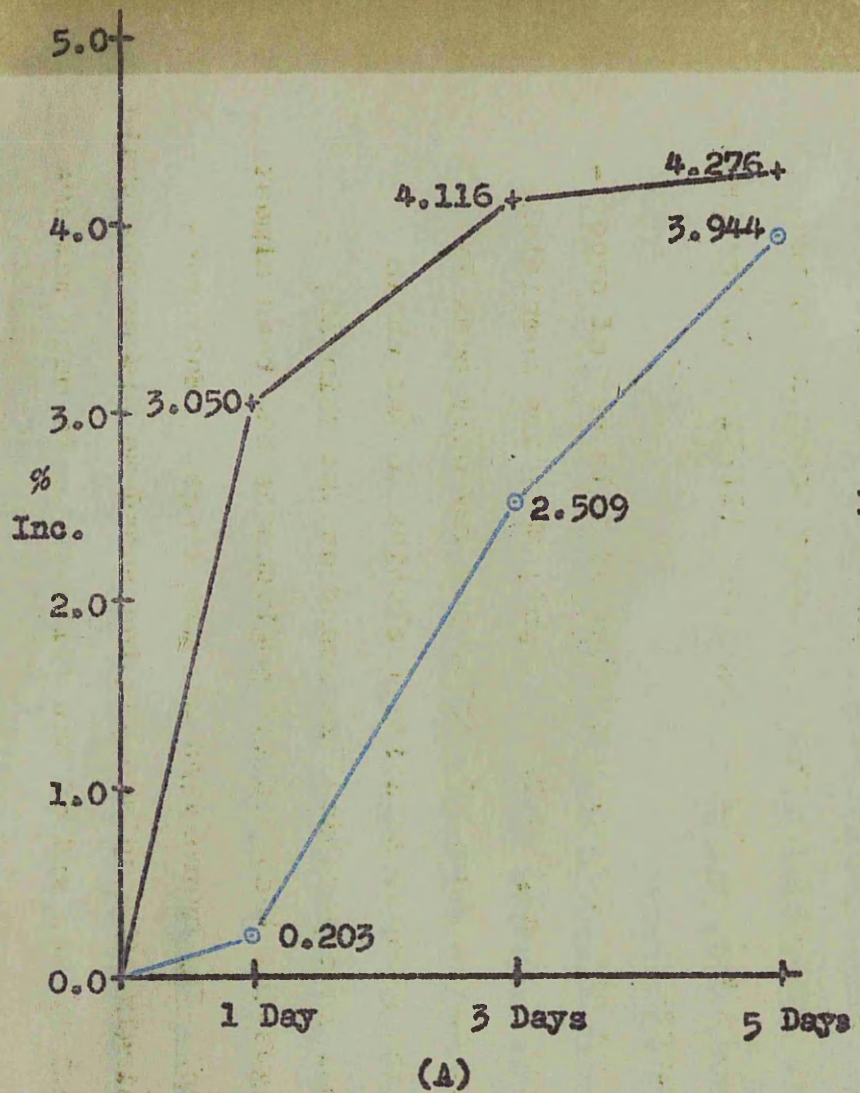
XXXVII. - 92.23%.

The relative activity of the bound and free umbelliferone was: 1.75 after 1 Day- 1.80 after 3 Days- 2.19 after 5 Days.

of the glucose moiety. It should be noted that it was not necessary to achieve quantitative recovery of the skimmin at each stage of the purification due to this use of the inverse isotope dilution technique.

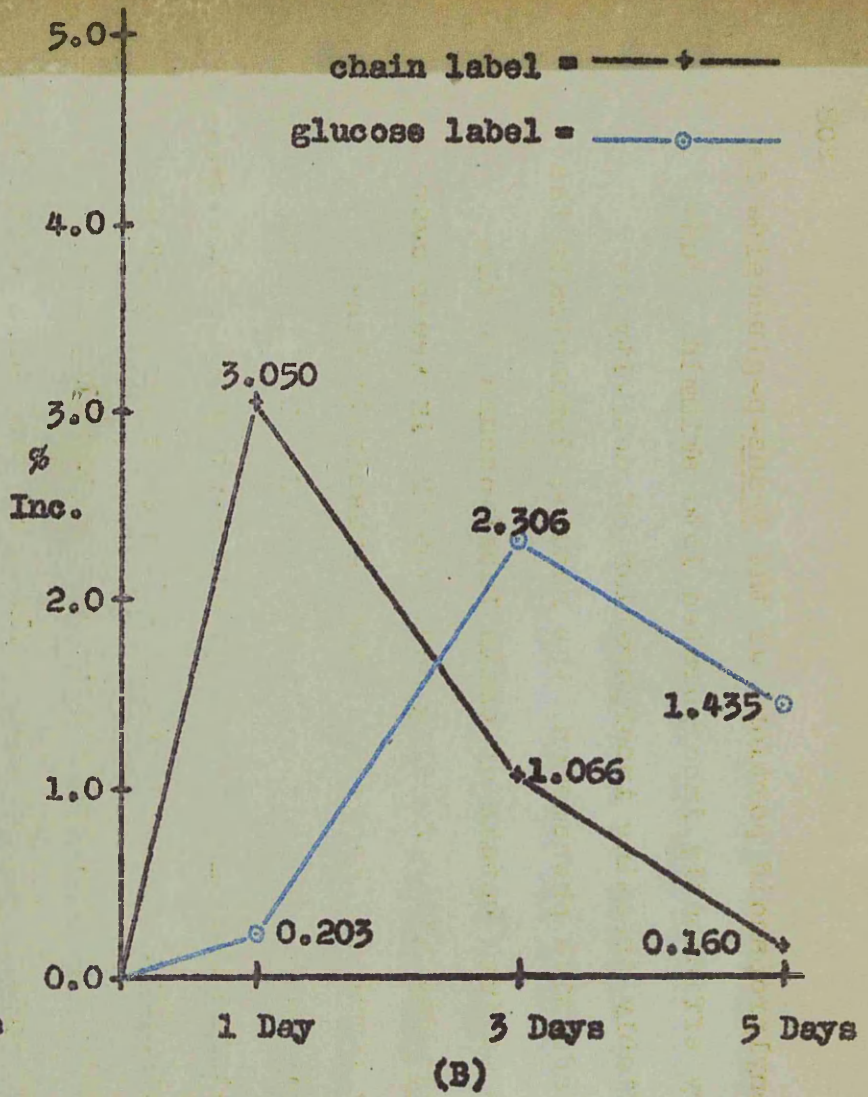
The results show that the chain activity of skimmin is consistently almost twice that of the recovered free umbelliferone, tending to confirm the previous hypothesis that umbelliferone is not a precursor of its glucoside in this sequence. However, although the small activity in umbelliferone could be accounted for by a limited hydrolysis of skimmin on disruption of the plant tissue, 9-8, the possibility that umbelliferone is the precursor of skimmin 8-9 and that the active hydroxy-compound has been greatly diluted by endogenous inactive umbelliferone cannot be eliminated. The results further show that the fed glucoside of p-coumaric acid cannot be incorporated into skimmin (1-2-3-4) as such, but rather that a hydrolysis of the glucoside must have taken place at some stage.

As anticipated, the chain label (and thus the



(A)

Double-label feedings - results.



(B)

Double-label feedings - results plotted as increments over previous period.

phenylpropanoid portion) or the trans-p-glucoside is very efficiently incorporated into skimmin. Justifiably treating incorporation of activity as an additive phenomenon, the results demonstrate that the maximal uptake of chain label occurs on the first day after feeding and that the increase over the remaining four days is progressively less (diagram). As previously discussed, this implies that the quantity of endogenous p-oxygenated cinnamic acid precursors must be very low as a result of a control of their synthesis in the normal plant. Once again, the dilution values of the chain activity from the fed glucoside are lower than the corresponding values for the aglycone (II-VII) in the later periods.

A most exciting feature of this set of experiments is that, at first sight, they are completely self-contradictory! After one day only some 7% of the glucose activity (relative to the chain label incorporation) is found on the skimmin, suggesting that the fed p-glucoside has been almost completely hydrolysed at some stage. Most confusingly, the uptake has increased to 61% of the chain label by the end of the third day. To make matters

worse, by the end of the fifth day, the relative incorporation has increased to over 92% of the chain label- which accords well with a direct hydroxylation of the p-glucoside without prior hydrolysis!

The observation that, as shown in the diagram, the maximal incorporation of the glucose label does not take place on the first day but rather in the second-third day period gave the first clue to this mystery. It is clear, incidentally, that only by the adoption of time-course studies has this interesting problem come to light. If the feedings had only lasted for one day, hydrolysis of the glucoside prior to hydroxylation would have been indicated, whereas if they had all been extended to five days, hydroxlyation of the p-glucoside itself would have fitted the results.

This demonstration of the fundamental unreliability of double-labelling studies without careful controls cuts at the basic assumption of such studies, that (apparent) retention of both labels necessarily proves the en bloc incorporation of the precursor.

A later feeding of p-glucosyloxycinnamic acid

FEEDINGS.

N. trans-p-Coumaric acid (2.61×10^6) 2.0 mgm-3.18×10^4.

XL.	Free acid	3.64%	1 Day - 1 stem.
	Bound acid	2.95%	

In spite of the feeding of twice the 'normal' quantity of precursor, some 45% of the recoverable trans-p-coumaric acid was present in a glucose-bound form.

O. trans-p-Glucosyloxycinnamic acid (2.17×10^6) 3.64 mgm-2.42×10^4.

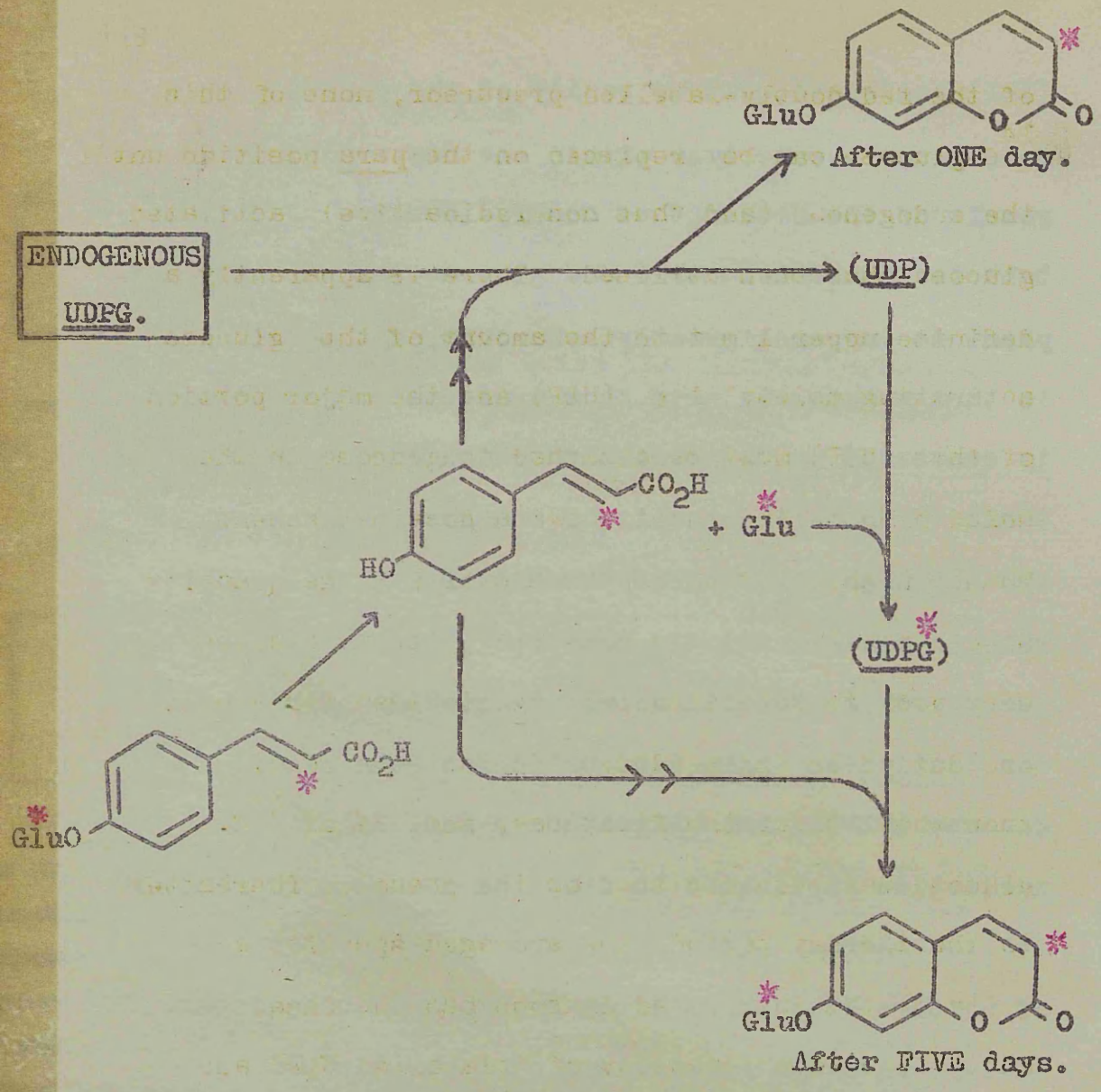
XLI.	Free acid	3.26%	1 Day - 1 stem.
	Bound acid	20.50%	
	Bound acid	0.65%*	

Only some 14% of the recoverable trans-p-coumaric acid was in the free form, suggesting that the hydrolysis of the glucoside is not as rapid as the glucosylation of the free compound. As compared with XL, the recovery of activity is almost four times greater which may demonstrate that the aglycone is more readily metabolised than the glucoside in reactions which do not lead to skimmin. Acid hydrolysis of the p-coumaric acid conjugates remaining after the emulsin hydrolysis gave only a small further recovery (0.65%*).

These recoveries represent those amounts of the fed precursors which remain in a soluble free or glucose-bound form. Clearly, the bulk of the compounds are in an insoluble, possibly esterified[148] form.

(XLI) has shown that up to 14% of the glucose moiety can become free within one day. Thus as a result of the feedings of doubly-labelled precursor, both the p-coumaric acid and glucose pools within the plant do increase greatly. However, the evidence is that p-coumaric acid thus produced is very rapidly converted to skimmin, a function of the extremely small 'turnover' quantity of endogenous precursor and the high activity of the hydroxylating enzymes. This is clearly not the case for glucose, since to react with a phenolic group the glucose must be in the 'activated' form i.e. uridine-diphosphate glucose (UDPG), the ubiquitous α-glycosyloxy nucleotide implicated in many enzymic glucose transfer reactions. UDPG is formed by the condensation of uridine triphosphate and α-D-glucose-1-phosphate (G-1-P) with the elimination of orthophosphate[171], and attempts to carry out the enzymic glucosylation of phenols with 'low-energy' donors such as G-1-P have been uniformly unsuccessful[152].

The best explanation of the remarkable relative increase in glucose uptake with time is that there is a significant pool of inactive UDPG already present in the hydrangea leaf and that, no matter how much labelled glucose is present from hydrolysis



Rationale of Glucose-label increase - Double-labelling experiments (XXXV - XXXVII).

The activity of the UDPG pool is initially zero and so, after ONE day, the net result is that almost no ^{14}C-glucose has been replaced on skimmin. The UDPG activity increases very markedly with time and after FIVE days, the overall effect is an apparent, but untrue, block incorporation.

of the fed doubly-labelled precursor, none of this ^{14}C-glucose can be replaced on the para position until the endogenous (and thus non-radioactive) 'activated glucose' has been utilised. There is apparently a definite upper limit to the amount of the 'glucose activating moiety' i.e. (UDP) and the major portion of this (UDP) must be attached to glucose in the unfed plant. The chart shows a possible scheme. Thus, in spite of the appearance of a large quantity of p-oxy-cinnamic acid precursor, the unlabelled UDPG pool is sufficiently large to last for almost one day after the feeding. In fact the results show that, after the first day, some 7% of ^{14}C-glucose has been replaced on the phenol. Thereafter, in the 2-3 day period, the averaged specific activity of the introduced glucose has increased due to the lower quantity of unlabelled UDPG and 54% of the glucose label relative to the incorporated chain has been so introduced. This is confirmed by the fact that the rate of skimmin synthesis in the 2-3 day period (1.07%) has dropped markedly compared to the first day (3.1%) and yet the relative incorporation of ^{14}C-glucose has increased some eight-fold. On the fourth and fifth days, a further

increment of 31% in the relative glucose label is apparent.

