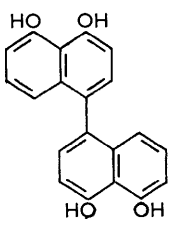
134. *Biosynthetic Pathways in* Daldinia concentrica.

By D. C. Allport and J. D. Bu'Lock.

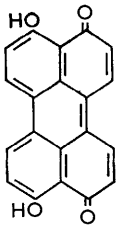
Various strains of *D. concentrica* have been studied in laboratory cultures and a series of aromatic metabolites has been isolated, including benzene derivatives with a C_2 or C_4 side-chain, such as the chromanone (V) (shown to be derived from acetate or a related compound), and mono- and di-methyl ethers of 1,8-dihydroxynaphthalene. Non-enzymic oxidation of 1,8-dihydroxynaphthalene has also been investigated. The combined evidence suggests that in the fungus the C_{10} benzene derivatives and 1,8-dihydroxynaphthalene are alternative metabolites of a common acetate-derived precursor, and that alternative metabolic fates of the 1,8-dihydroxynaphthalene are methylation and oxidation, by way of the binaphthyl (I) to polymeric quinones and the perylenequinone (II), as previously described.

As already described, the Ascomycete *Daldinia concentrica*, parasitic upon ash trees, produces in its sporophores a chromogen 4,5,4',5'-tetrahydroxy-1,1'-binaphthyl (I), which is oxidised *in situ* to 3,10-dihydroxyperylene-4,9-quinone (II) and to black polymeric pigments firmly bound to the cell-wall substance.[1] The sporophores with which that work was carried out were collected in the field; such sporophores are not produced by laboratory cultures of the fungus, which were our next object of study. In culture, *D. concentrica* is a somewhat variable species, the differences being partly sexual and partly genetic. Thus a series of 25—30 single-spore isolates grown on malt–agar plates produced mycelium varying from black or greenish-black through various grey shades to a patchy grey and white. The majority were heavily pigmented, produced black conidia freely, and blackened the agar medium, but a few pale strains did not form conidia or blacken the medium so intensely, and some observations on these strains are noted subsequently.

Our main work on *D. concentrica* in culture was carried out with four strains from the collection of the Forest Products Research Laboratory; these had been subjected to



(I)



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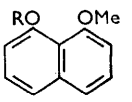


(III) R = Me
(IV) R = H

repeated sub-culturing and no longer produced conidia or pigment. One of these, strain 26C, forms a white mycelium on malt–agar, with some black pigment on ageing. Crude ethanol extracts of fresh cultures showed a distinctive spectrum, reminiscent of the chromogen (I) but not identical with it, and when strain 26C was grown on a larger scale the material responsible for this spectrum was readily isolated from the mycelium and

[1] Allport and Bu'Lock, *J.*, 1958, 4090.

from the medium. It proved to be neutral and was resolved into 1,8-dimethoxynaphthalene (III) and smaller quantities of the monomethyl ether (IV) (also neutral because of hydrogen-bonding).

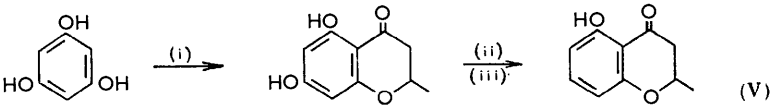
Another strain, $26A_1$, superficially resembles 26C but gives different chemical tests and even on ageing forms only a brownish pigment. Extracts from these cultures gave a strong ferric chloride reaction, and when the strain was grown on a larger scale the medium contained a mixture of phenols. A small fraction was removed from ether extracts by sodium hydrogen carbonate solution; the main component was then most easily isolated by steam-distillation. Further purification gave a solid, $C_{10}H_{10}O_3$, m. p. 30—33°, forming a mono-oxime and mono-(2,4-dinitrophenylhydrazone). The chromophore of the substance (ultraviolet absorption, Table 1) was identified as that of a dihydroxybenzoyl derivative,

TABLE 1. *Ultraviolet absorption spectra* (mμ; log ε *in parentheses*).

	$\lambda_{max.}$ in ethanol							$\lambda_{max.}$ in alkali					
(VII)	225	(4·10)	270	(4·04)	342	(3·47)		241·5	(4·10)	285	(3·93)	390	(3·70)
(VI)	222·5	(4·10)	270	(4·00)	342	(3·45)		241·5	(4·11)	285	(3·93)	387	(3·67)
(V)	270	(3·97)	347	(3·49)				242	(4·06)	280	(3·89)	380	(3·50)
(IX)	227	(4·72)	305	(3·84)	320	(3·84)	334 (3·91)			Not measured			
(IV)	298	(3·86)	315·5	(3·82)	330	(3·83)				Spectrum unchanged			
(III)	227	(4·79)	298	(3·93)	310	(3·80)				} Spectrum unchanged			
	316	(3·90)	325	(3·67)	330	(3·93)							
(VIII)	225	(4·31)	233 *		252	(4·09)	325 (4·19)	236	(4·32)	258	(4·10)	298 (3·28)	370 (3·60)

