Studies on the Biosynthesis of Phenols in Fungi

CONVERSION OF [¹⁴C]ORSELLINIC ACID AND [¹⁴C]ORCINOL INTO FUMIGATOL BY *ASPERGILLUS FUMIGATUS* I.M.I. 89353

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1. Sodium [¹⁴C]acetate was incorporated into orsellinic acid and fumigatol by *Aspergillus fumigatus*. 2. [¹⁴C]Orsellinic acid was prepared biosynthetically. It was converted almost entirely into fumigatol and fumigatin within 2 days of supplementation of the medium. The apparent decrease in incorporation after a longer period of growth was due to decomposition of radioactive fumigatol and the production of relatively unlabelled material. The addition of orcinol to these cultures decreased the conversion of [¹⁴C]orsellinic acid into fumigatol. [¹⁴C]-Orsellinic acid was incorporated into 3,4-dihydroxytoluquinol in both sets of cultures. 3. [¹⁴C]Orcinol was prepared from [¹⁴C]orsellinic acid after acid hydrolysis. It was also very effective as a precursor of fumigatol (60% incorporation). 4. The specific activity of fumigatin was lower than that of fumigatol at early stages of growth (4–5 days after inoculation) with all the labelled substrates that were tested. This indicated that fumigatin arose from fumigatol after oxidation in the medium. 5. The presence of orcinol in the medium greatly stimulated the incorporation of radioactivity (presumably derived from the ¹⁴CO₂H of orsellinic acid) into the isoprenoid compounds, ergosterol and ubiquinone, in the mycelium.

Many phenolic compounds are synthesized by fungi from one molecule of acetyl-CoA plus a number of malonyl-CoA units, e.g. 6-methylsalicylic acid (I) (Bu'Lock, Smalley & Smith, 1962), orsellinic acid (II) (Mosbach, 1961), javanicin, a dihydroxynaphthaquinone derivative (Gatenbeck & Bentley, 1965), and islandicin (1,4,5-trihydroxy-2-methylanthraquinone) (Gatenbeck, 1962). Some of these substances are secreted into the culture medium. Fumigatol (III) and its oxidation product, fumigatin, are synthesized in excellent yield from [¹⁴C]acetate by *Aspergillus fumigatus* (Packter & Glover, 1965a) and are presumably also formed via the 'acetate-plus-malonate' pathway.

It has been suggested that the phenolic acids, 6-methylsalicylic acid and orsellinic acid, may be intermediates in the biosynthesis of other aromatic compounds in fungi (Birch, 1961; Bentley, 1962). Indeed, it has recently been shown that 6-methylsalicylic acid is converted into fumigatol and fumigatin in *A. fumigatus* (Packter, 1965). It can also be hydroxylated at C-4 to form orsellinic acid, a normal metabolite in this organism. The results described by Packter (1965) indicated that 6-methylsalicylic acid was initially converted into another substance before it could be incorporated into fumigatol and fumigatin, and it seemed probable that orsellinic acid was the true precursor.

As orsellinic acid and fumigatol are both synthesized from C₂ units derived from acetate, it was decided to determine the specific activities of these metabolites with sodium [¹⁴C]acetate as labelled substrate, at various stages of growth. In addition, [¹⁴C]orsellinic acid was prepared biosynthetically and was tested directly for precursor activity of fumigatol and fumigatin in *A. fumigatus*. [¹⁴C]-Orcinol was formed by decarboxylation of [¹⁴C]-orsellinic acid and was similarly tested.

EXPERIMENTAL

Organisms. The fungi used during this work were obtained from the Commonwealth Mycological Institute (Kew, Surrey) and were: *A. fumigatus* (I.M.I. 89353) and *Penicillium barnense* (I.M.I. 40590). They were cultured as described previously (Packter, 1965).

(I) R = H
(II) R = OH

(III) R = O·CH₃
(IV) R = OH

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Isolation of metabolites. Orsellinic acid, fumigatin and fumigatol were isolated from *A. fumigatus* and resolved from each other by column chromatography on silicic acid-Celite (2:1, w/w). They were estimated spectrophotometrically by using their E values at λ_{max} 300, 263 and 285 $m\mu$ respectively. Orsellinic acid was further purified on thin-layer plates of Kieselgel G (E. Merck A.-G., Darmstadt, Germany) and finally crystallized from chloroform after dilution with carrier material. Fumigatin was rechromatographed, crystallized from cyclohexane and acetylated. Fumigatol was rechromatographed, oxidized to fumigatin and in most instances converted into the acetyl derivative (Packter, 1965). Orcinol, if present, would be eluted with light petroleum-ether (3:2, v/v) and resolved from fumigatin after removal of the latter with cyclohexane. It is stable in autoclaved medium for at least 48 hr.