A straightforward calculation of the activity of the glucose moiety of the skimmin extracted after the successive periods shows that in the first day it is 0.07 times as active as the incorporated chain label, in the second and third days it is ca. 2.3 times as active, while in the fourth and fifth days it is 9.7 times as active. It is clear that the concept of 'pool replacement' accounts well for the apparently contradictory results of this time-course investigation of simultaneous uptake of two ^{14}C precursors. The diagram showing a plot of the incorporation into the chain and sugar portions in each time period proves, in complete accord with the above rationale, that the period of maximal uptake of ^{14}C-glucose takes place after that of the chain label.

It is possible that a slow exchange of radio-glucose into preformed skimmin may contribute.

Although the results of the competition experiments provide some evidence that the glucoside of p-coumaric acid is a preferred precursor to the aglycone, the observed difference could be due to a different rate of transport within the plant system to the site of oxidation. Thus if the p-glucoside were

'COMPETITION' FEEDINGS.

L. trans-p-Coumaric acid (2.61×10^6) 1.92 mgm-3.06×10^4

plus 4.11 mgm trans-p-glucosyloxycinnamic acid.

XXXIII.	1.83% (45)	1 Day.

As above but 1.83 mgm p-coumaric acid (2.91×10^4 cpm) were fed with 3.88 mgm of its glucoside.

XXXVIII.	3.83% (12)	3 Days.

trans-p-Glucosyloxycinnamic acid (2.17×10^6) 3.64 mgm-2.42×10^4

plus 1.76 mgm of trans-p-coumaric acid.

XXXIV.	3.88% (31)	1 Day.

As above but 4.02 mgm of the glucoside (2.68×10^4) were fed with 1.86 mgm of the aglycone.

XXXIX.	4.49% (15)	3 Days.

So far from showing the anticipated lowering of uptake of one of these precursors, these results suggest that these compounds may stimulate each other's incorporation. Since no significant drop is observed, it is possible that the glucoside and aglycone are converted to skimmin in different sites within the leaves.

transported to the hydroxylation organelle more quickly than the aglycone, the fact that it may have to be hydrolysed before hydroxylation could be outweighed. Although the aglycone may well be the true substrate, the glucoside would tend to occupy the precursor pool. Now it is a curious fact that in these competition feedings (XXXIII, XXXIV, XXXVIII and XXXIX) the incorporation of activity has not been lowered in spite of the presence of twice the normal amount of phenylpropanoid precursors (cf XXI). This is decidely unexpected and suggests very strongly that fed β-glucoside and aglycone are transported to two different sites within the leaves.

The inference is therefore that there are indeed at least two sites of biosynthesis of skimmin within the leaves. One of them utilises the β-glucoside and the other the aglycone. The double-labelling experiments prove that, even at the site which uses the glucoside, it is not incorporated en bloc. Since p-coumaric acid is the first product of the enzyme sequence, and since glucode esters are frequently formed in plant tissue, the status of the glucoside appears doubtful.

2.3.3. Possible Mechanisms for 'META'-Hydroxylation.

The stage has now been reached where a discussion of the possible hydroxylation mechanisms seems worthwhile. Much current interest attaches to the mechanisms of phenolic coupling reactions in the elaboration of aromatic compounds by living systems. Valuable reviews[134-5,172-9] have collated the formidable body of evidence proving the free radical nature of many of the observed enzymic and *in vitro* couplings. The first stage is frequently the generation of phenol radicals which are stabilised by resonance with the mesomeric forms in which the odd electron resides on the ring *ortho* or *para* to the oxygen function.

This formation of radicals assumes the presence of a phenolic grouping in the molecule undergoing coupling but clearly the hydroxylation of aromatic rings as such must take place to produce the phenolic substrates for further reactions. An important case, previously mentioned is the *para*-hydroxylation of phenylalanine to yield tyrosine in liver tissue, a reaction which has attracted a great deal of attention.[39e]

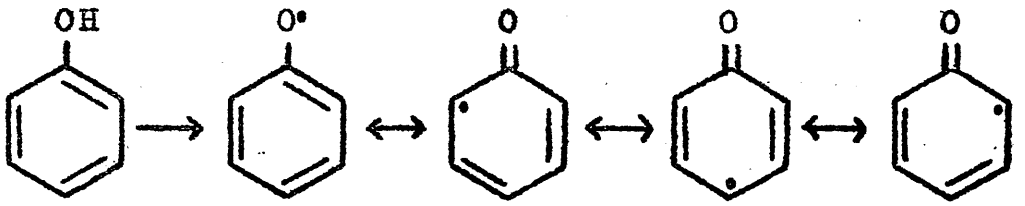
The mammalian enzyme shows the characteristics of the 'mixed function oxidases' in that both molecular oxygen and an external reducing agent are required to accomplish the hydroxylation and furthermore that only one atom of the transferred oxygen appears in the product. The first report of phenylalanine hydroxylase from higher plant tissue was made recently by Nair and Vining.[67] They purified the enzyme some 66-fold from spinach leaves and were able to show that its requirements are substantially similar to the liver enzyme. Since the formation of tyrosine in plants is usually accomplished by the transamination of p-hydroxyphenylpyruvic acid, it may be surprising that this plant possesses the activity characteristic of animal tissue. In fact, this is possibly a manifestation of a secondary pathway within spinach leaves to deal with any untoward increase in the concentration of phenylalanine without the production of trans-cinnamic acid. Previously, Nair and Vining[180] had prepared a crude unstable enzyme preparation from the same source that catalysed the hydroxylation of trans-cinnamic acid to yield p-coumaric acid. The

preparation exhibited an absolute requirement for an external electron donor and it appears that a sulphydryl group participates in the reaction. This hydroxylase is again similar to the liver enzyme and its action may be visualised as a transfer of oxygen while the acid is held (as a thioester?) on the enzyme surface.

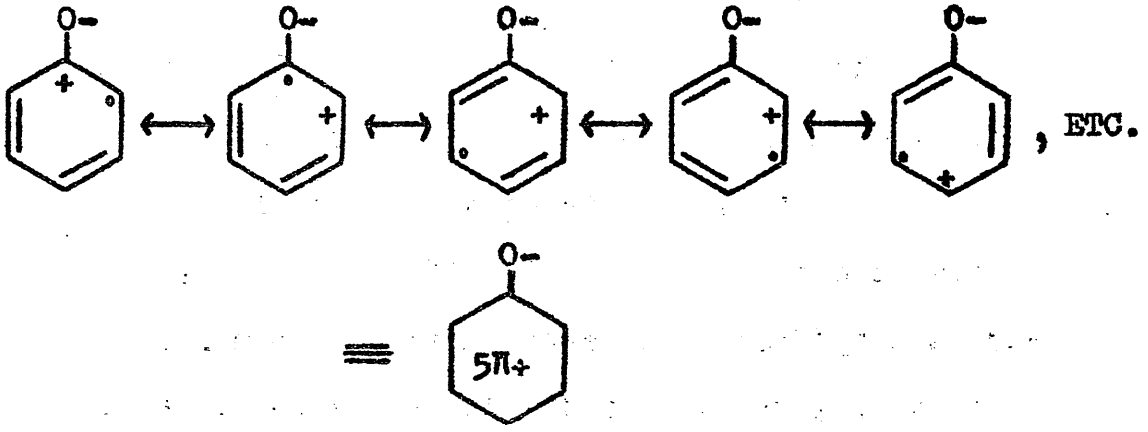
Conflicting reports exist as to the timing of hydroxylation of cinnamic acid in the formation of chlorogenic acid (60). It appears that in potato tuber tissue[181-2] cinnamic acid is esterified with quinic acid <u>before</u> hydroxylation while in tobacco leaves[114] the esterification does not apparently take place until <u>after</u> the formation of p-coumaric acid. In any event, p-coumaryl quinate is the substrate for the second hydroxylation, so that the hydroxylation necessary in the biosynthesis of skimmin may also require an ester substrate. The acid could be esterified with glucose, quinic acid, coenzymeA, or a thio-enzyme. The intermediacy of a phosphate anhydride is not ruled out. It will be seen that the mechanism preferred by the author does not depend on an ester substrate but leaves the question of possible activation open.

p-Coumaric acid will be described for convenience, but it must be emphasised that, as with many of the steps in this pathway, the true nature of the activated intermediates is unknown.

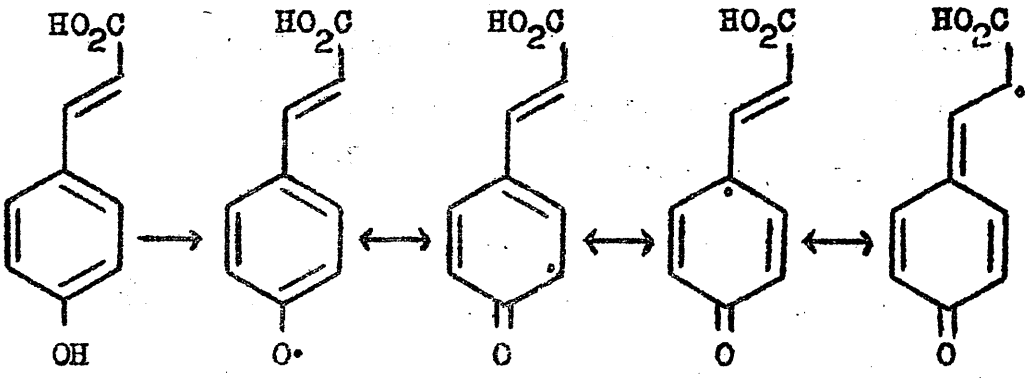
While it is true that aromatic hydroxylation meta to established groups does occur e.g. in the formation of meta-tyrosine derivatives from phenylalanine by fungi[128,183], and of 3-hydroxy-phenylpropionic acid from cinnamic acid by a bacterium[184], these particular syntheses do not involve substitution meta to an oxygen function. The fact that the competition experiments suggest that the glucoside of p-coumaric acid is a preferred precursor could be interpreted as circumstantial evidence favouring a direct meta-hydroxylation provided that total hydrolysis of cis-DGC occurs at a later stage. In the absence of data on the relative transport rates of glucoside and aglycone, such a scheme cannot be eliminated. In highly sophisticated studies on aromatic free radicals, Müller[185] has postulated the contribution of polar non-classical radicals to the ground state, including several with the odd electron meta to the oxygen



'Classical' radical forms.



Polar 'non-classical' radical forms (Müller[185]).

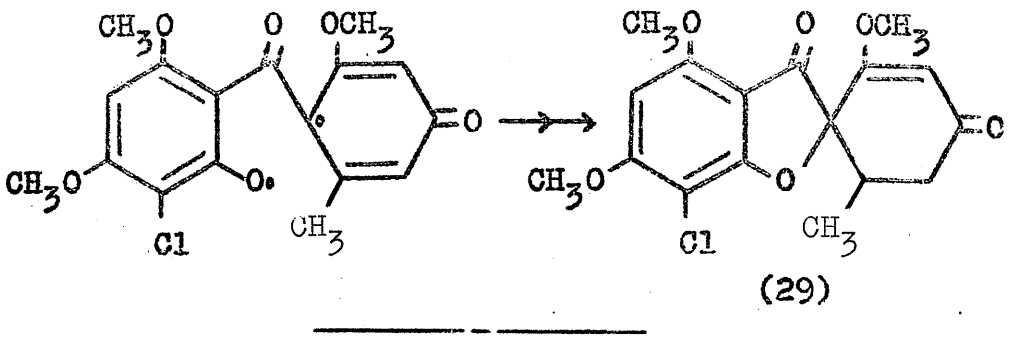


X

p-Coumaryl free-radical mesomers.

function while *in vitro* studies with complexed metal ions in ascorbic acid solutions (cited in[142]) suggest that direct *meta*-hydroxylation can occur. Such *in vitro* reactions are frequently taken (on slender evidence) as adequate models for enzymic hydroxylations.

The free radical hydroxylation mechanism favoured by the present author requires that the hydroxylation substrate be p-coumaric acid or an ester derivative. It is possible that it may be transported to the site of oxidation as a p-glucoside and hydrolysed to permit radical formation, which will give rise to the three mesomeric radical forms. By analogy with Müller's work, the maximal free-electron density is likely to be at the position *para* to the oxygen function i.e. *x* may be the major contributor to the resonance form. The preferred formation of *para*-radicals is attested by a wealth of experimental evidence[172-3,177,185-6] and has been implicated in the biosynthesis of many coupled phenolic compounds e.g. griseofulvin (29), Pummerer's ketone (73), usnic acid (74), and the morphine alkaloids (75). In addition, *para*-radicals have been shown to take part in certain



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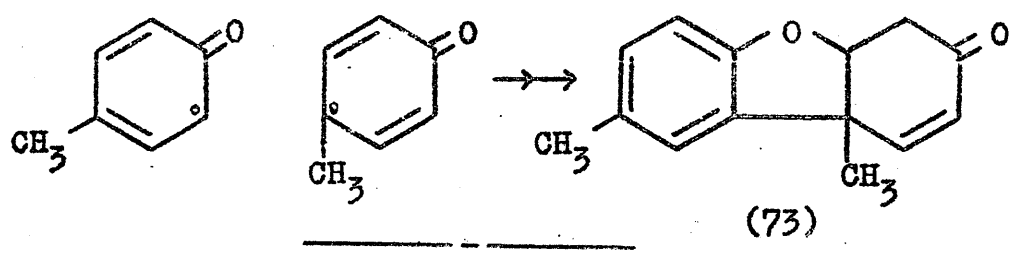

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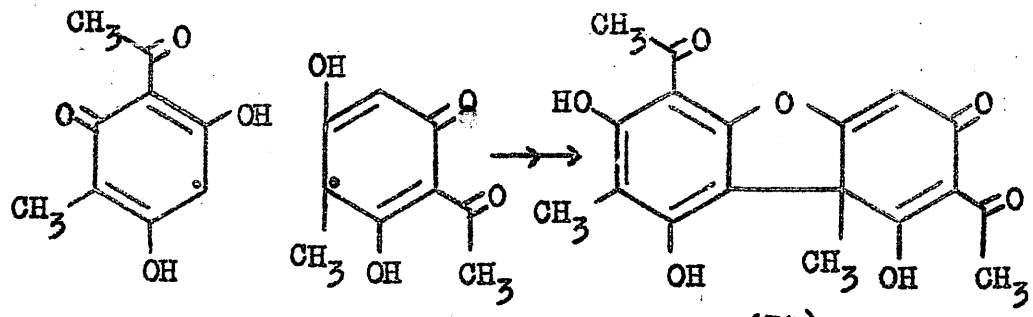

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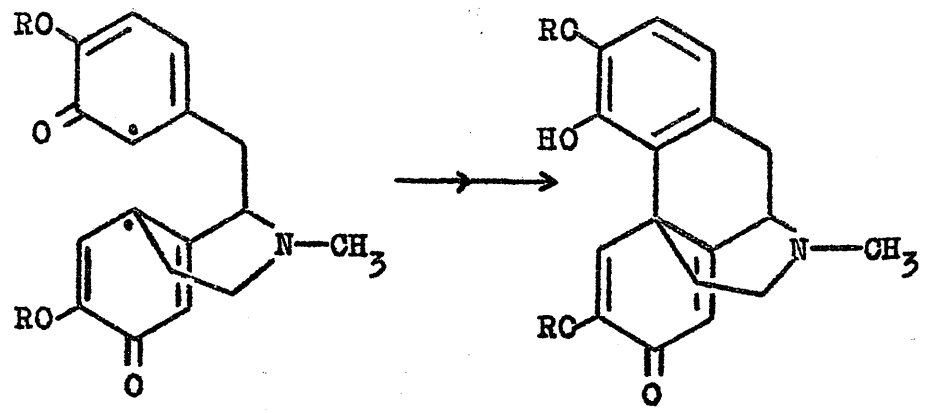