* Inflection

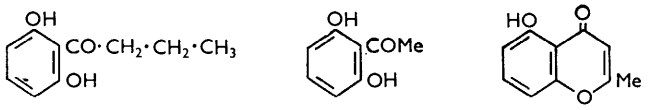
but unlike 2,6-dihydroxyacetophenone (the most readily available model) the compound titrated only as a very weak acid; its infrared spectrum showed a hydrogen-bonded carbonyl band with no distinct hydroxyl-group absorption. Fused with potassium hydroxide, under increasingly vigorous conditions, the compound gave successively 2,6-dihydroxyacetophenone, γ-resorcylic acid, and resorcinol; heating it with 15% aqueous potassium hydroxide gave 2,6-dihydroxyacetophenone in good yield. The compound gave positive Gibbs and Zimmerman tests and under modified Kuhn–Roth conditions gave acetic acid as the only volatile acid. It was therefore formulated as 5-hydroxy-2-methylchromanone (V). This was synthesised by the route shown; the natural and the synthetic product showed identical spectroscopic properties and formed the same 2,4-dinitrophenylhydrazone. The natural product is optically inactive.



(V)

Reagents: (i) $(Me{\cdot}CH{=}CH{\cdot}CO)_2O$–$AlCl_3$. (ii) p-$C_6H_4Me{\cdot}SO_2Cl$. (iii) Raney Ni.

The involatile residue from the steam-distillation of the chromanone showed a similar ultraviolet absorption spectrum, and by chromatography was resolved into its main components, 2,6-dihydroxybutyrophenone (VI) and 2,6-dihydroxyacetophenone (VII) (identified in each case by comparison with authentic materials).



(VI) (VII) (VIII)

When, after three weeks on the original (glucose–salts–maize steep) medium, mycelial mats of $26A_1$ were supplied with fresh 4% aqueous glucose, small amounts of the naphthalenes (III) and (IV) were produced in addition to compounds (V), (VI), and (VII). Similarly the chromanone (V) and butyrophenone (VI) were found in small

amounts with naphthalene derivatives (III) and (IV) in cultures of 26C. Thus the differences between the two strains are quantitative rather than qualitative.

From one batch of replacement cultures of strain $26A_1$, when the steam-involatile fraction containing (VI) and (VII) was worked up, a small yield of a third substance was obtained. The amount available was insufficient for complete characterisation and the material, m. p. 73—76°, was contaminated with compounds (VI) and (VII). However the ultraviolet absorption spectrum and the principal features of the infrared spectrum were those of 5-hydroxy-2-methylchromone (VIII) and this tentative identification was confirmed by paper chromatography.

Some chromones comparable to (VIII) are known as natural products, but chromanones such as (V) have not previously been recorded. In *D. concentrica* the three compounds (VIII), (V), and (VI) might represent successive stages of reduction, the effective side-chains being acetoacetyl, β-hydroxybutyryl (or crotonyl), and butyryl respectively, a sequence similar to that in fatty acid synthesis. The optical inactivity of the chromanone (V), even when isolated by mild procedures of solvent extraction and chromatography, suggests that it is formed from, or in equilibrium with, an open-chain precursor.

The compounds (V)—(VIII) are all of a type which can formally be built up by linear condensations of four or five " acetate-derived " C_2 units, and this hypothesis was tested