Another hydroxyquinone (referred to as compound 'Q') and the corresponding quinol have been isolated from certain cultures of *A. fumigatus* incubated with ^{14}C -labelled 6-methylsalicylic acid (Packter, 1965) or [^{14}C]orsellinic acid (Table 3). The structure of this substance had not been fully established but its properties are very similar to those of fumigatin. It therefore seemed probable that it was 3,4-dihydroxytoluquinone. Accordingly, this quinone was synthesized by treating fumigatol (100 mg.) with 2 ml. of HI and red phosphorus at 100° for 15 min. The reaction products were extracted with ether. The resulting extract was chromatographed and the quinones were eluted with light petroleum-ether (11:9, v/v). Fumigatin was removed by solution in cyclohexane and the residual material was crystallized from cyclohexane-benzene (1:1, v/v) to give purple clusters of 3,4-dihydroxytoluquinone (5% yield). This substance was identical with compound 'Q' as evidenced by colour reactions with (a) NaOH and (b) ethyl cyanoacetate, and by their respective ultraviolet- and infrared-absorption spectra (Packter, 1965).

Unsapoifiable lipid, ergosterol and ubiquinone were also isolated as described previously (Packter & Glover, 1965a; Packter, 1965). Ergosterol was estimated spectrophotometrically by using its E value at 282 $m\mu$ (Mercer & Glover, 1961).

All substances were purified to constant specific activity. [^{14}C]Orsellinic acid was prepared biosynthetically from

sodium [$1\text{-}^{14}\text{C}$]acetate (The Radiochemical Centre, Amer-sham, Bucks.). This was added to the culture medium of *P. barnense* after 7 days' growth and incubation was continued for a further 7 days. [^{14}C]Orsellinic acid was isolated and purified by crystallization to give anhydrous crystals, m.p. 183–184° and specific activity 4030 counts/min./ μmole . Some of this material was diluted with carrier and the total was then crystallized from chloroform. [^{14}C]Orcinol was obtained from this material (specific activity 1505 counts/min./ μmole) after reaction with N-HCl for 30 min. The $^{14}\text{CO}_2$ was passed into 10 ml. of N-NaOH and counted as BaCO_3 . The residual solution was diluted with water and extracted with ether to give a white solid with an ultraviolet spectrum identical with that of orcinol. Inflections at 262 or 300 $m\mu$ were absent, indicating that no orsellinic acid remained. The [^{14}C]orcinol was purified by crystallization from benzene and the anhydrous crystals had m.p. 107° and specific activity 1220 counts/min./ μmole . These results confirm the findings of Birkinshaw & Gowlland (1962) and Mosbach (1960) that [^{14}C]orsellinic acid obtained in this way was equally labelled in C-2, C-4, C-6 and the carboxyl group (C-8) (Table 1).

Spectrophotometry. Ultraviolet-absorption measurements were carried out in cyclohexane ('special for spectroscopy'; British Drug Houses Ltd., Poole, Dorset) or ethanol (refluxed over KOH and Zn, and then redistilled) in a Hilger and Watts Ultrascan.

Assay of radioactivity. Radioactivity was measured by counting portions of the various fractions on planchets in

Table 1. *Decarboxylation of orsellinic acid*

Experimental details are given in the text. The numbers of the labelled carbon atoms are given in parentheses.

	Specific activity	
	(counts/min./ μmole)	(counts/min./ $\mu\text{g. atom of }^{14}\text{C}$)
Orsellinic acid (C-2, C-4, C-6 and C-8)	1505	376
Orcinol (C-2, C-4 and C-6)	1220	407
BaCO_3 (C-8)	355	355

Table 2. *Incorporation of sodium [$1\text{-}^{14}\text{C}$]acetate into orsellinic acid, fumigatin and fumigatol*

Sodium [$1\text{-}^{14}\text{C}$]acetate ($5\mu\text{C}$; specific activity $2\text{-}9\mu\text{C}/\mu\text{mole}$) was added to each of nine Roux bottles containing 200 ml. of Raulin-Thom medium after 3 days' growth; three bottles were subsequently removed at intervals. All values are expressed as totals/600 ml. of medium. The specific activity of orsellinic acid has been corrected for the addition of carrier material.