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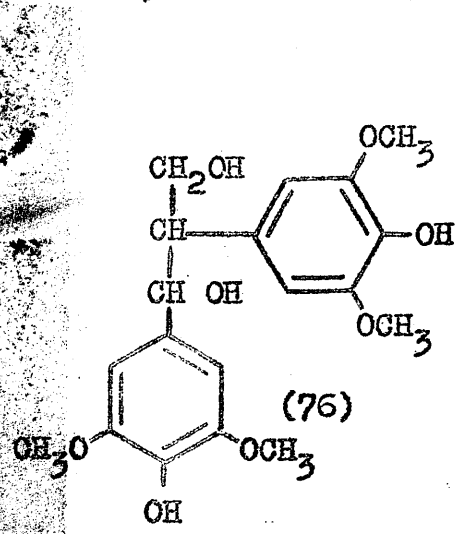

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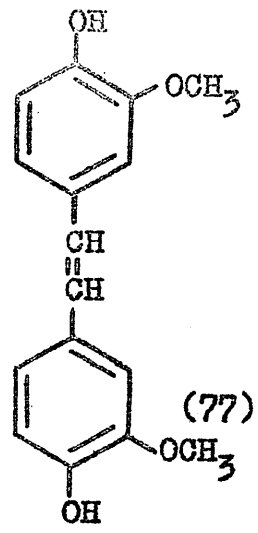

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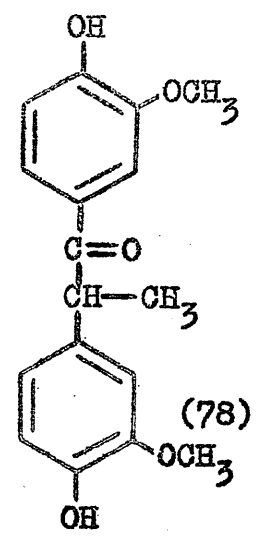

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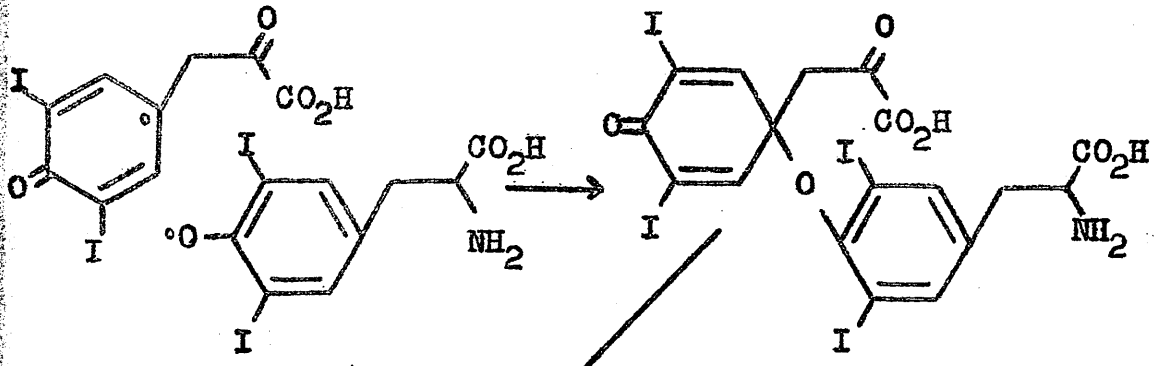

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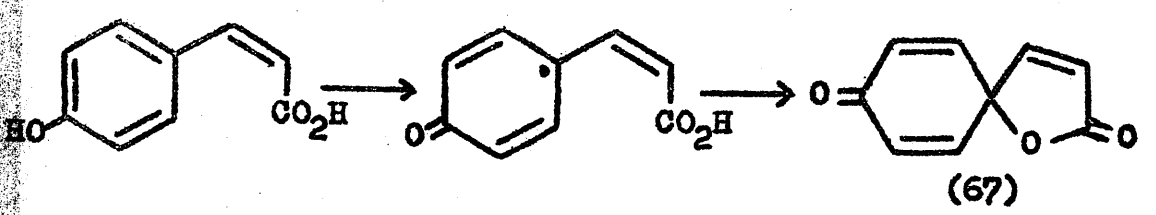
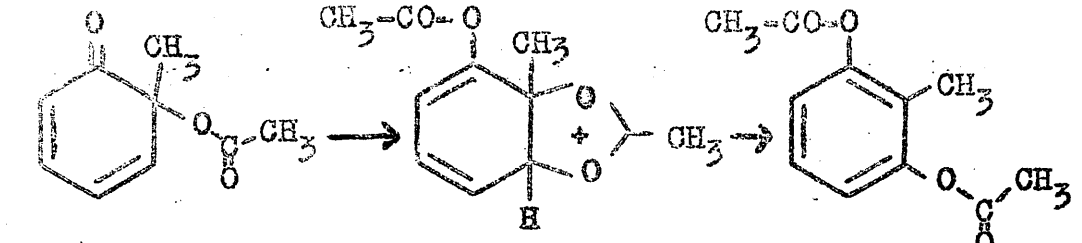


(67)

of the enzymic condensations of phenylpropanoid monomers in the production of lignin[175,187,221], the polymeric structural constituent of the cell walls of woody plants. Thus the compound (76) has been obtained by mild hydrolysis of beech lignin[188] while (77) and (78) have been obtained from spruce lignin.[189] The formation of these three compounds has been explained in terms of coupling between the quinone-methine and para-radical forms of the poly-oxygenated cinnamyl alcohols shown to be the precursors of lignin in vivo and in vitro.[175,221] The evident loss of a three-carbon side-chain in the production of these compounds is paralleled in the biosynthesis of thyroxine (79) from two diiodo-tyrosine units.[190] Freudenberg[188] has demonstrated the existence of the mesomeric forms of the radical from coniferyl alcohol by electron-spin resonance spectroscopic studies of the peroxidase-hydrogen peroxide dehydrogenation reaction and has proved that their contribution yields a surprisingly stable radical ($t_{\frac{1}{2}}$ = 45 secs.).

In passing, it is clear that the formation of the spirolactone (67) used in the tracer experiments



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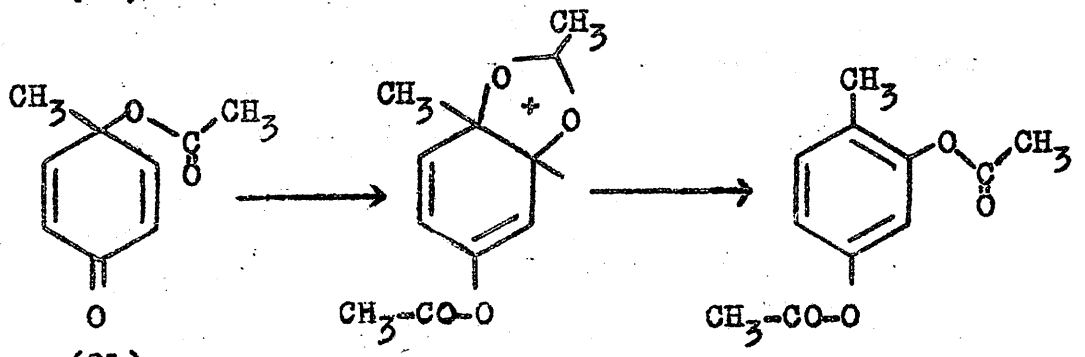
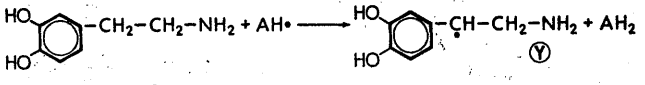
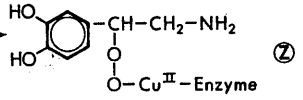


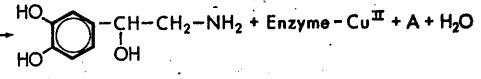
(81)

1. Enzyme-Cu^{II} + AH_2 ⟶ AH• + Enzyme-Cu^{I} + H^+

2. Enzyme-Cu^{I} + O_2 ⟶ Enzyme-Cu^{II}-O-O• (Ⓧ)

3.  (Ⓨ)

4. Ⓧ + Ⓨ ⟶  (Ⓩ)

5. Ⓩ + AH_2 + H^+ ⟶ 

Dopamine + AH_2 + O_2 ⟶ Norepinephrine + A + H_2O

AH_2 = Ascorbate AH• = Ascorbate free radical A = Dehydroascorbate

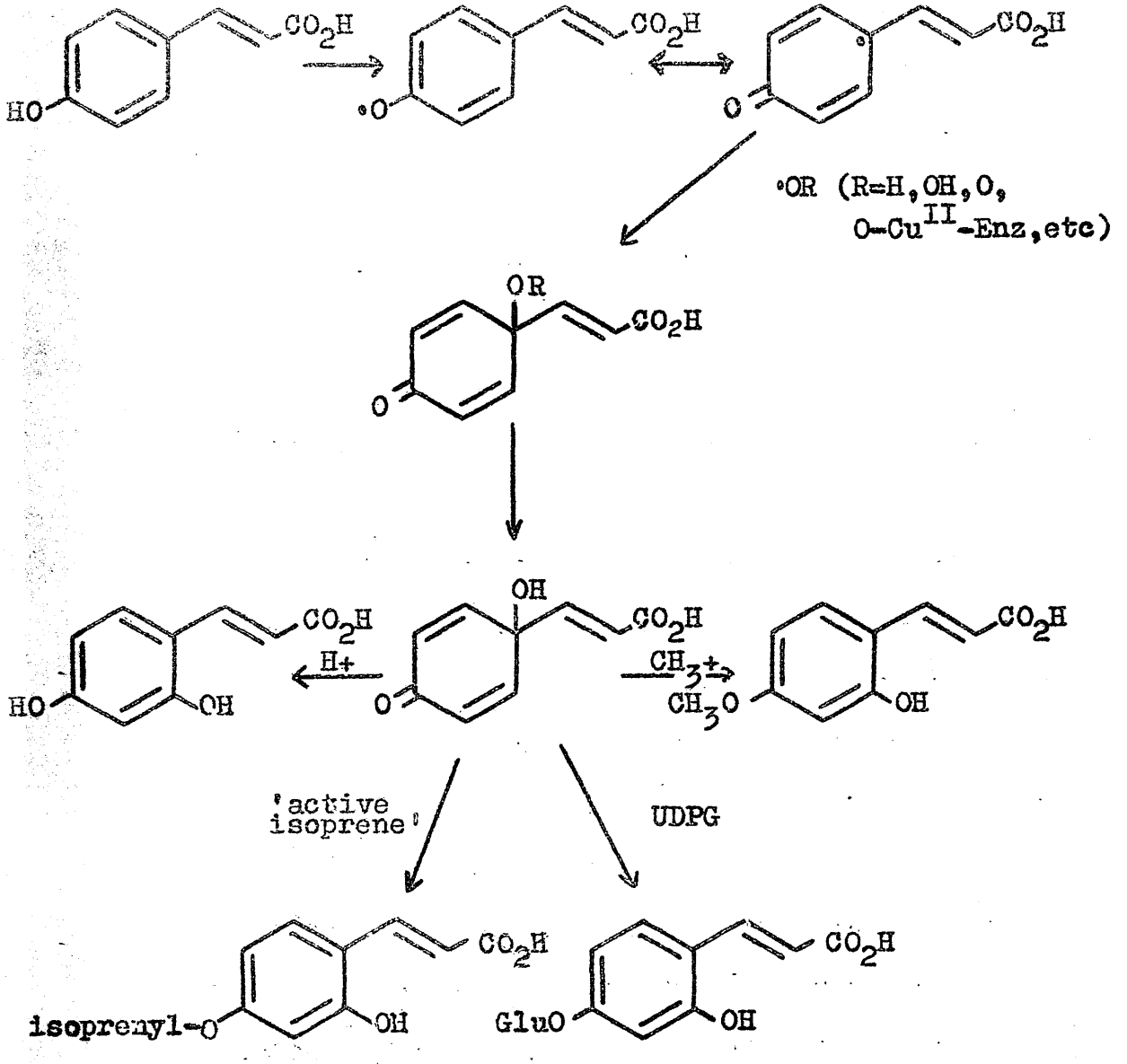
Postulated Mechanism for Dopamine Hydroxylation[194].

This mechanism accords fully with the known requirements of the 'mixed function oxidases'.

by the electrolysis of solutions of cis-p-coumaric acid may well be the result of coupling between a para-radical and the carboxyl group.

An interesting parallel for the postulated overall meta-hydroxylation process is evident in the rearrangement of the o-quinol acetate (80) derived from o-cresol by treatment with lead-tetra acetate in acetic acid.[191] The acid-catalysed rearrangement gives rise to a resorcinol derivative and thus ortho-substitution plus rearrangement has the gross effect of meta-substitution. Still closer parallels are to be found in the well-documented rearrangements (see [192]) of p-quinols e.g. (81) and their derivatives under certain conditions to give resorcinol fundamental nuclei.[193] Many of these rearrangements apparently involve cyclic polar intermediates, which of course are not excluded from the possible biological steps.

As previously mentioned, many of the phenol oxidases are known to be copper-containing enzymes and require ascorbic acid as a co-factor.[135] Two rec reports on the nature of the reaction mediated by dopamine β-hydroxylase, which has been purified



'Junction Oxidation' rationale for apparent meta-hydroxylation of phenolic precursors.

The direct process is another possibility.

from bovine adrenal medulla, are of great interest here. Thus Goldstein and his co-workers[194-5] have proposed the scheme shown on the basis of their electron-spin resonance studies, while Friedman and Kaufman[196] have proved that the copper within the enzyme undergoes cyclic reduction and oxidation ($Cu^{I} \rightleftharpoons Cu^{II}$) during the hydroxylation. It is further true that dopamine β-hydroxylase is a 'mixed-function oxidase', i.e. it requires an external electron donor (here ascorbic acid) and transfers molecular oxygen, only one atom of which appears in the product.

The admittedly speculative application of the scheme for dopamine hydroxylation to the problem in hand may yet contain an element of truth and therefore is shown in the diagram.