TABLE 2. *Degradation of labelled 5-hydroxy-2-methylchromanone.*

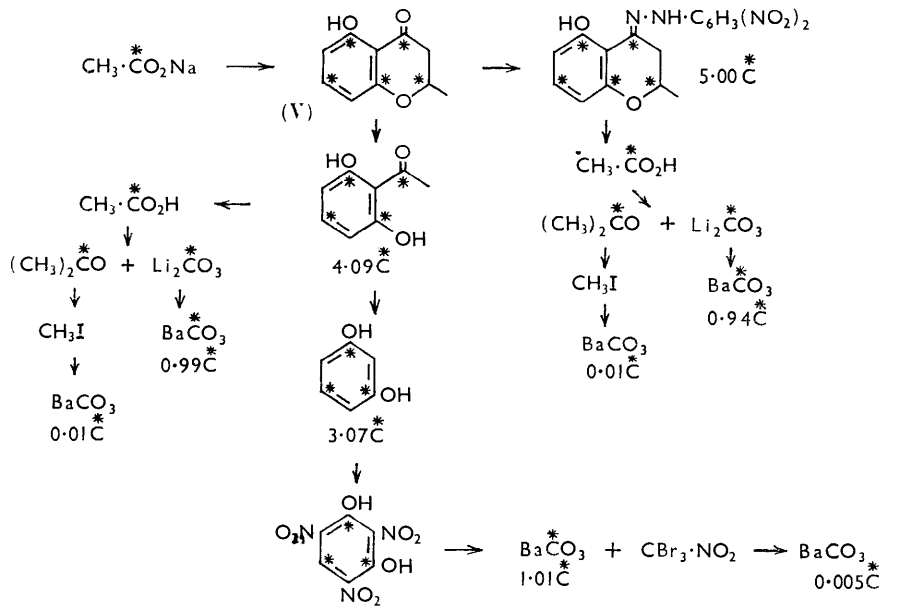
Compound	Mol. wt. × counts/min. (corr.)	No. of " active " C	Compound	Mol. wt. × counts/min. (corr.)	No. of " active " C
(V) as 2,4-dinitrophenyl-hydrazone	$1{\cdot}46 \times 10^6$	5·00	$BaCO_3$ from $C_{(3)}$ of (V)	$2{\cdot}11 \times 10^3$	0·01
$BaCO_3$ from $C_{(2)}$ of (V)	$2{\cdot}74 \times 10^5$	0·94	Resorcinol	$8{\cdot}95 \times 10^5$	3·07
,, Me of (V)	$3{\cdot}16 \times 10^3$	0·01	$BaCO_3$ from bromopicrin reaction	$2{\cdot}94 \times 10^5$	1·01
(VII) from (V)	$1{\cdot}19 \times 10^6$	4·09	$BaCO_3$ from bromopicrin	$1{\cdot}41 \times 10^3$	0·005
$BaCO_3$ from $C_{(4)}$ of (V)	$2{\cdot}87 \times 10^5$	0·99			

in the case of the C_{10} compound (V). Preformed mycelium of $26A_1$ was supplied with 4% aqueous glucose containing [1-^{14}C]acetate; after 12 days the chromanone (V) was isolated and found to have incorporated about 9% of the total ^{14}C administered. When the compound was degraded by the route shown, through 2,6-dihydroxyacetophenone and resorcinol, the radioactivity was found to be associated solely with the alternate carbon atoms of the molecule, as expected and as shown in the scheme (cf. also Table 2). The degree of labelling was uniform within the limits of error.

The carbon skeleton of the chromanone (V) is clearly equivalent to that of the naphthalene (III), and our observations in fact suggest that the benzene and the naphthalene derivatives are alternative metabolites of a common precursor. Thus cultures such as $26A_1$ appear to be relatively deficient in the enzymes required for complete cyclisation of the C_{10} chain, which would explain why they do not readily produce the black pigments derived from the binaphthyl (I). On the other hand strain 26C, also non-pigmented, possesses the enzymes necessary for the synthesis of the naphthalene ring in (III) and (IV); in this case the failure to produce pigment seems to be due to deficiency in an oxidase enzyme. Thus we found that malt–agar cultures of 26C are without visible action on added 1,8-dihydroxynaphthalene (IX), whereas pigmented strains all convert this into black pigment. On the other hand, cultures of $26A_1$, not producing detectable amounts of naphthalenes, are nevertheless capable of oxidising the added phenol (IX).

The differences between various types of culture can apparently be summarised as follows. In the " normal " or " wild-type " (conidial) strains naphthalenes are synthesised but are fairly rapidly removed by oxidation to pigment. In strains such as $26A_1$ the synthesis of the naphthalene system is relatively slow, though it may proceed in certain conditions (replacement cultures) giving (III) and (IV) and pigment, whilst the bulk of the material which would otherwise appear as naphthalenes is liberated as the benzene

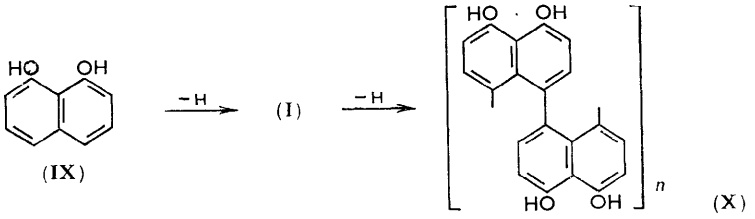
derivatives (V)—(VIII). In strains such as 26C naphthalenes are synthesised more rapidly than they are oxidised, and the surplus appears as (III) and (IV). This general picture was confirmed in the series of single-spore isolates: in the non-conidial strains,



production of the benzene derivatives was greatest when the formation of pigment was least.