Group	Total incubation time (days)	pH of medium	Ether extract (mg.)	Total activity (counts/min.)	Orsellinic acid (mg.)	Specific activity (counts/min./ μmole)		Specific activity (counts/min./ μmole)		Specific activity (counts/min./ μmole)	Total activity in fumigatol and fumigatin (counts/min.)
						Fumi-gatol (mg.)	Fumi-gatol (mg.)	Fumi-gatol (mg.)	Fumi-gatol (mg.)		
1	4	2.8	108	213 000	0.3	1025	65	306	2.1	276	122 000
2	5	2.8	170	181 000	0.5	109	105	255	4.7	252	157 500
3	6	3.0	258	143 000	0.5	55	131	151	4.0	153*	125 490

* The specific activity of 3,4-dihydroxytoluquinone was 94 counts/min./ μmole .

a Nuclear-Chicago Corp. gas-flow counter (25% efficiency). Equal amounts (usually 0.2 ml.) were estimated spectrophotometrically. Samples were counted at infinite thinness and corrected for background. All specific activities are expressed as counts/min./ μ mole.

Melting points. These were determined in unsealed capillary tubes in a Gallenkamp melting-point apparatus and are uncorrected.

RESULTS

Incorporation of sodium [1-¹⁴C]acetate into orsellinic acid and fumigatol. The values for the specific activities of orsellinic acid, fumigatol and fumigatin at different time-intervals are given in Table 2. The [¹⁴C]acetate was rapidly incorporated into these metabolites. The specific activity of orsellinic acid isolated from group 1 was considerably higher than that of fumigatol. At later stages of growth, the specific activity of orsellinic acid fell to about one-twentieth of this value. Most of the activity present in the ether-extractable material of groups 2 and 3 was concentrated in the fumigatol and fumigatin fractions (approx. 90%). The specific activity of fumigatol was somewhat higher than that of the quinone in group 1 but they were identical in groups 2 and 3. A small amount of 3,4-dihydroxytoluquinone (0.8 mg.) was isolated from the medium of group 3; its specific activity was lower than that of fumigatol.

These results were all reproduced in a confirmatory experiment.

Conversion of [¹⁴C]orsellinic acid into fumigatol. In a preliminary experiment, 10 mg. of [¹⁴C]orsellinic acid was added to each of three Roux bottles after 3 days' growth and incubation proceeded for a further 3 days. Fumigatol and fumigatin were isolated and both were highly labelled; their combined radioactivities accounted for over 80% of that found in the ether-extractable material. The incorporation of label into these metabolites was 80% (after correction for loss of the ¹⁴CO₂H group). 3,4-Dihydroxytoluquinone was also present and its specific activity was lower than that of fumigatin.

Next, the incorporation of [¹⁴C]orsellinic acid into fumigatol and fumigatin was measured after 5 and 8 days of incubation. Further, as the first stage in the conversion of orsellinic acid into fumigatol might be decarboxylation to orcinol, the possible dilution effect of this substance on incorporation was also tested. Details of the metabolites isolated from the medium are shown in Table 3. Considering groups 1 and 2, which were harvested 2 days after supplementation of the medium, both fumigatol and fumigatin were highly active. The specific activity of fumigatin, however, was lower than that of fumigatol. Orsellinic acid was isolated from these groups and its specific activity in both cases had been markedly decreased from its initial value.

Table 3. *Effect of orcinol on the incorporation of [¹⁴C]orsellinic acid into fumigatol*

Group	Ether extract (mg.)	Total activity of ether extract (counts/min.)	Total activity of aqueous residue (counts/min.)	Orsellinic acid (mg.)	Specific activity (counts/min./ μ mole)	Fumigatol (mg.)	Specific activity (counts/min./ μ mole)	Fumigatin (mg.)	Specific activity (counts/min./ μ mole)	Total activity in fumigatol and fumigatin (counts/min.)	Incorporation of orsellinic acid into fumigatol and fumigatin (%)	3,4-Dihydroxytoluquinol (mg.)	Specific activity (counts/min./ μ mole)
1	258	372900	—	0.7	336	134	450	3.9	393	363560	95	7	292
2	320	319300	—	0.8	220	156	312	3.2	202	290290	77	6	241
3	245	132900	155300	0.2	—	97	185	3.5	181	109720	29	4	56
4	302	126360	98500	0.2	—	95	133	3.0	138	74350	19	2	58

Orcinol (25 mg./200 ml.) was added to each of three Roux bottles in groups 2 and 4 respectively after 3 days' growth. [¹⁴C]Orsellinic acid (7 mg.; specific activity 4030 counts/min./ μ mole) was added to each bottle 4 hr. later. Incubation proceeded for a further 2 days (groups 1 and 2) or 5 days (groups 3 and 4). All values are expressed as totals/600 ml. of medium. The specific activity of the orsellinic acid isolated from the medium has been corrected for the addition of carrier material. The incorporation values have been determined on a basis of 1 μ g. atom of ¹⁴C. The pH of the medium of groups 1, 2, 3 and 4 was 2.2, 2.2, 4.2 and 4.8 respectively.