What is postulated here is that a substitution of effective hydroxyl radical <u>para</u> to the established oxygen function of a p-coumaric acid with a subsequent 1→2 shift of the introduced hydroxyl is a plausible route for the apparent <u>meta</u>-hydroxylation. The first step is the generation of a phenol radical. A coupling of the <u>para</u>-radical

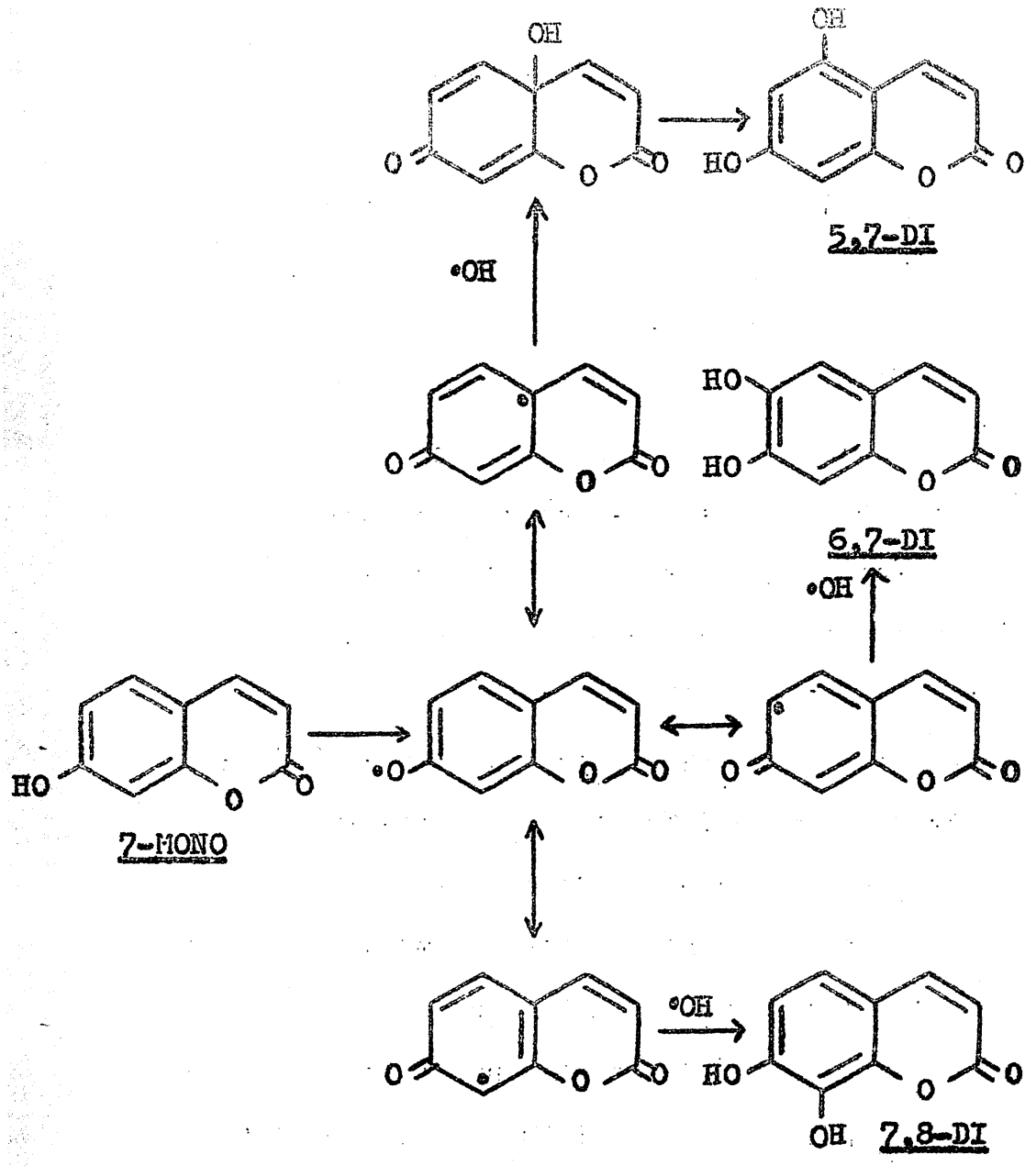
mesomeric form with hydroxyl, hydroperoxyl or enzyme-metalloperoxyl free radical will produce the p-quinol derivative. The 1→2 shift of the oxygen function before or after the removal of the blocking OH or enzyme-metallo-oxide groups, with a concomitant aromatisation of the ring can be readily seen to give rise to the meta-hydroxylated phenylpropanoid compound without the necessity of a direct meta-process. The first step may be termed 'junction oxidation' and clearly the evidence favouring the existence, and indeed the predominance, of para-radical mesomeric forms of phenol radicals also enhances the plausibility of this critical step. It is true that the p-quinol form could undergo a 1→2 carbon shift — this would yield a 2,5-dioxygenated cinnamic acid, a process of potential interest for the biosynthesis of gentisic and homogentisic acids.

If the p-quinol is indeed the first product of p-coumaric acid hydroxylation in the biosynthesis of skimmin in hydrangea, the necessary rearrangement with the simultaneous intervention of UDPG would result in the formation of the p-glucoside of

umbellic acid without the prior existence of free umbellic acid in the sequence. This would explain Brown's anomalous radiotracer[107] results without resort to enzyme-bound forms or differential transport of fed precursors. Furthermore, the analogous intervention of the biological methylation reagent S-adenosylmethionine would yield HMC, the herniarin precursor in lavender, without the formation of p-methoxycinnamic acid, which Brown has questioned as a 'natural' step in herniarin biosynthesis.[106] The interesting possibility therefore exists that a single (p-quinol) intermediate is the precursor of two oxygenated coumarins.

2.3.b. Speculative Applications of Hydroxylation Mechanism; Rationale of Skimmin Formation.

It may be claimed that the results reported here provide strong evidence as to the overall pathway for the biosynthesis of umbelliferone in Hydrangea macrophylla. It is clear that, contrary to previous theories, a mechanistically unfavourable aromatic hydroxylation, at least in effect, is part of the scheme for the biosynthesis of two 7-mono-



Extension of 'junction oxidation' scheme to the elaboration of poly-oxygenated coumarins from preformed umbelliferone.

oxygenated coumarins. This is supported by the work of Brown et al.[107]

The present demonstration that trans-2,4-dioxygenated cinnamic acid derivatives can be produced by a hydroxylation of a p-coumaric acid may be relevant to the formation of certain natural products which appear to be derived from shikimic acid and yet possess meta-oxygenation reminiscent of acetate-derived molecules.

In the complete absence of radiotracer studies on the formation of the 5,7-oxygenated coumarins, it may yet be postulated that closely analogous processes of radical formation and eventual rearrangement could produce this oxygenation pattern. (dia). The route from preformed umbelliferone is somewhat preferred. Possible support of this scheme is implicit in the sole published report on the biosynthesis of furanocoumarins. The experiments carried out by Floss and Mothes[13] showed that umbelliferone was by far the best precursor of the furocoumarins (64a,b.65a,b) when 1-^{14}C-cinnamic acid, 2-^{14}C-coumarin and 2-^{14}C-umbelliferone were fed to root cultures of

Pimpinella magna. Coumarin was very poorly utilised by comparison with the other two precursors Although the activity of each isolated furo-coumarin was unfortunately not given in this preliminary communication, it appears that the two 5,7-dioxygenated compounds bergapten (65a) and isobergapten (64a) were radioactive after the umbelliferone feeding. The furan ring is very probably formed from a cyclised isopentenyl substituent.[198]

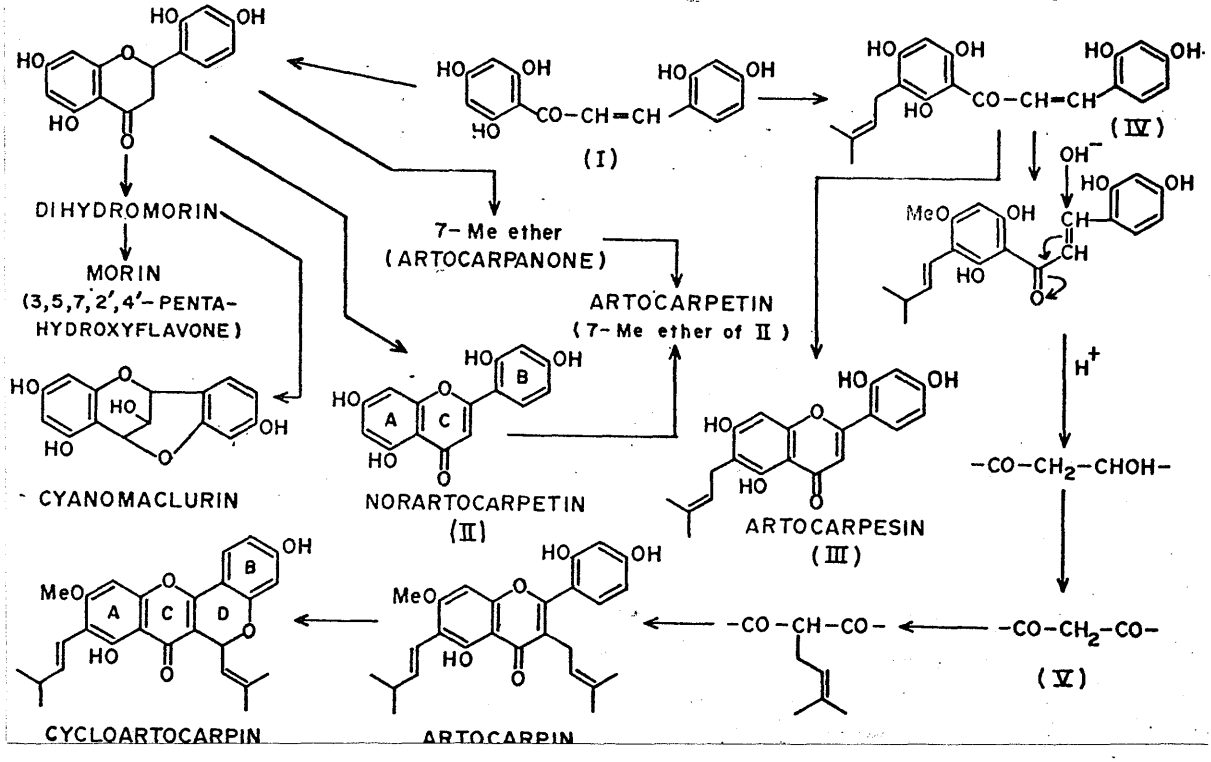
It is possible that radical formation from umbelliferone could give rise to the 6,7 and 7,8-dioxygenated coumarins (dia). However, the 6,7-oxygenated coumarins scopololetin and cichoriin are apparently formed from 3,4-dioxygenated cinnamic acids[114,115] i.e. that the oxygenation pattern is established before the cyclisation. By contrast, Kindl and Billek[117] have shown that a 7,8-dioxycoumarin,'hydrangetin', is formed preferentially from umbelliferone in Hydrangea macrophylla, while ferulic acid is not incorporated at all.

The furanocoumarins psoralen[199] and bergapten[200] have been found as the corresponding coumarinic acid glucosides in plants. In

addition, over 60% of the psoralen and bergapten present in the leaves of the true fig, Ficus carica var. 'Brown Turkey', have been isolated from a glucose-bound form in the present work. This suggests that polyhydroxylation and addition degradation of the isopentenyl substituent could occur before lactonisation, in conflict with the previous interpretation. A feasible, if unattractive, alternative is that the lactone ring may be cleaved after the elaboration of substituents and the ortho-hydroxyl glucosylated.

In a preliminary study of the metabolism of the indiarubber plant, Ficus elastica, var. 'decora', 2-^{14}C-p-coumaric acid was incorporated into the umbelliferone produced by this plant but only to some 0·03%, almost certainly a result of the extreme difficulty of feeding to this latex-forming species.

Ranging still further afield, very many naturally-occurring phenylpropanoid derivatives are known which contain a 2,4-di- or 2,4,5-tri-oxygenated aromatic ring. Previously, this 'anomalous' oxygenation pattern has been accounted for by



Postulated biosynthesis of Artocarpus flavonoids[201].

assuming the derivation of the phenylpropanoid precursor by ring-opening of a coumarin.[30,31b] While this is a feasible process, it is clear that the prior formation of 'umbellic' or 'aesculic' acid precursors followed by condensation with e.g. three effective C_2 units would produce the same result. In fact, such an extension of the present work has been postulated by Venkataraman et al.[200] in their consideration of the probable biosynthetic routes to the Artocarpus flavones. (dia).

In the experiments reported here, an attempt has been made to elucidate the mechanisms of formation of umbelliferone in hydrangea. An equally significant problem is - Why is it made at all?

The role of phenolic and other compounds in the disease resistance of plants is a field of great theoretical and practical interest and many excellent reviews are available which summarise current concepts of their action.[39b,40,202-6] As a widely distributed group of natural products the coumarins, in particular coumarin itself,[70] have been studied in this connection. In many

cases,[15,16] they have been implicated as factors for growth-regulation and germination inhibition, as well as natural 'antibiotics'. Before discussing the possible action of skimmin in hydranges, it seems appropriate to discuss the modes of solute transport in plants.[207-211]

In woody plants two basic transport systems are present, the xylem and the phloem. Both derive from the cambium, the thin layer of actively-growing cells between the bark and the wood which extends from the root tips to the leaves. The xylem life-cycle moves inwards into the wood to form the water-conducting conduits and it consists of dead cells whose walls have been greatly strengthened. This is done by the deposition of cellulose fibrils and lignin in the dying cells to make a 'reinforced concrete' structure imparting rigidity to the plant. The phloem, in contrast, develops from the cambium outwards and consists of living cells joined end to end, and connected via tiny pores in the end walls. They are thus termed 'sieve tubes'. Unlike the xylem, the phloem of all species is highly susceptible to injury when the

sieve tubes RAPIDLY shut off the flow of liquid. For example, certain components of the cytoplasm may instantly plug the sieve plates when pressure is released and callose, a polysaccharide, closes the sieve pores more permanently.

The xylem is believed to be mainly responsible for the bulk transport of dilute mineral solutions from the roots to the leaves[207] while the phloem has been implicated as the main carrier of photosynthetic products and other compounds from the leaves to the roots and the shoot tips.[208] This greatly simplified picture is complicated by the fact that food substances have been shown to move in both phloem and xylem and that such food substances not only move to the roots via the phloem - they make a circuit.[210] Furthermore, the possibility of interchange of materials between the two systems has been studied and shown to operate in certain cases.[212-13]

On feeding hydrangea by cotton wick, it may be postulated that the phloem tubes are promptly closed by their defence mechanisms and that the xylem is the main transport route of the intro-

duced liquid to the leaves. Once at the leaves, the evidence of feeding XXXII suggests that a rapid conversion of p-coumaric acid to skimmin takes place. The function of skimmin in the leaves is a matter of conjecture. It could conceivably act as an ultra-violet screen for the vital processes (in fact umbelliferone derivatives are frequently used in sun-tan oils). Since the evidence is that any injury of the leaf tissue results in the release of free umbelliferone, it is quite possible that the formation of skimmin is a latent anti-fungal or other defence mechanism. Rather than produce the free phenol in quantity, which might interfere with the normal operation of the cell, the readily translocated glucoside would be as efficient in conjunction with endogenous glucosidases.

It must be confessed that several of the plants were attacked by red-mites and by the powdery mildew Oidium hortensiae, a common parasite of hydrangea under glass. They were saved by chemical spraying. Hydrangea leaves infected with the fungus were examined under ultra-violet light (350 mμ) after drying and compared with dried

healthy foliage. A striking difference existed between them in that a strong blue-fluorescent zone surrounds the fungal lesions. This fluorescence is intensified by treatment with alkali, cf. umbelliferone, and since the healthy leaves showed only a slight yellow fluorescence (from skimmin?) on similar treatment, it may be argued that umbelliferone has been released from its bound form as a result of fungal attack. In fact, a quantitative analysis of the infected leaves showed that at least 97% of the umbelliferone is in the free form.

In confirmation of the previous hypothesis that the leaves are the main site of skimmin synthesis, it should be pointed out that the weight loss of healthy leaves on drying was rather more than 90%, so that, in feeding XXXII, ca. 130 mgm. dry weight of leaf tissue has produced 0.58% incorporation. Because the stems (0.66% / 4.3 gm. - fresh) and roots (0.05% / 16.0 gm.-fresh) lose far less weight on drying, this result emphasises the difference in synthetic ability between them and the leaves, which definitely appear to be the

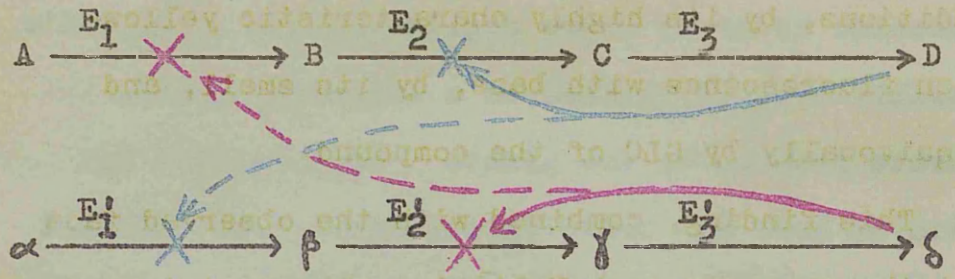
main synthetic site for skimmin. The stem incorporation probably results from a deposition of skimmin in the older phloem tissue.