Though 1,8-dihydroxynaphthalene (IX) has not itself been detected in *D. concentrica*, the isolation of its mono- and di-methyl ether from cultures unable to metabolise it oxidatively strongly suggests that it is a metabolic intermediate and that in the pigmented cultures, and in the wild sporophores, it is oxidised enzymically by way of the binaphthyl (I) to the pigments described in our earlier paper. Such coupling reactions can sometimes be effected non-enzymically; in any case a study of the oxidation of compound (IX) seemed of some intrinsic interest since, whilst this diol cannot yield a simple quinone, it was observed to autoxidise rather readily.

Paper chromatography and absorption spectra were used to characterise the products. Oxidation of the diol (IX) with neutral ferricyanide was thus found to give a range of substances, the simplest being the binaphthyl (I), which was always accompanied by material of similar properties but apparently of higher molecular weight. The extent of polymerisation seemed to depend upon the stage at which the product was precipitated



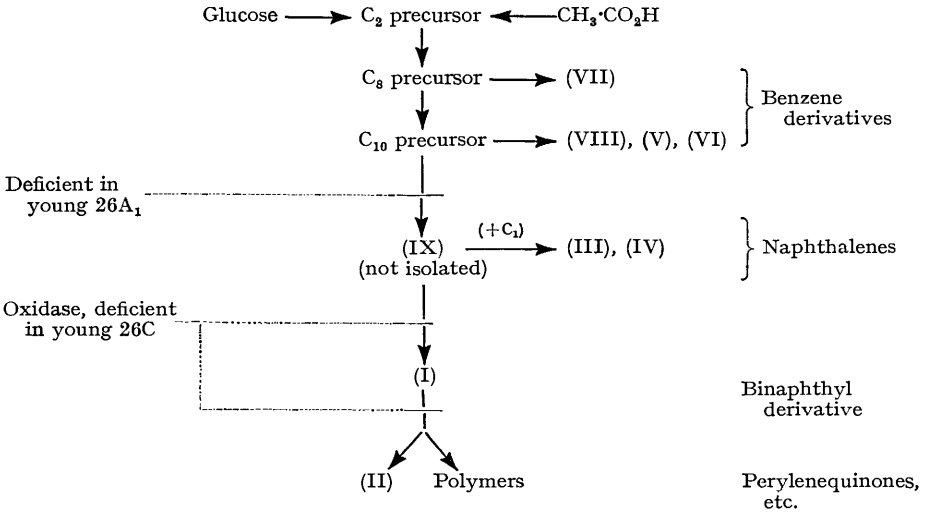
(IX) (X)

from the methanol–water mixtures used as solvent; as the proportion of methanol was increased, and precipitation delayed, the consumption of ferricyanide increased, the yield of binaphthyl (I) fell, and the yield of products with a lower R_F increased. In hot aqueous solutions, or with higher concentration of methanol, or in alkaline solution, or on prolonged

storage, the consumption of ferricyanide rose even further and the products were black polymers.

There are thus three types of product, *viz.*, the binaphthyl (I), the less oxidised polymers, and the more oxidised polymers. The less oxidised polymers are produced initially as grey or light green precipitates, with an infrared spectrum very like those of (I) and (IX); these seem to owe most of their colour to slight quinhydrone formation since they are largely soluble in methanol or ether, leaving a small residue of dark insoluble material. The solutions show an ultraviolet absorption spectrum like that of (I); this shows that the polymerisation can only involve α-positions of the naphthalene ring since β-substituted naphthalenes show quite different spectra.[2] The less oxidised polymers thus have structures resembling (X), in which coplanarity of the naphthalene units will be severely hindered, so that the system has the same effective chromophore for $n \geqslant 1$. The more highly oxidised polymers are dark green or black, insoluble in methanol or concentrated sulphuric acid, but readily reduced by dithionite to products resembling the less oxidised polymers. In this they resemble the cell-bound pigment of the *D. concentrica* sporophores described previously.

It may be asked, in view of this picture of the oxidation process and the theory that it is effectively reproduced in *D. concentrica* sporophores, why the binaphthyl (I) should be present in the sporophores at all, since these are so heavily pigmented that its further oxidation can obviously occur. The most probable explanation is that as the cells develop, the prevailing oxidation potential is eventually maintained at such a level that, whilst oxidation of (IX) to (I) can proceed, further oxidation of the product is inhibited. This effect may well be produced by the accumulated pigment itself, since this will contain a reservoir of reversibly reducible quinonoid units, the individual redox potentials of which will not be identical (because of steric effects and varying degrees of conjugation). Such polymers give flat curves in oxidation–reduction titrations, *i.e.*, they exert a " redox poising " effect; in the present system the final potential might well be intermediate between those for the oxidations of (IX) and (I).[3]



Metabolic pathways in D. concentrica.

Our studies with *D. concentrica*, wild and in culture, can be combined to give a picture of the secondary metabolism of this species which is summarised in the scheme. At least

[2] Cf. Daglish, *J. Amer. Chem. Soc.*, 1950, **72**, 4859.