The specific activity of 3,4-dihydroxytoluquinol was lower than that found for fumigatol. In estimating the amount of this substance present, it was assumed that approx. 15% had been converted into the quinone (i.e. similar to the yield obtained in the oxidation of fumigatol). Over 90% of the activity in the ether-extractable material was present in the fumigatol and fumigatin fractions.

A different pattern of results was obtained in groups 3 and 4. The total activity present in the ether-extractable matter was much less in these groups and this was confirmed by the lower specific activities of fumigatol and fumigatin. Moreover, the aqueous medium possessed considerable activity. Insufficient orsellinic acid was detected to permit purification.

The specific activities of fumigatol and fumigatin isolated from the orcinol-supplemented groups were

lower than the corresponding values in groups 1 and 3 (Table 3). The synthesis of 3,4-dihydroxytoluquinol was not affected.

Determination of radioactivity in some unsaponifiable lipids in the mycelium. Most of the radioactivity present in the unsaponifiable lipid fraction was due to ergosterol (Table 4). Ubiquinone was also labelled and possessed about the same specific activity as ergosterol. The residual radioactivity in the unsaponifiable matter was presumably due to the presence of other unidentified sterols.

The incorporation of radioactivity into isoprenoid-derived compounds was greatly stimulated by orcinol (groups 2 and 4).

Conversion of [¹⁴C]orcinol into fumigatol. The results obtained in this experiment are given in Table 5 and were similar to those determined for [¹⁴C]orsellinic acid. [¹⁴C]Orcinol was very effec-

Table 4. *Incorporation of activity from [¹⁴C]orsellinic acid into some unsaponifiable lipids in the mycelium*

Experimental details are given in Table 3. The specific activity of ubiquinone has been corrected for the addition of carrier material.

Group	Unsaponifiable lipid		Ergosterol				Ubiquinone		
	Weight (mg.)	Total activity (counts/ min.)	Weight (mg.)	Total activity (counts/ min.)	Specific activity		Weight (mg.)	Specific activity	
					(counts/ min./ μmole)	(counts/ min./ μg. atom of ¹⁴ C)		(counts/ min./ μmole)	(counts/ min./ μg. atom of ¹⁴ C)
1	123	235	15.1	151	4	0.7	0.69	0	0
2	220	4690	9.9	695	24	4.0	0.61	20	2.0
3	252	950	48.1	624	5	0.8	0.80	4	0.4
4	265	6950	40.4	4440	44	7.3	0.92	77	7.7

Table 5. *Conversion of [¹⁴C]orcinol into fumigatol*

[¹⁴C]Orcinol (5 mg.; specific activity 1220 counts/min./μmole) was added to each of six Roux bottles after 2.5 days' growth. Incubation proceeded for a further 2 and 4.5 days (groups 1 and 2 respectively); each group consisted of three Roux bottles and the results are expressed as totals/600 ml. of medium. The pH of the medium of groups 1 and 2 was 2.3 and 2.4 respectively.

	Group 1			Group 2		
	Weight (mg.)	Total activity (counts/min.)	Specific activity (counts/ min./μmole)	Weight (mg.)	Total activity (counts/min.)	Specific activity (counts/ min./μmole)
Medium						
Ether extract	229	50620	—	300	22500	—
Aqueous residue	—	< 5000	—	—	24100	—
Fumigatol	103	43570	71	130	14560	20
Fumigatin	3.6	1190	55	4.4	506	19
Orcellinic acid	0.7	0	0	0.5	0	0
Mycelium						
Unsaponifiable lipid	90	900	—	139	790	—
Ergosterol	18	0	0	29	0	0

tively converted into fumigatol and fumigatin (57 and 1.5% respectively) within 2 days of supplementation of the medium (group 1). The specific activity of fumigatin was again lower than fumigatol at this stage of growth (compare group 1 in Table 2 and groups 1 and 2 in Table 3). Nearly 90% of the radioactivity in the ether-extractable material was located in the fumigatol and fumigatin fractions. Orsellinic acid was isolated but, as expected, possessed no radioactivity. Orcinol was not present in significant amounts (less than 0.1mg.). Similar results were obtained for group 2. This group, however, contained less radioactivity than the first, but the aqueous medium after ether extraction was appreciably radioactive.