Translocation of skimmin from the leaves most probably takes place via the living phloem tissue.[212] Since sucrose is frequently the main component of phloem sap,[208] as judged by sophisticated tapping experiments using aphids, it could be that the addition of the glucose group to umbelliferone makes it more soluble in the phloem sap and simultaneously makes its shape approximate to that of sucrose. In any event, skimmin is brought to the roots and, although it has been shown that coumarin glucosides can be recovered from bleeding xylem sap[214-16] i.e. by a recycling process, it appears that skimmin may accumulate within the root tissue. The story does not end there however, since an extraction of the free and bound umbelliferone from hydrangea root has shown that almost half is present as the free compound. This may be explained by assuming the presence of a fairly active glucosidase within or on the surface of the roots (already proved for certain plants[217]). The

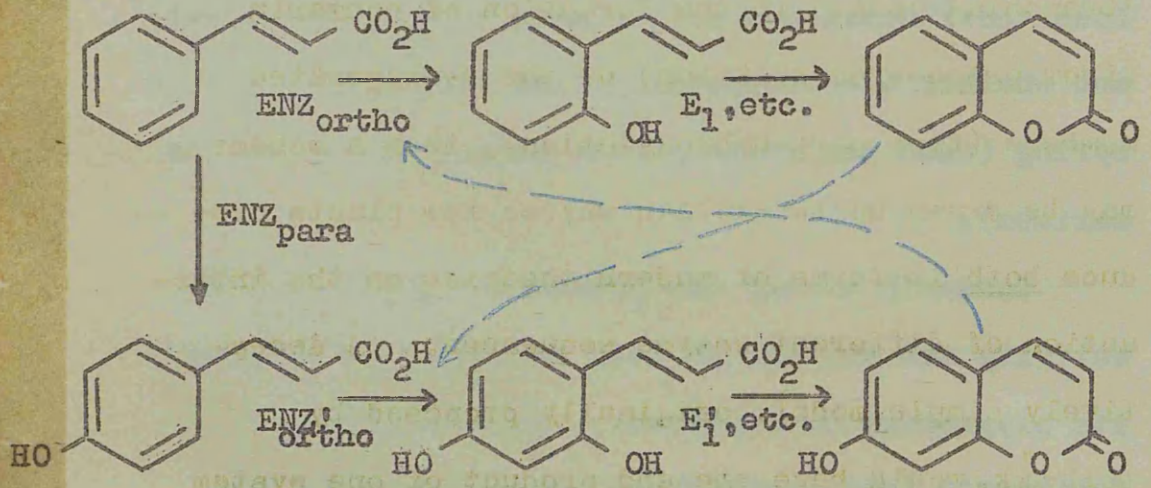
action or soil micro-organisms may also result in the hydrolysis of skimmin. A minor point is that our hydrangea plants had been propagated by cuttings and therefore the bulk of the 'root' tissue was morphologically 'stem'. This being the case, the concentration of umbelliferone (free/ bond) in true root tissue was not certain. However, this was easily rectified by an estimation of the proportion of this compound present in the adventitious roots from a cutting. The result was almost identical (ca. 1 mgm/gm fresh weight) with that previously found. In passing, this concentration is extremely high and suggests that skimmin is not physiologically active in the plant tissue. The ultimate fate of the hydroxycoumarin has been examined by extraction of the 'Peralite' in which a hydrangea had grown for almost two years. Only a very small amount of umbelliferone was found even close to the roots with possibly a trace of skimmin. This accords with previous findings that, although scopoletin may be a 'root exudate', umbelliferone is much less liable to enter the soil.[215] What then, is the purpose of skimmin in the roots? The concentration of this

glucoside is by no means negligible and it is quite possible that it is used as a secondary carbohydrate reserve by the plant. The observed 1:1 ratio of free and bound umbelliferone may reflect the carbohydrate requirements of the actively growing hydrangea. It would be of great interest therefore, to follow the free-bound ratio throughout the year to find whether a significant drop occurs in the late autumn, (when the dying leaves lose their nutrients to the main body of the plant) and whether a corresponding rise takes place in the spring (when the carbohydrate demand may be at its maximum).

Coumarin itself has pronounced physiological activity and differs in this respect from many of its oxygenated derivatives. Accordingly, a deliberate (and successful) search was made for this compound in hydrangea extracts. It was found in trace quantities in both leaf and root extracts and in each case the glucose-bound form, presumably coumarinyl glucoside, predominated. The constitution of the free material was proved by TLC comparison with authentic coumarin under the standard



Enzyme sequence interaction showing feed-back inhibition of early steps by later metabolites. It is a 'fine' control of the sequence. The repression of enzyme formation is a 'coarse' control.



Possible inhibition system for the formation of coumarin and its oxygenated derivatives, e.g. in H.alba, ENZ'_{ortho} is either absent or masked, and in H.macrophylla ENZ_{ortho} is almost totally inhibited. In lavender, neither sequence is apparently made inoperative.

conditions, by its highly characteristic yellow-green fluorescence with base, by its smell, and unequivocally by GLC of the compound.

This finding, combined with the observed fact that the coumarin-rich Melilotus alba contains no trace of oxygenated forms, permits certain speculations on the nature of the enzymic control of the biosynthesis of this class of natural products. If trans-cinnamic acid is indeed the 'branching point' in the formation of coumarin (from ortho-hydroxylation) or the 7-oxygenated members (from para-hydroxylation), then a scheme may be drawn up to explain why so few plants produce both in terms of modern theories on the interaction of different enzyme sequences. A deceptively simple model, originally proposed by Delbrück, would have the end product of one system inhibiting an enzyme involved in another[218] (dia). Since the inhibition is mutual, the first reaction to get started would permanently inhibit the other. Alternatively, a system of repression of enzyme formation[219] may operate. This differs from 'end-product inhibition' which involves the repression

of formation or action of the enzymes of a given sequence. In the postulated model the end-products act on the regulators of systems other than their own. A scheme setting out possible interactions for the biosynthesis of coumarins is shown.

The apparent branching point in the formation of coumarin and its oxygenated derivatives is the first hydroxylation of trans-cinnamic acid. It can be argued that in M.alba the para-hydroxylation step leading to the umbelliferone system is inhibited in some way and that coumarin itself is the only lactone formed. Conversely, in hydrangea ortho-hydroxylation as a first step may be almost totally inhibited, although the ortho-hydroxylation of trans-p-coumaric acid is not affected and accordingly, the 7-oxygenated coumarin is formed. The situation in lavender is more complex since both pathways operate. Either the inhibition systems described are not significantly active, or the sites of synthesis of the coumarin and herniarin produced may be separated one from the other by some physiological barrier. At all odds, such

possibilities seem worthy of examination.

Although much has been learned on the biosynthesis of umbelliferone in hydrangea, enzymological and cytological studies appear essential to gain information on the many remaining problems e.g. the precise mechanism of each step, the nature of the activated intermediates, the spatial organisation and the control systems of the operating enzymes. Further, it is clear that many problems of potential interest to plant physiologists and pathologists arise from the observed constitution of hydrangea.

Part 2.4. Experimental.

As before, the page numbers given before each description refer to the use of the data obtained in the Results and Discussion (2.2).

P.148. Preparation of 1-^{14}C-labelled precursors.

A. Malonic acid (100 mg) was added to an open vial of 1-^{14}C-malonic acid (sodium salt). The weight of radiomalonate was ca. 3 mgm containing 0.1 mc activity. Water (0.5 ml) was added and the solution allowed to equilibrate at room temperature for one week. The aqueous phase was then transferred to the reaction flask and evaporated to dryness. To the dried residue was then added p-hydroxybenzaldehyde (110 mg) followed by dry pyridine (3 ml) and piperidine (3 drops). The mixture was heated on the steam bath for 2 hours, during which the suspension dissolved and vigorous evolution of carbon dioxide took place. Concentrated hydrochloric acid (10 ml) was added and then water (50 ml). The resulting solution was extracted with 3 portions of ether (60 ml each), the extract washed once with brine and itself extracted with 5% sodium bicarbonate solution (3x60 ml). This basic solution was acidified with dilute hydrochloric acid and extracted with ether (3x90 ml). The organic extracts were dried and reduced

to dryness yielding trans-p-coumaric acid m.p. 208-11°* (lit.[141] 210-13°) in a high state of purity and in ca. 80% of the theoretical yield. Its purity was convincingly established by IR and UV spectroscopy in comparison with authentic material as well as mixed melting point and chromatographic mobility. This material was usually diluted to 350-400 mg quantity with inactive acid before further preparations and feedings.

* = 1-^{14}C-labelled.

B. cis-p-Coumaric acid was prepared from the trans-isomer by irradiation of its ethanolic solutions for 24 hours with a Hanovia mercury vapour lamp. After this period, the solution was removed from the quartz flask and reduced to dryness. The residue was then dissolved in boiling water (ca. 10 ml/gm) and allowed to cool overnight. This resulted in the crystallisation of essentially all the trans-acid present while the more soluble cis form remained in solution. This operation was carried out in a filter beaker and so the direct filtration of the solution was a simple matter. The crystals were recycled and the solution extracted with ether to produce cis-p-coumaric acid* m.p. 126-8°, with spectral properties fully consistent

with the cis-form and demonstrably pure by chromatographic analysis.(lit.m.p.126-7° [222]). Yield 30-35%.

C. The 2-^{14}C-spirolactone was prepared from the labelled cis-p-coumaric acid by Dr.Meyers by his reported technique[123]. This involves the electrolysis of aqueous solutions of the cis-acid, extraction of the neutral products and fractional sublimation and repeated crystallisation to yield the required compound m.p.116-7° as stout colourless prisms. A refinment of the reported technique - the addition of small amounts of ammonium chloride to the solution - greatly improved the yield (from 1-2% to 4-5%).[142]

These syntheses were repeated during the course of this research to give the corresponding 2-^{14}C-labelled coumaric acids and the 3-^{14}C-spirolactone, for the hydrangea feedings. In every case, the activity of each precursor was measured by the method described in section 2.1.d.(p.136).

P.149. Feedings to Lavandula (Munstead).

The 1-^{14}C-labelled precursors were fed to the plants via the roots as described and absorbed as far as possible. After six days, they were extracted as detailed and the coumarins separated by the combined TLC and chemical techniques. The amounts of material

recovered were as follows:

TRANS TRIAL. UMB 0.5 mgm HER + COU 10.6 mgm. The plant material weighed 7 gm.

TRANS. UMB (ex Herniarin) 3.3 mgm.
COU 3.6 mgm.
Plant material weighed 23.3 gm.

CIS. UMB (ex Herniarin) 5.6 mgm.
COU 4.4 mgm.
Plant material weighed 35.3 gm.

SPIRO. UMB (ex Herniarin) 10.2 mgm.
COU 4.2 mgm.
Plant material weighed 28.2 gm.

The fractional sublimation was carried out on the mixture of coumarin and umbelliferone from the demethylation. Coumarin was obtained pure at 95-100° and umbelliferone at 120-160° (0.02mm Hg). Their purity was established by TLC comparison with authentic material.

The purified precursors and final products from these feedings were counted by Dr.Meyers in a Nuclear-Chicago windowless gas-flow counter (courtesy of the Biochemistry Department) and the overall results are shown in the text (p.151).

The degradation of spirolactone-derived umbelliferone is detailed overleaf.

P.152. Degradation of Spirolactone-derived Herniarin (as umbelliferone).

After appropriate trials, the umbelliferone (9.8 mgm) ultimately derived from the spirolactone feeding was shaken for one hour in a sealed tube with 23.2 mgm of yellow mercuric oxide and sodium hydroxide (80 mgm) in water (1 ml) in order to effect its conversion to umbellic acid (trans-2,4-dihydroxycinnamic acid). The suspension was centrifuged and the fluorescent green solution decanted. The solid residue was washed with water (1 ml) and the combined liquids acidified with conc. hydrochloric acid (0.5 ml), thereby discharging the fluorescence. After a few minutes, umbellic acid began to crystallise from the solution which was therefore cooled and allowed to stand for several hours. The crystals were removed by centrifugation, dried at 100°C under vacuum, and weighed - 5.1 mgm (47% yield).

This umbellic acid was decarboxylated by heating with copper bronze (40 mgm) and quinoline (3 ml). The reaction train consisted of the decarboxylation flask (dipping into a Woods metal bath) coupled to two small bubblers each containing 3.5 ml of 0.2N carbonate-free sodium hydroxide solution. A slow, controlled flow of nitrogen was passed through the train during the

process to sweep the carbon dioxide formed directly into the traps. The gas was evolved over a period of one hour while the temperature was rising from 170° to 230° and the reaction flask was maintained at 230° for a further hour to complete the decarboxylation.

To each bubble trap (containing the carbon dioxide as dissolved sodium carbonate) just sufficient of a carbonate-free solution of barium hydroxide (1N) was added to precipitate all the carbonate as the very insoluble barium salt. The trap remote from the reaction flask contained only a small amount of the salt which was combined with the centrifuged washed precipitate from the first trap. The total material was washed twice with water, twice with ethanol, twice with ether and then desiccated. Yield = 6.1 mgm, corresponding to 109% of the original compound. Even although this indicated that a small amount of aerial carbon dioxide had been taken into the product, the activity of the barium carbonate, which represents the activity of the lactone carbonyl of the spirolactone-derived herniarin, was over 91% of the umbelliferone thus demonstrating that only slight randomisation of the 2-^{14}C label of the spirolactone had taken place.

Synthesis of standard coumarins.

Umbelliferone and its 7-methyl ether, herniarin, were prepared by the condensation of malic acid with the appropriate resorcinol derivative. The condensation reagent was conc. sulphuric acid and thus constitutes an application of the normal Pechmann synthesis[223] of coumarins.

Aesculetin (6,7-dihydroxycoumarin) was obtained by hydrolysis of the commercially-available aesculin, the 6-β-D-glucoside.

Skimmin, the β-D-glucoside of umbelliferone, was prepared by the silver oxide-catalysed condensation of acetobromoglucose with the free phenol followed by selective removal of the blocking acetyl groups with aqueous barium hydroxide solution. This was identical with the reported procedure.[167] Its purity was established by TLC on cellulose powder.

P.159. Origin of Hydrangea macrophylla varieties.

The three cultivars 'Bouquet Rose','Blue Bird' and 'Blue Wave' were obtained from:

T.Hilling & Co.Ltd., Chobham,WOKING,Surrey.

P.163. GLC analysis of recovered trans-p-coumaric acid.

The crystalline material remaining from the recovery of trans-p-coumaric acid following the

feeding of trans-cinnamic acid and the para-oxygenated phenylpropanoid precursors was identical in all respects with the authentic compound. Its purity was demonstrated by GLC of the methyl ester in each case, when no traces of contaminants were observed under the following conditions:

Column: 4' x ¼" glass tubing. Gas Flow: 40 ml/min.
Support: 100-120 mesh acid-washed silanised Gas-Chrom P.
Stationary Phase: 7% F-60/1% Polymer Z.
Temperature: 175° - Flash heater 225°.

Under these conditions, the Rt values of standard compounds were:

methyl p-coumarate	=	17.1 mins.
methyl cinnamate	=	1.9 mins.
coumarin	=	4.0 mins.
methyl p-methoxy-cinnamate	=	7.3 mins.
ethyl o-coumarate	=	16.2 mins.
herniarin	=	15.9 mins.
psoralen	=	25.2 mins.
bergapten	=	64.3 mins.
xanthotoxin	=	"
umbelliferone	=	80.5 mins.

The retention times of the more polar compounds were so great that it was found necessary to use the conditions as described above with an increased gas

flow rate - 80 ml/min. The Rt values found were:

herniarin	-	10.5 mins.
psoralen	-	16.3 mins.
bergapten	-	43.5 mins.
xanthotoxin	-	"
umbelliferone	-	50.5 mins.

For an improved assessment of the purity of certain preparations of coumarin, from plant and fungal extracts, the conditions were as above (with gas flow 40 ml/min) but with an operating temperature of 150° resulting in an Rt value of 10.1 mins. (cf[224])

The identity of the naturally-occurring coumarins was regarded as established when: the Rf values on paper chromatograms, Kieselgel G and cellulose powder TLC in several solvents (adsorption and partition systems); the characteristic fluorescence with base or hydrolysis with emulsin; the Rt values on GLC analysis and, not least, the smell (for coumarin and herniarin) were consistently and closely comparable with those of authentic standard compounds. The UV spectra and characteristic λmax shift with base were also employed for this end.

P.164. The Synthesis of trans-2-^{14}C-cinnamic acid.

This necessary precursor was prepared in a

straightforward by the condensation of malonic acid labelled in the 2-position with benzaldehyde.

Thus malonic acid (21.8 mgm) and benzaldehyde (22 mgm-0.02 ml) were dissolved in pyridine (0.75 ml) and a trace of piperidine added. The mixture was heated on the steam bath for 5 hours during which the initial gas evolution ceased. The solution was acidified with conc. hydrochloric acid (5 ml) and the resulting precipitate of cinnamic acid removed by extraction with 3 x 5 ml portions of ether. The weight of the crude material produced after reduction of the acid-washed, dried ether extract was 22.9 mgm, corresponding to a yield of 73.8%. This was crystallised twice from water to give the pure compound (14.0 mgm)- yield 45.1% based on malonic acid - with m.p. 130-32° (lit. 133°) undepressed by the pure authentic trans-cinnamic acid.