[3] Figge, " Biology of Melanomas," New York, Acad. Sci., 1948, p. 405; Bu'Lock, *Chem. and Ind.*, 1952, p. 739; Cassidy, Ezrin, and Updegraff, *J. Amer. Chem. Soc.*, 1953, **75**, 1615.

two of the reactions in this scheme are subject to characteristic enzyme deficiencies, the feature which has made possible the reconstruction shown; it should be noted that in this case, unlike the well-known uses of blocked mutant micro-organisms, the deficiencies are relative and not absolute.

D. concentrica is one of the few examples of a system in which both partial and complete aromatisation of an " acetate-derived " structure has been observed. However we would not infer that the chromanone (V) or the chromone (VIII) is itself a precursor of the naphthalene (IX); similarly the C_8 compound (VII) is not a precursor of the C_{10} compounds. That exogenous (VII) is taken up but is not a precursor of (V) was shown experimentally; of the ^{14}C supplied as (VII), less than 1% was incorporated into (V). Thus the intermediates in the aromatisation processes remain undetected; however, our observations do show that not only the benzenoid compounds (V)—(VIII), but also the naphthalenes (III) and (IV), the binaphthyl (I) and the perylene (II), all ultimately have a common origin in " acetate," a fact not necessarily implied by their structural features alone.

It is commonly held that the great variety of compounds which can be isolated from laboratory cultures of micro-organisms are pathological by-products with little or no relevance to the metabolism of the same organisms growing in their " natural " habitat. This assertion can seldom be tested experimentally, particularly with the very numerous Imperfect Fungi of which the mature reproductive forms have not been identified. However, in the case of *D. concentrica*, the non-conidial strains of which would be classified as Imperfecti if their origin was not known, there is a very close and significant relation between the metabolites from laboratory cultures and the constituents of the naturally grown fruit-bodies; the various forms studied, ranging from highly defective vegetative strains through conidial strains to the fully sexual " wild " state, display varying aspects of the same metabolic process.

Experimental

Extraction of Cultures of D. concentrica *Strain* 26C.—In a typical procedure, the fungus was grown on 750 ml. of glucose–salts–corn-steep medium [4] for 2 weeks. The mycelium (20 g.) was then separated from the glass wool used as a support, and dried at 30°. The dried mycelium was powdered and extracted exhaustively (Soxhlet) with light petroleum (b. p. 40—60°) followed by ether. The petroleum extract was evaporated and the residue distilled in steam. The distillate was extracted with ether, and the ether extract evaporated to a mixture of oil and crystals (0·05 g.). This was triturated with light petroleum and recrystallised repeatedly from ether and ethanol–water, to give 1,8-dimethoxynaphthalene (III), m. p. and mixed m. p. 158—161° (for ultraviolet absorption see Table 1). The infrared absorption was identical with that of synthetic material prepared by the method of Buu-Hoï and Lavit.[5]

The ether extract from the mycelium was washed with aqueous sodium hydroxide and water and evaporated, giving an oil (0·8 g.) which partly crystallised; the crystals were separated and recrystallised, giving a further quantity of the diether (III) (0·01 g.). The residual oil, of characteristic fruity odour, contained traces of the ether (III) together with an ester or lactone (C=O band at 1740 and 1770 $cm.^{-1}$, OH bands at 3400—3600; $\lambda_{max.}$ *ca.* 270 mμ).

The culture-medium (4 l.) was extracted with ether, and the extract worked up as for the ether extract from the mycelium; the process afforded the diether (III) (0·03 g.) and more of the unidentified oil (*ca.* 0·25 g.). The oil remaining on evaporation of the mother-liquors was sublimed at 20 mm./100°. The sublimate was identified by paper chromatography as 8-methoxy-1-naphthol (IV). The alkaline washings were acidified and extracted with ether; evaporation of the extract and steam-distillation gave the chromanone (V) (see below) (0·04 g.). In the non-volatile residues from the steam-distillation, the butyrophenone (VI) (see below) was identified by paper chromatography. Other unidentified phenols were also present.

Extraction of D. concentrica *Strain* $26A_1$.—The cultures were prepared as above; in a

[4] Robbins, Kavanagh, and Hervey, *Proc. Nat. Acad. Sci., U.S.A.*, 1947, **33**, 171.
[5] Buu-Hoï and Lavit, *J.*, 1956, 2412.

typical procedure the culture medium (15 l.) was separated, filtered, and extracted with ether (3 × 1 l.); the combined extracts were washed with aqueous sodium hydrogen carbonate. The ether was then evaporated and the residue steam-distilled; the distillate was extracted with ether, and the extracts were evaporated, giving 5-hydroxy-2-methylchromanone (V) (*ca.* 1·0 g.), obtained after purification by counter-current distribution between 1 : 1 ethanol–water and n-hexane as pale yellow needles, m. p. 30—33° (ultraviolet absorption, Table 1; C=O absorption band at 1630, 1650 cm.$^{-1}$, no sharp OH band) [*oxime*, m. p. 158·5—159·5° (uncorr.) (Found: C, 62·5; H, 5·6; N, 7·0. $C_{10}H_{11}O_3N$ requires C, 62·2; H, 5·7; N, 7·2%); 2,4-*dinitrophenylhydrazone*, dark red needles, m. p. 243° (corr.) (Found: C, 53·5; H, 4·1; N, 15·3; OMe, 0. $C_{16}H_{14}O_6N_4$ requires C, 53·6; H, 3·9; N, 15·6%)].