Trace amounts of 3,4-dihydroxytoluquinone (0.4mg.) were detected in group 1.

The unsaponifiable lipid in groups 1 and 2 did not possess appreciable radioactivity. Ergosterol was not radioactive.

DISCUSSION

The results described in Table 2 are consistent with the view that orsellinic acid is a normal intermediate in the biosynthesis of fumigatol from acetyl-CoA. In group 1, the specific activity of orsellinic acid was much higher than that of fumigatol, but in the other groups at later stages of growth it fell below that value. However, at this time, the pool of [^{14}C]acetyl-CoA would have been greatly diluted by endogenously produced material and substances subsequently formed from acetyl-CoA would have a lower specific activity. Accordingly, as only 0.3mg. of orsellinic acid was present in the medium after 4 days' growth, the secretion of a further milligram, for example, of unlabelled substance during the fifth and sixth day of incubation would decrease the specific activity considerably. It is evident from the comparative amounts and specific activities of orsellinic acid that the mycelium is freely permeable to this substance. This is confirmed by the results given in Table 3. By contrast, the situation with fumigatol is quite different. The cultures in group 1 had already secreted an appreciable amount of fumigatol into the medium, and non-radioactive material synthesized later would not affect the specific activity to the same extent. Presumably, fumigatol does not re-enter the mycelium.

[^{14}C]Orsellinic acid is converted almost entirely into fumigatol within 2 days (Table 3). It is also incorporated into 3,4-dihydroxytoluquinol (IV), but on all occasions the specific activity of this substance was appreciably lower than that of fumigatol. The addition of orcinol to the medium effectively diluted out the incorporation of orsellinic acid into fumigatol. The synthesis of 3,4-dihydroxy-

toluquinol was not affected. This is in marked contrast with the effect of orcinol on the conversion of [^{14}C]labelled 6-methylsalicylic acid into fumigatin and 3,4-dihydroxytoluquinone (Packter, 1965). The incorporation into fumigatin was decreased to nearly zero and at the same time the cultures were stimulated to synthesize 3,4-dihydroxytoluquinone, with a very high specific activity. Significant quantities of 3,4-dihydroxytoluquinol or the corresponding quinone are only present when the medium has been supplemented with orsellinic acid or 6-methylsalicylic acid together with orcinol. These results suggest that 3,4-dihydroxytoluquinol is not an intermediate in the biosynthesis of fumigatol.

The apparently diminished incorporation into fumigatol and 3,4-dihydroxytoluquinol over longer periods of incubation may be explained by the fact that these metabolites decompose in aqueous solution into ether-insoluble compounds. Indeed, the residual medium, after extraction with ether, possessed a considerable amount of radioactivity (Tables 3 and 5). Presumably this was due mainly to the presence of breakdown products of fumigatol.

[^{14}C]Orcinol is also very effectively and quickly converted into fumigatol (60% incorporation). Ergosterol, isolated from the mycelium, is unlabelled, and so presumably none of the [^{14}C]orcinol has been metabolized to [^{14}C]acetyl-CoA.

The results therefore indicate that orsellinic acid is formed from acetyl-CoA and then decarboxylated. The orcinol thus produced may be hydroxylated and *O*-methylated to form fumigatol. However, the results expressed in Tables 3 and 5 do not rule out the possibility that orsellinic acid may be hydroxylated initially to produce a trihydroxy acid, for example, which could be the true precursor. Orcinol has not been detected in the culture medium of *A. fumigatus* but it has been found, together with orsellinic acid, 5-methylorsellinic acid and 6-methylsalicylic acid, in the medium of mutants of *Aspergillus terreus* and in shake-cultures of the wild type (Curtis, Harries, Hassall & Levi, 1964). Further, it has been established that orsellinic acid is decarboxylated by *Gliocladium roseum* (M. W. Steward & N. M. Packter, unpublished work).

A previous report has already suggested that [^{14}C]orsellinic acid might be incorporated into fumigatin in *A. fumigatus* (Pettersson, 1963). However, the fumigatin in this work was not obtained in a crystalline state. The medium was oxidized before extraction and fumigatol was not isolated. Further, the spectral properties quoted for fumigatin do not agree with those established by Packter & Glover (1962) for the pure crystalline material.