P.164. Feedings I-XVI, XVIII-XX.

The synthesis of the 2-^{14}C-labelled precursors required for these experiments followed the routes previously established (P.238).

In these tables the data presented are ancillary to those set out in the text. They are intended to complete the description of the results of each

feeding by giving an indication of the nature of the stems and leaves fed, the amount of recovered umbelliferone, the number of cpm recovered. By comparing this information with the overall results of each experiment, it is readily seen that green (i.e. non-lignified) shoots produce much less umbelliferone than the woody shoots but that this smaller quantity of hydroxy-coumarin is much more active (less-diluted). The net result is fully consistent with the apparent fact that the green, actively-growing leaves are the site of synthesis of skimmin and that the skimmin is deposited in the woody stems and the roots.

The results of series A, B, C and E are set out in the tables facing p.164. The terms G and W refer to green or woody shoots respectively.

Feeding.	Stems.	Fresh Weight.	UMB. Weight.	Cpm rec'd.
I	2W	11.3 gm	9.1 mg	287
II	2W	10.0 gm	4.2 mg	785
III	2W	11.2 gm	2.5 mg	506
IV	2W	11.5 gm	4.8 mg	830
V	2W	8.0 gm	2.2 mg	535
VI	2W	11.5 gm	5.0 mg	960
VII	2W	11.0 gm	3.5 mg	1002

	Feeding.	Stems.	Fresh Weight.	UMB Weight.	Cpm rec'd.	
	VIII	2W	8.5 gm	-	-	
	IX	2W	7.0 gm	3.1 mg	96.4	
B.	X	2G	8.0 gm	0.74 mg	65	
	XI	2W	12.0 gm	4.0 mg	50.8	
	XII	2W	10.0 gm	2.8 mg	127	
	XIII	1G,1W	5.0 gm	1.66 mg	227	
			- - - - -			
	XIV	2W	12.0 gm	0.59 mg	56.8	
C.	XV	2W	7.5 gm	-	-	
	XVI	2W	9.0 gm	0.81 mg	78.5	
			- - - - -			
	XVIII	2W	6.0 gm	1.34 mg	8.6	
E.	XIX	1G,1W	9.5 gm	4.34 mg	13.1	
	XX	2W	11.5 gm	4.1 mg	20.4	
			- - - - -			
	P.168. Feedings XXI-XXV.					
F.	XXI	2W	8.0 gm	0.28 mg	36.5	Free
			- - - - -	1.66 mg	269	Bound
H.	XXV	1G,1W	9.0 gm	0.36 mg	13.9	Free
				3.74 mg	139	Bound
			- - - - -			
	XXII	2W	9.5 gm	4.3 mg	678	
G.	XXIII	2W	10.2 gm	3.45 mg	1130	
	XXIV	1G,1W	6.2 gm	1.90 mg	882	
			- - - - -			

P.170. Synthesis of the β-glucoside of 2-^{14}C-p-coumaric acid.

The labelled trans-p-coumaric acid (100 mg) was dissolved in methanol (25 ml) and, after the addition of 3 drops of conc. sulphuric acid, the mixture was heated under reflux for 4 hours. The solution was poured into water (100 ml) and the suspension extracted with ether (3 x 100 ml). The ether extracts, after washing with 5% sodium carbonate solution (200 ml) and drying over sodium sulphate, were evaporated to give methyl p-coumarate m.p.127-134° (lit.m.p.137°) in quantitative yield. The methyl ester (107 mg), aceto-bromoglucose (675 mg) and black silver oxide (500 mg) were suspended in quinoline (2 ml). The mixture was shaken for 30 minutes, then allowed to stand overnight. After trituration with 25% aqueous acetic acid, the mixture was poured into ethanol (100 ml).

Hydrochloric acid was added until this solution was slightly acid and the precipitated silver salts were removed by filtration. Evaporation of the filtrate produced methyl tetraacetylglucosyloxycinnamate m.p. 158-160° (lit.[153] 160-161°). This material was taken up in methanol (25 ml) and saturated barium hydroxide (12 ml) added. After shaking this suspension for 18

hours, the crystals had almost completely dissolved and the solution was adjusted to pH 4 with sulphuric acid. The barium sulphate thus formed was removed by centrifugation and the filtrate evaporated. The residue was crystallised from methanol to yield trans-2-^{14}C-p-β-D-glucosyloxycinnamic acid (42 mg), m.p.190-193.5° (lit.[153] 194-195°).

P.171. Synthesis of the β-glucoside of 2-^{14}C-O-coumaric acid and its aglycone.

A. To a mixture of helicin (salicylaldehyde β-glucoside) (49.2 mgm) and 2-^{14}C-malonic acid was added pyridine (1 ml) and 3 drops of aniline. The reaction mixture was heated at 75° for 7 hours and the cooled reaction mixture added dropwise to 25 ml of anhydrous ether with stirring. The precipitated gum was collected in a Craig tube and crystallised once from ethanol yielding 43 mgm of product m.p. 238-241°(lit.[80] 241.5-243.5°). This was recrystallised from water twice and the product dried at 160° under vacuum, yielding the pure compound m.p.243-5°. The radiochemical yield was 34% and the chemical yield of trans-o-glucosyloxycinnamic acid was 28.8 mgm or 26.2% based on malonic acid.

P.171. Feedings XXVI-XXXI.

	Feeding.	Stems.	Fresh Weight.	UMB Weight.	cpm rec'd.
	XXVI	1G,1W	8.5 gm	1.85 mg	24.6
I.	XXVII	1G,1W	13.0 gm	2.36 mg	24.6
	XXVIII	2W	6.5 gm	2.34 mg	23.8
	XXIX	1G,1W	11.0 gm	1.83 mg	14.4
J.	XXX	1G,1W	6.5 gm	1.98 mg	16.5
	XXXI	2W	8.0 gm	2.41 mg	20.0

P.172. Feeding XVII.

D.	XVII	1G,1W	11.5 gm	4.9 mg	1360

P.174. Feeding XXXII.

XXXII	1W	1.27 gm	0.41 mg	162 Leaves
		4.31 gm	2.07 mg	185 Stem
		16.03 gm	16.78 mg	14 Roots

P.177. Chromatography of Phenylpropanoid Compounds.

The preliminary studies on hydrangea extracts were carried out using ascending paper chromatography on Whatman No.1 paper with 2% acetic acid as eluent. In this respect, the system closely resembled that employed by Fujita and Furuya[225] in their studies on coumarins. The table overleaf includes the data obtained for these compounds using TLC on Whatman

CC41 crystalline cellulose powder. This system appears most promising for the analysis of polar materials and for the general classification of polarity groups.

	2% HAc on paper	5% HAc-TLC cellulose	AAW-TLC cellulose
Coumarin	0.70	0.83	0.92
Aesculetin	0.31	0.48	0.70
* Aesculin	0.59	0.73	0.22
Scopoletin	0.34	0.54	0.79
* Scopolin	0.66	0.78	0.25
6-Hydroxy-coumarin	0.48	0.61	0.86
Herniarin	0.57	0.71	0.92
Cinnamic acid	0.51	0.57	0.90
p-Coumaric acid	0.37	0.50	0.86
+ Caffeic acid	0.61, 0.29	0.78, 0.53	0.91, 0.76
Ferulic acid	0.33	0.50	0.82
o-Coumaric acid	0.40	0.64	0.85
+* Chlorogenic acid	0.77, 0.57	0.86, 0.76	0.35

The entries marked * indicate that these are 'monosaccharide' derivatives. They migrate slowly in the AAW system.

The entries marked + show spots (higher Rf) apparently due to the respective cis isomers.

The combined data on p.177 and 253 proved to be invaluable for the characterisation of the naturally-occurring compounds, when the individual fluorescence and base reactions were taken into account.

The adoption of a technique of two-dimensional elution of cellulose powder TLC plates with/without the same solvent and with/without emulsin hydrolysis between the separate runs has been of central importance in the studies on the 'bound' forms of umbelliferone in hydrangea. Thus skimmin was first identified in the plant extracts by its low Rf in AAW (suggesting a mono-saccharide) and by its rapid emulsin hydrolysis (β-D-glucoside) to yield umbelliferone (second elution with 5% HAc.). Since its fluorescence, base reaction and UV spectrum were identical with those of the authentic compound, the combined evidence appears unequivocal. Further, conclusive, evidence that the main bound form is indeed skimmin is implicit in the results of the double-labelling experiments (XXXV-XXXVII) since the observed incorporation of activity was into the skimmin purified after the feedings.

cis-DGC was identified by its very low Rf in AAW (suggesting a diglucoside), its slow emulsin hydrolysis (thus not a coumarin disaccharide) and, most significantly,

by its remarkable transformation to skimmin on irradiation (thus only one glucose unit at para-position). The precise analogy available with the lactonisation of o-coumarinyl glucoside leaves very little doubt that the structure of this minor bound form of umbelliferone is cis-DGC, particularly in view of its identical chromatographic mobility with the synthetic compound.

P.177. Bound forms of umbelliferone.

In a preliminary analysis of the umbelliferone present in hydrangea, the solution remaining after removal of the free and glucose-bound forms was made strongly acid with conc. hydrochloric acid and heated under reflux for 4 hours. It was found that the ether-extractable material recovered from the cooled solution contained only a very small quantity of umbelliferone, negligible by comparison with the previous extracts. This constituted a demonstration that no significant fraction of the hydroxycoumarin is present in an acid-labile form.

P.183. UV Isomerisation Studies of Cinnamic acids.

Each compound (2 mgm/100 ml methanol) was irradiated with a Hanovia mercury vapour lamp and 2 ml aliquots removed at 2 minute intervals for the first half-hour and half-hourly thereafter. It was found

that prolonged heating under reflux had no effect on the initial λmax and I_0max of these compounds, provided that light was excluded. The individual aliquots were immediately shielded from light and the I_0max measured on a Unicam SP800 ultra-violet spectrophotometer. Since the cis-forms of the three compounds exhibit I_0max values consistently lower than the trans-forms, it is clear that the observed progress curves do represent the summation of the I_0max values for the mixtures of isomers formed as a result of the irradiation. In order to permit a direct comparison of the curves, the zero-time value of each has been assigned as 1.00 (using 2 mm-path length cells). This has involved only a small multiplication factor for each set of results and does not constitute a false presentation.

In these studies on methanolic solutions, I_0max, which is directly related to the εmax of the trans-cis mixtures, was the factor measured. In confirmation of these results, o-coumaric acid glucoside was irradiated in aqueous solution and the observed λmax plotted in a precisely similar manner. The curve produced was superposable on that obtained by the I_0max technique.

P.201. Synthesis of p-U-^{14}C-glucosyloxy-cinnamic acid.

A. 0.1 mc of U-^{14}C-β-D-glucose (2.11 mg) was diluted with unlabelled anhydrous β-D-glucose (303 mgm) and anhydrous sodium acetate (155 mgm), dissolved in dry acetic anhydride added. (1.4 ml), The mixture was heated on the steam bath for 4 hours and the clear solution resulting was then added dropwise to 6 ml of hot water in a round-bottomed tube. The oily suspension was vigorously swirled and shaken for 10 minutes and then allowed to cool slowly. The product, β-D-pentaacetyl glucose, crystallised as fine colourless needles. After cooling to 0° overnight, the frozen liquid was melted and the crystals filtered off at 0°, washed repeatedly with cold water, and dried at 65° under vacuum for 12 hours. The pentaacetate (605 mgm, 91.5%) melted at 122-126° as compared to the reported value of 131°. Since this low melting point was noted by Fischer[170] and since the IR spectrum of this product was superposable on that of the authentic material, it was used for the subsequent preparation without further purification.

B. The β-D-pentaacetyl glucose (600 mgm) prepared as described above was suspended in a 1:1 w/v solution

(2 ml) of hydrobromic acid in acetic acid and shaken overnight. The solution was diluted with chloroform (6 ml) and washed with ice-cold water (2 x 6 ml), and brine (6 ml). After drying over anhydrous sodium sulphate, the solvent was removed under vacuum to yield acetobromoglucose (573 mgm) as a mass of tan needles m.p.83-87° (lit.m.p.88-89°). Once again, this product was used for the succeeding step without further purification.

C. The condensation of U-^{14}C-acetobromoglucose with unlabelled methyl p-coumarate was carried out by the method established by Mauthner[153].

U-^{14}C-acetobromoglucose (572 mgm) and methyl p-coumarate (240 mgm) were dissolved in acetone (2.0 ml) and 0.55 ml of a 2.5N aqueous solution of sodium hydroxide added dropwise with shaking. After 1 hour at room temperature, the solvent was removed under vacuum and the oily product thoroughly washed with cold water, dissolved in methanol (8 ml) and decolourised with charcoal. Reduction of the solution to 4 ml bulk and cooling gave a first crop of the required tetraacetylglucosyl methyl ester as needles m.p.159-61° (lit.[153] 160-161°). This was used for the next step in the required synthesis since its weight

was 106 mgm, sufficient for the present purpose. A further 59 mgm of pure product was isolated from the mother liquors.

D. The tetraacetylglucosyl methyl ester (105 mgm) was dissolved in methanol (5 ml) and a 6% aqueous solution of barium hydroxide added (5 ml). The reaction mixture was shaken overnight and then saturated with carbon dioxide. The precipitated barium carbonate was removed by centrifugation and the supernatant brought to pH4 with sulphuric acid. The barium sulphate formed was also removed by centrifugation and the clear colourless solution reduced to dryness under vacuum. The residue was subjected to preparative TLC on 0.5 mm-thick cellulose powder with 5% HAc (twice) and AAW (twice). After each elution, which was carried out on plates 50 cm in length, the band corresponding in Rf with the required glucoside of p-coumaric acid was eluted with methanol finally producing a semi-crystalline residue consisting of the pure compound as judged by analytical TLC. This residue was crystallised three times from methanol yielding trans-p-U-^{14}C-glucosyloxy-cinnamic acid (18.7 mgm) m.p.191-194^{o}(lit.[153] m.p. 194-5^{o}).

P.207. Double-Labelling Experiments - XXXV-XXXVII.

The object of this section is to set out all the results of these highly significant studies of the metabolism of p-oxygenated cinnamic acid precursors in hydrangea. It includes the method employed for computation of the numerical data.

To produce the necessary 2-^{14}C and U-^{14}C-glucose labelled p-glucoside of p-coumaric acid, 6.10 mgm of the 2-^{14}C-labelled compound with relative specific activity 2.17 x 10^6 cpm/mmole (1.352 x 10^4 cpm) was dissolved in 0.6 ml of water with 6.26 mgm of the U-^{14}C-glucosyl compound with relative specific activity 2.051 x 10^6 cpm/mmole (1.312 x 10^4 cpm) and 5.35 mgm of sodium bicarbonate. The solution was administered to the three hydrangea plants (0.1 ml to each of two stems) and the cpm fed to each plant as the given labels are shown above in parenthesis.

The chain-label activity of the fed glucoside, and also the glucose-label activity, were calculated in a straightforward manner:

Chain-label $\frac{6.10 \times 2.17 \times 10^6}{12.36} = \underline{1.07 \times 10^6}$

Glucose label $\frac{6.26 \times 2.05 \times 10^6}{12.36} = \underline{1.04 \times 10^6}$

XXXV 2G - 2.5 gm fresh weight.

Free UMB - 0.20 mgm. Activity = 1.365×10^4

Cpm rec'd = 16.9

Incorp'n = 0.125%

¼ Bound UMB - 0.70 mgm. Activity = 2.389×10^4

Cpm rec'd = 103

Incorp'n = 0.763%

From the weight of ¼ Bound UMB (i.e. skimmin hydrolysate) the total weight of skimmin remaining in solution is calculated = 4.23 mgm. The weight of diluting skimmin added was 10.08 mgm and, after TLC purification, the amount of skimmin left of the 14.31 mgm available for chromatography was estimated as 10.17 mgm by UV spectroscopy.

Prior to crystallisation to constant activity, a further 40.30 mgm of inactive skimmin was added, so that 50.47 mgm was subjected to crystallisation.