The residue after steam-distillation was extracted with ether, and the ether evaporated. The resulting brown oil (1·9 g.) was passed in benzene through a short column made up from a previously boiled slurry of "Florisil" (Floridin Co., U.S.A.) in benzene. The eluate was evaporated, taken up in 1 : 3 benzene–light petroleum (b. p. 60—80°), and put on to a similar column. Elution with this solvent mixture gave a fraction *A* and was followed by elution with a 1 : 1 mixture giving a fraction *B*.

Fraction *A* gave a partly-crystalline residue which was purified by repeated recrystallisation from benzene–light petroleum and methanol–water, affording 2,6-dihydroxybutyrophenone (VI) as yellow needles, m. p. and mixed m. p. 116·5—118° (Found: C, 66·7; H, 6·9. Calc. for $C_{10}H_{12}O_3$: C, 66·5; H, 6·7%) (absorption, Table 1; C=O band at 1630 cm.$^{-1}$, OH band to 3250 cm.$^{-1}$). Fraction *B* gave a partly crystalline residue which after trituration with light petroleum gave 2,6-dihydroxyacetophenone (VII) as yellow needles, m. p. and mixed m. p. 154—158° (absorption, Table 1; C=O absorption band at 1630 cm.$^{-1}$, OH band to 3280 cm.$^{-1}$). Authentic specimens of the ketones (VI) and (VII), prepared by the methods of Russell, Frye, and Maudlin,[6] were spectroscopically identical with the natural products and gave no depression of the m. p.

When the culture medium in 14-day cultures of *D. concentrica* $26A_1$ was replaced by fresh 4% aqueous glucose, and the cultures were incubated for a further 14 days, extraction and working up by essentially the above procedure gave the same products, together with 1,8-dimethoxynaphthalene (III), obtained from that part of the steam-volatile material which was not extracted from ether by aqueous 10% sodium hydroxide, and 8-methoxy-1-naphthol (IV), obtained as an alkali-insoluble constituent of the residues from steam-distillation and characterised after sublimation by paper chromatography.

In one set of similar cultures, chromatography of the fraction containing the ketones (VI) and (VII) afforded a third substance, obtained as yellow needles, m. p. 72—76°, whose ultraviolet and infrared spectra were very similar to those of 5-hydroxy-2-methylchromone (VIII). Paper chromatography showed the material to contain some of the ketones (VI) and (VII) together with (VIII); compound (VIII) was differentiated from (V), which has the same R_F value, by its colour reactions on the paper.

Characterisation of the Chromanone (V).—The chromanone gave a grey-green colour with ferric chloride in ethanol, a positive Gibbs test [7] with 2,6-dichlorobenzoquinone *N*-chloroimide, and a positive Zimmermann test [8] with *m*-dinitrobenzene. On electrometric titration the compound behaved like *o*-hydroxy- and unlike 2,6-dihydroxy-acetophenone. Oxidation under modified Kuhn–Roth conditions [9] gave acetic acid as the only detectable volatile acid. The compound was heated with 50% aqueous potassium hydroxide in ignition tubes for periods of time varying from 5—10 min., the residues then acidified, extracted with ether, and examined by paper chromatography with 0·5N-hydrochloric acid as eluant; successive products were identified as 2,6-dihydroxyacetophenone, γ-resorcylic acid, and resorcinol.

Synthesis of the Chromanone (V).—To a solution of anhydrous aluminium chloride (10·2 g.) and dry phloroglucinol (5·4 g.) in redistilled nitrobenzene (75 ml.), crotonic anhydride (5·0 g.) and further aluminium chloride (15 g.) were added with shaking in ½ hr.; after being kept at room temperature for 2 days, the whole was poured into iced dilute hydrochloric acid. The nitrobenzene was separated and the aqueous phase extracted twice with ether; the nitrobenzene and ether extracts were combined and solvents removed in steam, after which the hot

[6] Russell, Frye, and Maudlin, *J. Amer. Chem. Soc.*, 1940, **62**, 1441.
[7] Gibbs, *J. Biol. Chem.*, 1927, **72**, 649.
[8] Zimmermann, *Z. physiol. Chem.*, 1935, **233**, 257.
[9] Bickel, Schmid, and Karrer, *Helv. Chim. Acta*, 1955, **38**, 664.

aqueous liquor was decanted from a brown oil and upon cooling deposited brown crystals. When twice sublimed at 170°/15 mm. these gave 5,7-*dihydroxy*-2-*methylchromanone*, m. p. 176—177° (spectrum, Table 1) (Found: C, 61·9; H, 5·4. $C_{10}H_{10}O_4$ requires C, 61·8; H, 5·2%).