The specific activity of fumigatol was higher than that of the quinone in group 1 of the [^{14}C]acetate

Scheme 1. Proposed sequence of reactions for the biosynthesis of fumigatol and formation of fumigatin by *A. fumigatus*.

experiment but their activities were equal in groups 2 and 3 (Table 2). This latter result is in agreement with that reported by Packter & Glover (1965a) for cultures of *A. fumigatus* incubated with [^{14}C]acetate for 3 days. The total incubation time was 7 days. In the experiment described in Table 2, the medium was pale orange when the [^{14}C]acetate was added; some fumigatin (and presumably fumigatol) therefore was present already. Accordingly, the specific activity of the small amounts of [^{14}C]labelled fumigatin produced by oxidation of fumigatol would initially be lowered. This effect would become less marked subsequently. A similar pattern of results was also obtained when [^{14}C]orsellinic acid or [^{14}C]orcinol was the labelled substrate. Packter (1965) has indicated that most of the fumigatin present in the medium could arise from oxidation of fumigatol. Some fumigatin, however, is secreted as such after 7 days' incubation.

Thus the results obtained during this work support the proposed pathway of fumigatol biosynthesis and fumigatin formation that is outlined in Scheme 1. No evidence is available as to which carbon atom of orcinol is hydroxylated first. The reported lack of incorporation of 4-[^{14}C]-methoxy-toluquinone into fumigatol (Packter & Glover, 1965b) is readily explained as a hydroxyl group at C-3 of the toluquinol ring would appear to be necessary for precursor activity.

Orellinic acid might also act as a precursor of gliorosein (Brian, Curtis, Howland, Jefferys & Raudnitz, 1951) in *G. roseum*. Gliorosein (1,6-dihydro form, established by its n.m.r. spectrum) (V) is slowly isomerized in the medium during incubation into the corresponding *p*-quinol, which is itself oxidized to form rubrogliocladiol (quinhydrone) and aurantiogliocladiol (quinone) (Steward

& Packter, 1965). A preliminary report by Birch (1961) has indicated that [5- ^{14}C]orsellinic acid (prepared synthetically) is not incorporated into aurantiogliocladiol. No experimental details were given. Recent studies in this Laboratory (M. W. Steward & N. M. Packter, unpublished work) have shown that [^{14}C]orsellinic acid is rapidly decarboxylated by *G. roseum* and an approximately equivalent amount of orcinol is secreted into the medium. This suggested that orcinol does not act as a precursor either. It is possible, however, that orsellinic acid and orcinol are involved in the biosynthesis of gliorosein *in vivo* and react as enzyme complexes. The formation of enzyme-bound intermediates in acetyl-CoA and malonyl-CoA condensation reactions has now been well established in fatty acid (Wakil, Pugh & Sauer, 1964) and mevalonic acid biosynthesis (Brodie, Wasson & Porter, 1964).

During the conversion of [^{14}C]orsellinic acid into fumigatol, the carboxyl group is eliminated as $^{14}\text{CO}_2$. At the same time, radioactivity is incorporated into the isoprenoid-derived compounds, ergosterol and ubiquinone, in the mycelium. Goodwin (1959) has demonstrated that $^{14}\text{CO}_2$ is fixed into β -carotene by *Phycomyces blakesleeanus* when leucine is present in the medium. Further, Packter & Glover (1962, 1965b) have established that small amounts of L-leucine (0.1–0.2 g./200 ml.)

stimulate the synthesis of ubiquinone in *A. fumigatus*. These observations are readily explained by the work of Coon, Kupiecki, Dekker, Schlesinger & del Campillo (1959), who indicated that β -methylcrotonyl-CoA, a product of leucine catabolism, may be carboxylated to give β -methylglutaconyl-CoA and β -hydroxy- β -methylglutaryl-CoA, a precursor of isoprenoid compounds. Ergosterol does not become labelled when ^{14}C -labelled 6-methylsalicylic acid is used as substrate (Packter, 1965). However, in this case only 5% is decarboxylated to *m*-cresol and 1–1.5% is converted into fumigatin and fumigatol. Further, these reactions do not occur until a late stage of growth. Accordingly, only a small amount of $^{14}\text{CO}_2$ is available for fixation into β -methylglutaconyl-CoA at a time when there would be little turnover of isoprenoid compounds.

No explanation can be given, as yet, for the apparent stimulation of incorporation of radioactivity into ergosterol and ubiquinone (and presumably other products of terpene biosynthesis) in the presence of orcinol.

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