The successive activity values after each crystallisation were: 1.285×10^5 cpm/mmole.

4.557×10^4 "

Calculated for original skimmin. 3.123×10^4 "

2.600×10^4 "

2.543×10^4 "

This last figure is not significantly different from

the penultimate and is thus taken as the result for:

Activity Bound UMB (Chain + Glu) = 2.543×10^4

Thus the activity of the recovered Glucose label equals $(2.543-2.389) \times 10^4 = \underline{0.154 \times 10^4}$ cpm/mmole, at once seen to be very much lower than the activity of the chain label.

Total skimmin = $\frac{4.23 \times 4}{3} = 5.64$ mgm.

Dilution value into Glucose label

$$= \frac{1.04 \times 10^6}{1.54 \times 10^3} = 674.4$$

Cpm recd in Glucose label $= \frac{1.54 \times 10^3 \times 5.64}{326.3}$

$= 26.6$ cpm.

% Incorporn-Glucose label $= \frac{26.6 \times 100}{1.312 \times 10^4}$

$= \underline{0.203\%}$.

From these data, the incorporation of chain activity into skimmin was calculated as $4 \times 0.763 = \underline{3.052\%}$, while the incorporation of glucose activity was $\underline{0.203\%}$.

- - - - -

XXXVI 2W - 2.3 gm fresh weight.

Free UMB - 0.30 mgm. Activity = 1.669×10^4

Cpm recd = 30.8

Incorp'n = 0.228%

- - - - -

¼ Bound UMB = 0.75 mgm. Activity = 3.008×10^4

Cpm rec'd = 139

Incorp'n = 1.029%

Thus total chain incorporation into skimmin equals $4 \times 1.029 = \underline{4.116\%}$.

From the weight of ¼ Bound UMB the total weight of skimmin remaining in solution is calculated = 4.53 mgm while the amount of added unlabelled skimmin was 10.16 mgm giving a total for purification of 14.69 mgm. After TLC the remaining skimmin was estimated as 10.99 mgm and a further 40.33 mgm was added to give a total of 51.32 mgm for recrystallisation to constant activity.

The successive activity values after each step were:

1.025×10^5 cpm/mmole.

5.412×10^4 "

Calculated for original skimmin.

4.801×10^4 "

$\underline{4.798 \times 10^4}$ "

Thus the activity of the recovered Glucose label equals $(4.798-3.008) \times 10^4 = \underline{1.790 \times 10^4}$ cpm/mmole.

$$\text{Total skimmin} = \frac{4.53 \times 4}{3} = 6.04 \text{ mgm.}$$

Dilution value into Glucose label

$$= \frac{1.040 \times 10^6}{1.790 \times 10^4} = 58.01$$

Cpm rec'd in Glucose label $= \frac{1.790 \times 10^4 \times 6.04}{326.3}$

$= 329.3$ cpm.

% Incorporn-Glucose label $= \frac{329.3 \times 100}{1.312 \times 10^4}$

$= \underline{2.509\%}$

- - - - -

XXXVII 2W = 2.0 gm fresh weight.

Free UMB - 0.16 mgm. Activity = 2.054×10^4

Cpm rec'd = 20.3

Incorp'n = 0.150%

- - - - -

½ Bound UMB - 0.52 mgm. Activity = 4.503×10^4

Cpm rec'd = 144.5

Incorp'n = 1.069%

Thus total chain incorporation into skimmin equals

- 4 x 1.069 = 4.276%.

From the weight of ½ Bound UMB the total weight of skimmin remaining in solution is calculated = 3.14 mgm while the amount of added unlabelled skimmin was 11.20 mgm giving a total for purification of 14.34 mgm. After TLC the remaining skimmin was estimated as 11.93 mgm and a further 40.77 mgm was added to give 52.70 mgm total for recrystallisation to constant activity. It hardly seems necessary to state that the

skimmin ultimately produced was pure as judged by TLC analysis, IR and UV spectra, melting point and mixed melting point with authentic material.

The successive activity values after each step were: 2.433×10^5 cpm/mmole.

Calculated for original skimmin. 1.161×10^4 "

8.565×10^4 "

8.441×10^4 "

Thus the activity of the recovered Glucose label equals $(8.441-4.503) \times 10^4 = 3.938 \times 10^4$ cpm/mmole.

Total skimmin $= \frac{3.14 \times 4}{3} = 4.29$ mgm.

Dilution value into Glucose label

$$= \frac{1.04 \times 10^6}{3.938 \times 10^4} = 26.4$$

Cpm rec'd in Glucose label $= \frac{3.938 \times 10^4 \times 4.29}{326.3}$

$= 517.4$ cpm.

% Incorporn-Glucose label $= \frac{517.4 \times 100}{1.312 \times 10^4}$

$= 3.944\%$.

After the crystallisations (five for XXXV, four for the others), the weight of skimmin produced was ca. 8 mgm.

In these experiments, care was taken to ensure

that the hydrolysis of the glucose-bound forms of umbelliferone by endogenous glucosidases is minimal. In view of this, it is striking that the free umbelliferone recovered amounted to some 6.7-9.1% by weight and 3.4-5.3% by activity of the recovered chain label.

This demonstrates that umbelliferone exists in hydrangea up to 93% as skimmin (neglecting cis-DGC). The relatively small difference between the activity of recovered free and bound umbelliferone is not entirely in harmony with the postulate that skimmin is the precursor of its aglycone. If such were the case, and if the endogenous glucosidases had indeed been denatured by the preliminary heating, a much greater activity of skimmin relative to the free phenol would have been anticipated. However the possibility still exists that the free compound, produced by a total hydrolysis process (p.203), may be the precursor of the glucoside and that the highly active umbelliferone first produced during the feedings has been diluted by the significantly large pool of endogenous inactive umbelliferone. This rationale is in fact 'logical' but further work is necessary to prove or disprove its validity.

P.210. Feedings XL-XLI.

N.	XL	1G	4.5 gm	ACID	1159 Free
					938 Bound
		- - - - -			
O.	XLI	1W	3.4 gm	ACID	789 Free
					4961 Bound
					157 Bound*

P.213. 'Competition' Feedings.

Feedings.	Steme.	Fresh Weight.	UMB Weight.	cpm rec'd.
XXXIII	2W	5.5 gm	1.16 mg	560
XXXVIII	2W	9.0 gm	0.80 mg	1115
L.		- - - - -		
XXXIV	2W	6.0 gm	1.93 mg	846
XXXIX	2W	8.4 gm	1.38 mg	1200

P.226. Feeding to Ficus elastica.

In this preliminary experiment a trial was made of the wick-feeding technique to a latex-producing plant. This proved to be extremely awkward since the point on insertion of the cotton wick into the leaf petiole was continually blocked and the wick itself clogged. In spite of this, 2 mg of trans-p-coumaric acid (2.80×10^4 cpm) were fed to Ficus elastica decora, the 'india-rubber plant', for one day. The umbelliferone contained in the 9 gm leaf was isolated

from a glucose-bound form - skimmin? - and purified by preparative TLC on Kieselgel G using 20% ethyl acetate- chloroform. This yielded umbelliferone (0.41 mg) containing 8.8 cpm. The result of the feeding may be expressed as 0.031% (665). This is very much inferior to the observed incorporation of this precursor into skimmin in hydrangea, possibly an indication of the inefficient transport of the fed material to the site of synthesis.

P.231. Analysis of infected hydrangea leaves.

The powdery mildew Oidium hortensiae was observed on a few of the plants in the growing box. The infected leaves were removed and dried (10 gm ultimately available). These leaves were powdered and extracted with 80% ethanol. The ethanol was removed and the cloudy-yellow aqueous solution remaining after cooling and filtration through glass-fibre paper extracted with ether to permit the isolation of any Free umbelliferone. After emulsin hydrolysis, the solution was again extracted with ether to remove the Bound umbelliferone. Both crude fractions were purified by preparative TLC and the strongly-blue fluorescent bands corresponding to the authentic compound excised. The subsequent estimation of the amounts of umbelliferone

present carried out by UV spectrophotometry.

The result was that the Free compound (0.68 mg) comprised over 97% of the total since the Bound amounted to less than 0.02 mg. In passing the total weight of free and bound umbelliferone present in these dried leaves was very much less than that expected on the basis of feeding XXXII (0.41 mg/ 1.27 gm of fresh leaves). It is apparent that the fungal attack has resulted in the release of the hydroxycoumarin from its bound form since healthy leaves showed very little free umbelliferone on similar treatment. Furthermore, it is possible that a rapid metabolism of such liberated umbelliferone may be a secondary result of attack.

P.232. Free-Bound ratio in Root tissue.

Hydrangea root tissue (1.94 gm fresh weight) was heated under reflux with 80% ethanol (500 ml) for 30 minutes. This resulted in an intensely fluorescent (green-blue) solution which proved to contain all the extractable free and bound umbelliferone. This was so despite the later blending and rigorous extraction of the root tissue and it suggests some specific physiological concentration of these compounds. From the first extract the free and bound umbelliferone

was isolated by the techniques described in the immediately previous discussion (p.268).

1.94 gm hydrangea root.

Free Umbelliferone = 0.56 mgm = 46.0%

Bound " = 0.66 mgm = 54.0%

Total " = 1.22 mgm.

P.233. Total umbelliferone in morphological 'root'.

Hydrangea root tissue, the adventitious rootlets removed from cuttings, were weighed (2.8 gm) and blended in 200 ml of 4N hydrochloric acid. The suspension was heated under reflux for two hours and then filtered. The solution was continuously extracted with ether for four hours and the umbelliferone in the extract isolated and purified by TLC as before. UV spectrophotometric analysis of the final methanolic eluate from the TLC plate indicated that the 2.8 gm of roots contained 2.38 mgm of free and bound umbelliferone, comparable with previous determinations e.g. feeding XXVII.

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Part 3. THE FORMATION OF COUMARINS BY PHYTOPHTHORA INFESTANS (Mont.) de Bary.

In this, the third project carried out as part of this thesis, the themes of the preceding sections - fungal metabolites, the biosynthesis of coumarins - are combined in the first reported study of the secondary metabolism of Phytophthora infestans (Mont.) de Bary, the causal organism of potato blight.

Regrettably, insufficient time remains to do full justice to the topic and apologies are indeed due to Dr.D.D.Clarke of the Botany Department whose advice and collaboration, together with the skilled technical assistance of Miss J.Brown and Miss M. McKenzie, have made this study possible.

In spite of this, the tracer studies on the typically 'plant' coumarins formed by this fungus when grown on a synthetic, and thus defined, medium suggest close parallels between the synthetic mechanisms of this 'primitive' fungus and certain higher plants. In view of the established physiological activities of coumarin and its oxygenated derivatives, it is possible that the formation of coumarins by both the host and the parasite may contribute to the control

and/or the development of the blight syndrome.

3.1. Introduction.

The emergence of potato blight in the years 1843-46 has been well-described by Austin Bourke[226]. In particular, the sufferings of the Irish population as a result of the damage to their staple food in the year 1846 have been vividly depicted[227]. The ineffectual and callous regard of the crisis by Russell's Government in London, combined with the protectionist agitation of the Tory farmers against the proposed repeal of the Corn Laws (which limited the entry of foreign grain into Britain), led directly to the death of a sizable fraction of the Irish people and to the emigration of many others. The socio-economic implications of this tragedy have not yet been fully worked out.

Although Ireland is the best-known area of damage, much of North America and Europe were struck by this disease at this time - starvation was widespread. Despite the efforts of generations of plant breeders, the potato blight fungus has remained one of the prime causes of crop loss. In 1917, for example, the German crop was heavily attacked resulting in the

so-called "Turnip Winter" which may have contributed to the demoralination of the German civilian population towards the end of the First World War.

In a demonstration of the continuing significance of this plant pathogen, Grainger[228] has estimated that in a single potato-growing area, the West of Scotland, blight, inter alia, was responsible for a real cash loss of £1,100,000 in the market value of the crop for the year 1956 - a loss of 28%!

Niederhauser and Cobb[229] have pointed out in a general review of blight control that 'among major food crops the potato is surpassed only by rice in total production but requires much less acreage. Therefore its nutritional yield per acre exceeds that of other staples.' Clearly, Phytophthora infestans presents a problem of prime significance in the necessary increase of food supplies associated with the present expanding world population.

In view of its long-acknowledged significance, the apparent fact that very little direct study of the metabolism of P. infestans has been carried out is extremely surprising. The reason may be that until recently it was not possible to culture this fungus on a synthetic - (and thus completely defined)-

medium. Such a technique is essential to prove the de novo synthesis of an observed metabolite by the fungus, as opposed to the elaboration of exogenous precursors. The medium developed by French[230] contains 2.5%w/v of glucose as the main carbon source and 0.1% asparagine as the nitrogen source. Phosphate is supplied by 0.05% potassium dihydrogen phosphate and the other components are magnesium sulphate heptahydrate 0.025% and thiamine (vitamin B_1) 1 ppm.

Another medium frequently employed for the growing of blight cultures is an extract of French Beans, prepared by blending 250 gm of the beans in 1 litre of water and filtering the suspension through celite. The fungus thrives on this extract but, as previously stated, its use leads to grave ambiguity in deciding on the true status of metabolites isolated from the ill-defined medium.

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Phytophthora infestans (Mont.) de Bary is a member of the Phycomycete group (tube fungi), which constitutes one of the most important plant pathogenic genera. In common with the other members of this wide-spread group, its actively-growing mycelium is aseptate (Part 1.1.) a convenient but sometimes

misleading distinction from the other main groups.

With the black stem rust of wheat (Puccinia graminis) potato blight shares the notoriety of being the world's most destructive plant disease[229]. In the field its main action is to destroy the foliage of the plant but, depending on the precise timing of the outbreak, extensive damage does occur to the more or less mature tubers. In order to achieve the dramatically sudden attack so characteristic of the disease, the atmospheric conditions and the ambient temperature must remain within quite narrow limits. Under the conditions of warm, damp weather required the fungus sporulates asexually on the potato leaf giving rise to motile, biflagellate 'zoospores' which can be carried for miles by moist air currents.

When a zoospore falls onto the potato leaf, it sends filaments through the epidermis which progressively invade the cells, destroying them in its progress.

On the tuber, a somewhat similar situation prevails but one highly characteristic symptom of blight (which was the initial stimulus for the work to be described) is that an intense blue fluorescence develops and this is associated with an accumulation

of chlorogenic acid (the quinic acid ester of caffeic acid) and of oxygenated coumarins. Here is the link between the two previous projects of this research. However viewed, the present demonstration that this pathogen has biochemical affinities with its host must be of potential significance in an analysis of the possible modes of action and control of the disease.

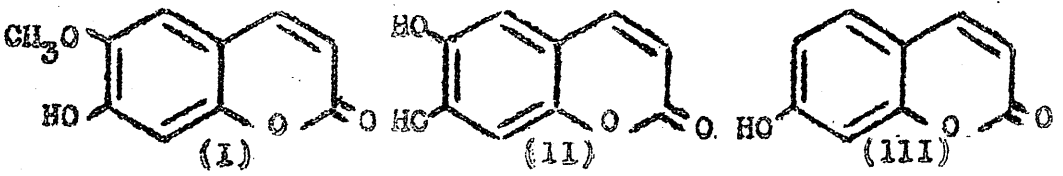
3.2. Results, Discussion and Experimental.

Since time is so limited, it appears most reasonable to present this section in the following manner. The results of the radiotracer studies will be set out in a reproduction of a proposed note by myself and Dr. D.D. Clarke while the associated experimental data will be shown in an appendix.

In actual fact, the origin of the present study was that the exhibition of the fluorescence induced in potato tuber tissue by P. infestans is a part of the course in Mycology organised by the Botany Department here. Since enquiry elicited the information that such fluorescence is a result of increased synthesis of phenylpropanoid compounds, the study now described was commenced.

/Note on the production of coumarins by P.infestans./

The tissue surrounding blight lesions in tubers of several varieties of potato fluoresces strongly in ultra-violet light and at least part of this fluorescence is due to an accumulation of oxygenated coumarins and their glucosides. Scopoletin (I) and scopolin accumulate to the greatest extent[1], but increases in aesculetin (II) and umbelliferone (III) also occur-see[2].