This product (1·0 g.) was heated with toluene-*p*-sulphonyl chloride (1·0 g.) in dry pyridine (5 ml.) for 1 hr., cooled, and poured into water; the product was recrystallised from ethanol–water and ethyl acetate–light petroleum, to give 5-*hydroxy*-2-*methyl*-7-*toluene*-p-*sulphonyloxychromanone*, m. p. 118—119° (Found: C, 58·5; H, 4·6. $C_{17}H_{16}O_6S$ requires C, 58·6; H, 4·6%). This derivative (150 mg.) in ethanol (10 ml.) was heated under reflux with freshly prepared Raney nickel (*ca.* 1·2 g.) for 3 hr., after which the ethanol was decanted and the nickel dissolved in hot concentrated hydrochloric acid. The solution was diluted and extracted thrice with ether, and the ether and ethanol solutions were combined, evaporated, and steam-distilled, to give crude 5-hydroxy-2-methylchromanone (V) as an oil which was converted into the 2,4-dinitrophenylhydrazone, m. p. and mixed m. p. 243°.

Synthesis of 5-*Hydroxy*-2-*methylchromone.*—The only published synthesis of this compound,[10] by the action of sodium carbonate on 3-acetyl-5-hydroxy-2-methylchromone, is not convenient. We obtained the compound as follows. To granular sodium (1·6 g.) was added, in 45 min., a suspension of the ketone (VII) (3·0 g.) in dry ethyl acetate (10 ml.) under nitrogen, with cooling; the mixture was heated at 100° for 45 min., then water was added, and the mixture acidified with acetic acid. The precipitated solid was extracted into ether, recovered, steam-distilled and recrystallised from aqueous ethanol, giving 5-hydroxy-2-methylchromone, m. p. 89—90° (lit.,[10] 92°) (absorption Table 1; $\nu_{max.}$ 1620, 1653 cm.$^{-1}$), giving a purple colour with ferric chloride in ethanol.

Radioactive Assay.—Specimens were counted with an end-window counter as infinitely thick samples (1 cm.^{2} cross-section), or as "infinitely thin" films (corrected for self-absorption). The statistical error of counting, corrected for background and lost counts, was always $<3\%$. The results in Table 2 are given as the products of the molecular weight and the counts/min., corrected as necessary for the experimental dilutions.

Incorporation of [1-^{14}C]*Acetate.*—Strain $26A_1$ was grown for 19 days on the full medium, which was then replaced by 4% aqueous glucose; after a further 4 days, sterile sodium [1-^{14}C]acetate (total 320 μc) was added to 4 flasks, and 12 days later the aqueous medium (5 l. including washings) was withdrawn and extracted with ether (1 × 1 l. and 4 × 500 ml.). The combined ether extracts were evaporated to 500 ml., washed twice with aqueous sodium hydrogen carbonate, and evaporated and the residue was distilled with steam. The chromanone (V) was isolated from the distillate by ether-extraction and purified by countercurrent distribution (n-hexane–aqueous ethanol); the yield was estimated spectroscopically (134 mg.). The product was then diluted 1 : 3 with pure material.

The diluted chromanone (107 mg.) was converted into the 2,4-dinitrophenylhydrazone, which was purified by chromatography in chloroform on bentonite–kieselguhr and recrystallised to constant activity. A portion of the product (50 mg.) was diluted with pure inactive material (368 mg.) and again recrystallised to constant activity. When counted as a "thin film," the observed activity was 148 counts per min. per mg., corresponding to *ca.* 9% incorporation of added acetate into the chromanone (V).

The diluted 2,4-dinitrophenylhydrazone was oxidised (Kuhn–Roth), giving acetic acid which was degraded by standard methods.[11]

Degradation of Labelled Chromanone (V) *by Alkali.*—The chromanone (100 mg.) was heated under reflux under nitrogen with 15% aqueous potassium hydroxide (10 ml.) for 2 hr., cooled, acidified with dilute hydrochloric acid, and extracted with ether; the extract was washed with aqueous sodium hydrogen carbonate and water, dried ($MgSO_4$), and evaporated, giving the acetophenone (VII) as a brown residue, purified by sublimation at 120—130°/15 mm. (yield 90%), trituration with light petroleum, and recrystallisation from water and chloroform to constant m. p. (154·5—155·5°) and activity. A portion (78 mg.) was diluted with pure inactive synthetic material (861 mg.) and again recrystallised to constant activity. The C atoms of the side-chain were isolated by Kuhn–Roth oxidation.[11]

The diluted ketone (VII) (300 mg.) was heated under nitrogen with potassium hydroxide (2 g.) and water (0·6 ml.) in a nickel crucible, for 5 min. at 160°, raised during 15 min. to 280° and for 70 min. at 280°; the almost colourless melt was dissolved in dilute sulphuric acid.

[10] Limaye and Kelkar, *Rasayanam*, 1936, **1**, 24; *Chem. Abs.*, 1937, **31**, 2213.
[11] Birch, Massy-Westropp, Rickards, and Smith, *J.*, 1958, 360.

The solution was extracted with ether (10 × 25 ml.) and the combined extracts dried and evaporated; the crude resorcinol was purified by sublimation at 110°/15 mm. (yield 90%) and recrystallised to constant m. p. (108·5—110°) and activity.

Degradation of Resorcinol.—The resorcinol (137 mg.) was dissolved in concentrated sulphuric acid (3 ml.) at 0°, and nitric acid (0·45 ml.; *d* 1·42) followed by nitric acid (0·3 ml.; *d* 1·50) were added dropwise with stirring. After 5 min., iced water (5 ml.) was poured in and after 15 min. at 0° the precipitate was collected, dissolved in methanol–chloroform (charcoal), and recrystallised from chloroform, giving styphnic acid of constant m. p. (174—175°) and activity (yield 76%). This was degraded to barium carbonate (from $C_{(2)} + C_{(4)} + C_{(6)}$ and $C_{(1)} + C_{(3)} + C_{(5)}$, separately) by the methods described for the bromopicrin degradation of 2,4,6-trinitrophloroglucinol by Birch *et al.*[11]

Incorporation of Labelled 2,6-*Dihydroxyacetophenone.*—When the undiluted labelled ketone (VII), obtained as above, was incubated (45 mg.) with preformed *D. concentrica* $26A_1$ mycelium on 4% aqueous glucose for 14 days, and the chromanone (V) isolated as already described, the product gave a thin-film count corresponding to an incorporation of $<1\%$. Because of the low activity no degradation was attempted.

Ferricyanide Oxidation of Naphthalene-1,8-*diol* (IX).—In a typical experiment, aqueous potassium ferricyanide (0·166 g. in 8·0 ml.) was added in one lot to the phenol (0·021 g.) in methanol (2·0 ml.), and the mixture kept under nitrogen in the dark for 15 min. The green precipitate was then filtered off and washed well with cold water. The filtrate and washings were combined and the ferricyanide was titrated under nitrogen.[12] The precipitate was taken up in methanol or ether, its ultraviolet absorption determined, and examined by paper chromatography on strips or discs with saturated butanol–aqueous ammonia or 15% aqueous acetic acid; the chromatograms were viewed under ultraviolet light and sprayed with diazotised *p*-nitroaniline. With a relatively dilute solution of the phenol (0·01 mg./ml.), 2—2·2 mol. of ferricyanide were consumed, the product being a black precipitate in which no binaphthyl (I) was detectable; with a stronger solution (15 mg./ml., in 7% aqueous methanol) only 1·35—1·4 mol. of ferricyanide were consumed, and the product, a green precipitate, contained detectable amounts of binaphthyl (I). The results of similar experiments are summarised on p. 657.

	R_F values				Colour with		R_F values				Colour with
	A	B	C	D	*p*-$NO_2 \cdot C_6H_4 \cdot N_2^+$		A	B	C	D	*p*-$NO_2 \cdot C_6H_4 \cdot N_2^+$
Resorcinol	0·74	0·68	0·79		Brown	(IX)	0·51	0·30		0·69	Purple
Resorcylic acid	0·66	0·55	0·82		Brown	(IV)	0·49			0·59	Grey-purple
						(V)				0·73	Deep red
(VII)	0·68	0·57	0·83	0·80	Lemon-yellow	(VIII)				0·68	Pale pink
(VI)	0·64	0·49		0·81	Lemon yellow	(I)	0·26				Grey-purple

A, 15% aqueous acetic acid; B, 0·5N-hydrochloric acid; C, butan-1-ol saturated with 6N-ammonia; D, 6N-ammonia saturated with butan-1-ol.

Paper Chromatography.—The R_F values recorded for various compounds above, and their colour reactions with diazotised *p*-nitroaniline followed by 20% aqueous sodium carbonate,[13] are summarised in the annexed Table.

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[12] Vogel, " Textbook of Quantitative Inorganic Analysis," London, 1947, p. 435.
[13] Swayne, *Biochem. J.*, 1953, **53**, 202.