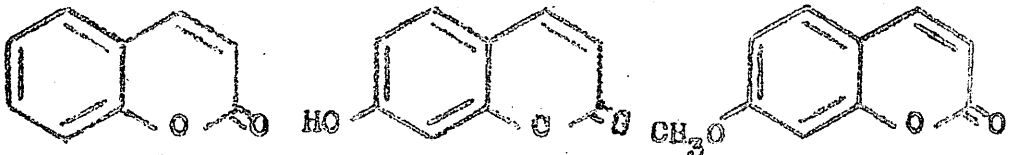
Coumarins from blight lesions.

Although the potato is capable of producing these compounds[3], and in fact coumarin itself[2], since they are present in uninfected tubers, the possibility exists that P.infestans can also produce them. This has now been shown to be the case.

When cultured on an extract of French Beans, prepared by blending 250 gm of beans in 1 litre of distilled water and filtering through celite, an isolate of race 4 of P.infestans grew well but no coumarins were detected either in the mycelium or in the culture filtrate. However, when grown on

a synthetic minimal medium[4], small amounts (ca.0.1 mgs /litre) of three coumarins were produced. Although P.infestans grows well on this synthetic medium, better growth occurs on the French Bean extract, and it may be that the formation of these compounds is stimulated by stress conditions as in certain higher plants[5-7].

The coumarins were isolated and shown to be coumarin (IV), umbelliferone (V) and herniarin (VI) by paper and thin-layer chromatography, characteristic fluorescence and reactions with base, R_f values in gas-liquid chromatographic analysis, ultra-violet spectra and, not least, smell (for coumarin and herniarin). The properties of the isolated compounds corresponded with those of authentic samples.



(IV) (V) (VI)

Coumarins produced by P.infestans.

Recent work with higher plants has clarified the biosynthetic routes from phenylalanine to the coumarins[8]. Accordingly, radiotracer experiments were carried out with with P.infestans to determine whether similar or different pathways are operative. They involved the administration of 150 μmole/litre of generally-^{14}C-

labelled L-phenylalanine and L-tyrosine and specifically 2-^{14}C-labelled trans-p-coumaric acid to one-day old surface cultures growing on the minimal medium.

Ten Roux bottles, each containing 200 ml of medium, were used for both amino-acid experiments, and six were used for the p-coumaric acid experiment. Hyphal fragments, obtained by lightly blending the mycelium of seed cultures in a Waring blender, were used for the inoculum. After eight days growth, the mycelium was extracted with acetone and the culture filtrate with ethyl acetate. The incorporations of activity into coumarin and umbelliferone were measured by inverse dilution analysis[2]. Thus, known weights (ca.12 mgm each) of unlabelled coumarin and umbelliferone were added to each organic extract and completely dissolved. The coumarins were then isolated from the extracts by repeated thin-layer chromatography on Kieselgel G using solvents (9:1 chloroform-methanol; chloroform) that effected their complete separation. Each was then eluted from the Kieselgel, fractionally sublimed, and crystallised to constant activity from water.

The incorporation levels into coumarin and umbelliferone are shown in Tables 1 and 2.

Precursor.	Cpm fed.	Percentage incorporation ($x10^{-2}$)		
		Mycelium.	Broth.	Total.
L-Phenylalanine	$3.502x10^{6}$	3.93	5.67	9.60
L-Tyrosine	$3.362x10^{6}$	0.170	0.316	0.486
p-Coumaric acid	$1.081x10^{5}$	0.264	0.315	0.579

Table 1. Incorporations into coumarin.

Precursor.	Cpm fed.	Percentage incorporation ($10x^{-2}$)		
		Mycelium.	Broth.	Total.
L-Phenylalanine	$3.502x10^{6}$	6.16	6.31	12.47
L-Tyrosine	$3.362x10^{6}$	0.298	0.060	0.358
p-Coumaric acid	$1.081x10^{5}$	2.30	5.83	8.13

Table 2. Incorporations into umbelliferone.

The herniarin present was not quantitatively measured but the pattern of its activity was identical with that of the umbelliferone. It should be recognised that the low incorporation values are due in part to the very small quantities of coumarins formed in the experiments.

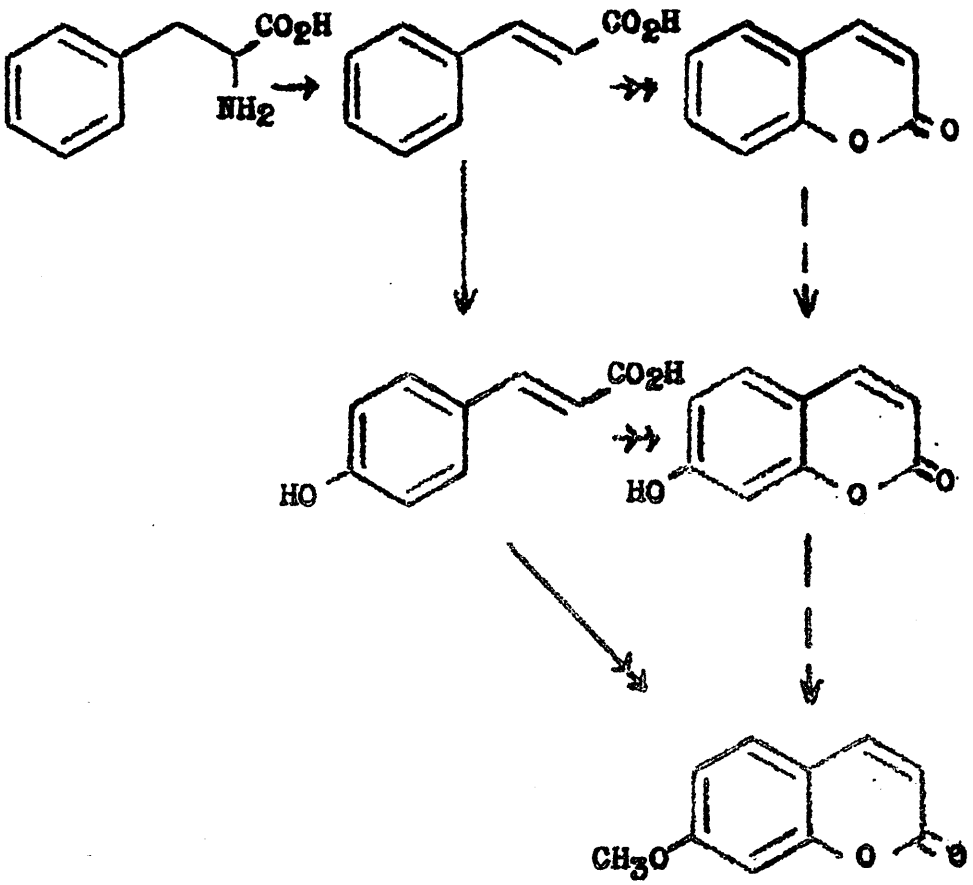
The results show that phenylalanine is a good precursor of coumarin and umbelliferone. They further indicate that p-coumaric acid is a good precursor of umbelliferone, but not coumarin, and that tyrosine is not significantly used for either. As stated, the pattern of incorporation into herniarin matches that into umbelliferone.

The involvement of trans-p-coumaric acid in the biosynthesis of both umbelliferone and herniarin indicates that the deamination of an aromatic amino-acid is part of the operating sequence. The negligibly small incorporation of tyrosine is good evidence that the pathway lies from phenylalanine to trans-cinnamic acid, which may then give rise to coumarin. The specific para-hydroxylation of cinnamic acid will result in trans-p-coumaric acid[19], the precursor of both umbelliferone and herniarin. Clearly, these compounds may be ultimately derived from shikimic acid, as

they are in higher plants[11].

The direct 7-hydroxylation of coumarin to give umbelliferone cannot be excluded, but the good incorporation of p-coumaric acid into umbelliferone suggests that the hydroxylation pattern is established at the cinnamic acid stage. The methylation of umbelliferone to give herniarin may occur but this would constitute a variant of the apparent pathway to herniarin in lavender[12]. The steps from the cinnamic acid stage to the coumarin lactone ring system may involve hydroxylation ortho to the side-chain, direct attack by the acid group, or spirolactone rearrangement[8]. These alternatives are under investigation.

These preliminary observations permit a definition of the overall pathways that may give rise to the coumarins in P. infestans (scheme) since control experiments have demonstrated that their presence is a result of the metabolic activities of the fungus , rather than a spontaneous cyclisation of of cinnamic acids[13]. Thus, P. infestans, using glucose as its main carbon source, is capable of total syntheses hitherto observed solely in higher plants[14]. Its observed secondary metabolism bears striking



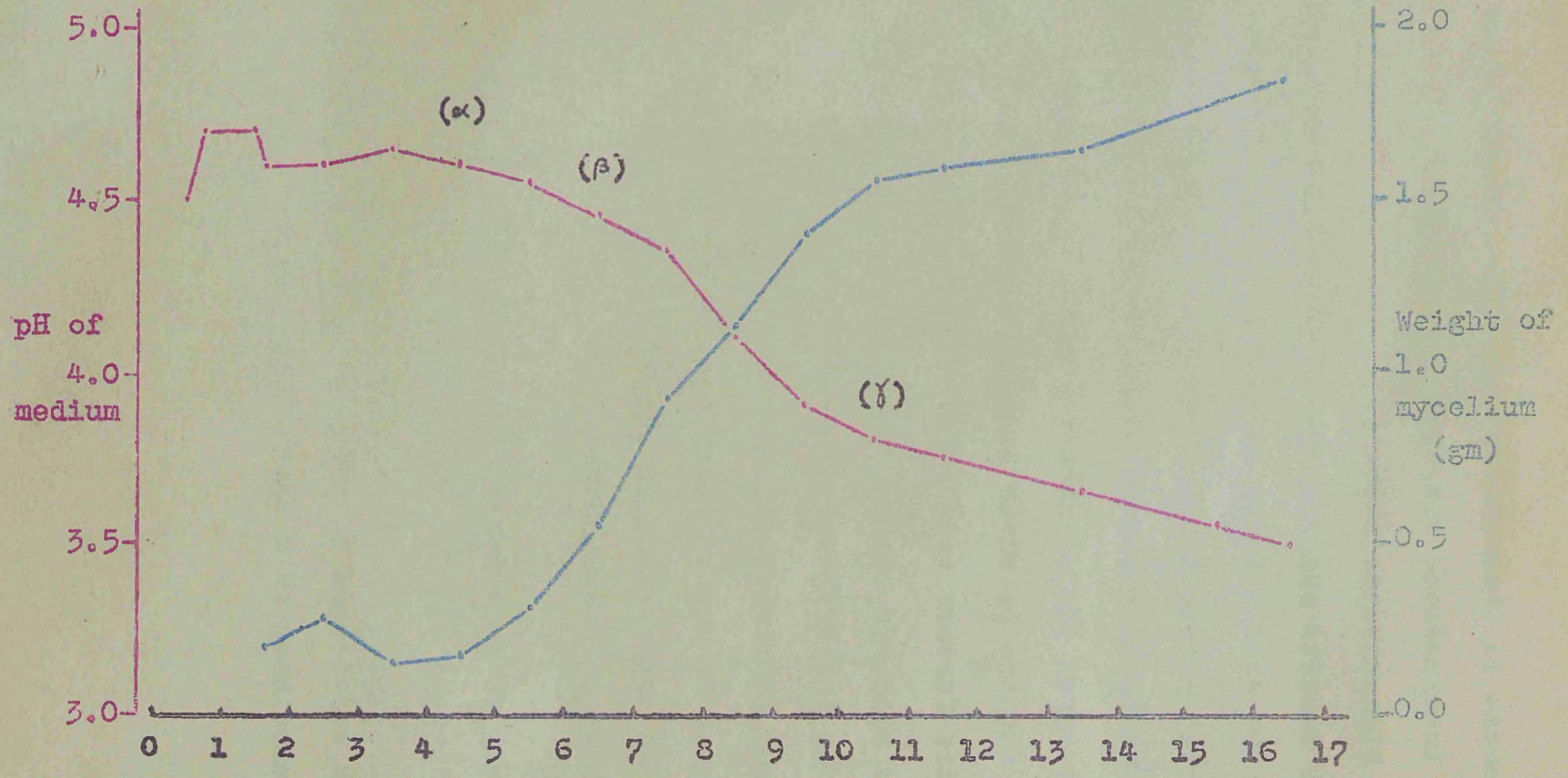
Possible routes to the coumarins in P. infestans.

resemblances to that of certain higher plants since similar intermediate compounds are involved in similar biosynthetic pathways.

The results suggest that P. infestans may contribute to the accumulation of coumarins which occurs in infected potato tubers. The apparent relationships of the synthetic mechanisms used by both host and parasite offer the possibility of an interaction between the two in which intermediates synthesised by one are further metabolised by the other e.g. cinnamic acids to coumarins. Although coumarin and certain of its oxygenated derivatives possess well-documented physiological activity[15,16], there is no evidence as yet to implicate them as primary factors in the control of the development of the host-parasite relationship.

/This note gives the results of the chemical study on P. infestans carried out by myself and Dr. Clarke./

The references to this note are given after the group associated with the overall section.



P.infestans growth curve. The ^{14}C-labelled precursors were fed on Day 2 and the coumarins extracted and purified on Day 10.

APPENDIX.

The diagram reproduced displays the results of a study of the timing of growth of *P. infestans* carried out in collaboration with Miss M. McKenzie. The mycelium in each case was dried by extraction with acetone for four hours or more and the weights given refer to the quantity of dried mycelium produced in five Roux bottles each containing 200 ml of minimal medium.

It is clear that, after a lag-period of four days during which the pH of the medium undergoes a rapid initial rise and fall, a linear growth phase which is correlated with a drop in the pH value occurs.

It is further clear that, after the tenth day, the growth rate and pH drop slacken significantly. This phase is associated with the marked browning of the mycelium and the medium characteristic of the terminal oxidation processes.

(α) Examination of the inoculum of blended hyphae on the fifth day clearly indicated that vegetative growth, (obligatory under these conditions) had commenced.

(β) On the seventh day, the mycelium had developed to the extent that it formed a full hyphal mat, albeit a fragile one.

(δ) On the eleventh day, the final browning reactions were easily discerned.

This study was of great significance in the control of the radiotracer experiments. As stated in the diagram, the radioactive compounds (injected into the sterile cultures through a Millipore filter) were fed on the Second day after the establishment of the seed cultures.

Chromatographic analysis of the successive extracts from these growings showed that a trace of umbelliferone was present from the beginning of the seed culture but that coumarin and herniarin did not manifest themselves until later.(ca. Day 5).

Gas-liquid chromatographic analysis of these extracts confirmed the finding and, in a wider sense, indicated that the original simple pattern of major metabolites was increasingly changed to a highly complex mixture only after the twelfth day i.e. at the beginning of the terminal oxidation phase.

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The compounds fed to P.infestans were:

L-phenylalanine -(aqueous soln.) U-^{14}C-labelled.

Activity = 1.144 x 10^7 cpm/mmole.

Quantity fed = 50.6 mgm in equal portions to 10 bottles.

Cpm fed = 3.502 x 10^6.

Activity recovered on mycelium = 13.7%

L-tyrosine - (aqueous soln. of hydrochloride)
Activity = 1.064×10^7 cpm/mmole. U-^{14}C-labelled.
Quantity fed = 66.0 mgm in equal portions to 10 bottles.
Cpm fed = 3.362×10^6.
Activity recovered on mycelium = 15.2%

trans-p-Coumaric acid.- (aqueous soln. of sodium salt)
Activity = 5.916×10^5 cpm/mmole. 2-^{14}C-labelled.
Quantity fed = 30.0 mgm in equal portions to 6 bottles.
Cpm fed = 1.081×10^5.
Activity recovered on mycelium = 12.2%.

- - -

As indicated above, from 12 to 15% of the fed activity was recovered apparently adsorbed on the dried, extracted mycelium. The possibility that it might be combined as a lignin appears unlikely since the tests for lignin gave no positive reaction.

The uniform recovery would support a physical process of adsorption onto a given surface area of mycelium.

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The feeding of these compounds to the fungus caused no significant alteration in the pH curve or in the weight of dried mycelium obtained at the end of each experiment.

References - Note on Fungal Coumarins (p.297 ff).

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