Biochem. J. (1967) **102**, 122

Studies on the Biosynthesis of Phenols in Fungi

BIOSYNTHESIS OF 3,4-DIMETHOXY-6-METHYLTOLUQUINOL AND GLIOROSEIN IN *GLIOCLADIUM ROSEUM* I.M.I. 93065

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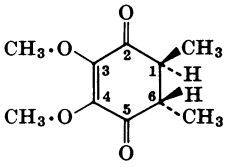
(*Received* 6 *May* 1966)

1. Gliorosein was obtained in excellent yield (150mg./200ml. of Raulin–Thom medium) from surface cultures of *Gliocladium roseum*. Its nuclear-magnetic-resonance spectrum showed conclusively that it is 1,6-dihydro-3,4-dimethoxy-6-methyltoluquinone. 2. Sodium [2-^{14}C]acetate was incorporated into gliorosein and the related products (3·3% conversion). The specific activities of these substances increased in the order gliorosein, 3,4-dimethoxy-6-methyltoluquinol, the related quinhydrone and quinone, indicating that gliorosein was the actual metabolite that was secreted and that the other compounds were derived from it in the medium. 3. 6-Methylsalicylic acid was not taken up by the mycelium and could be recovered unchanged. Orsellinic acid was decarboxylated by *G. roseum* and an equivalent amount of orcinol was secreted into the medium. The methyl esters of 6-methylsalicylic acid and orsellinic acid were both hydrolysed by an esterase present in the mycelium. Some of the 6-methylsalicylic acid thus produced was secreted into the medium and the orsellinic acid was decarboxylated. 4. Washed mycelium of *G. roseum* converted aurantiogliocladin and 3,4-dimethoxy-6-methyltoluquinol quantitatively into gliorosein within 18hr. More critical experiments with ^{14}C-labelled substrates demonstrated that 3-hydroxy-4-methoxy-6-methyltoluquinol and 3,4-dimethoxy-6-methyltoluquinol, and their respective quinones, were effectively incorporated into gliorosein and related products (49, 68, 30 and 57% respectively). 5. The following sequence of reactions is proposed for the biosynthesis of gliorosein: acetyl-CoA + 3 malonyl-CoA + *S*-adenosylmethionine → 5-methylorsellinic acid → 3-hydroxy-4-methoxy-6-methyltoluquinol → 3,4-dimethoxy-6-methyltoluquinol → gliorosein. 6. Since gliorosein is optically active (dextrorotatory), the final tautomerization reaction leading to its formation must be enzyme-catalysed.

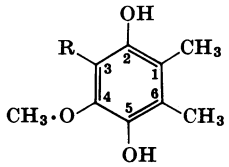
Brian, Curtis, Howland, Jefferys & Raudnitz (1951) isolated three antibiotics of similar structure from the medium of *Gliocladium roseum* and named them aurantiogliocladin, rubrogliocladin and gliorosein. Vischer (1953) characterized them as 3,4-dimethoxy-6-methyltoluquinone (IV), the related quinhydrone (III) and 1,6- or 3,4-dihydroaurantiogliocladin respectively. The production of 3,4-dimethoxy-6-methyltoluquinol (II) would be essential for the formation of quinhydrone and it has recently been detected in cultures of *G. roseum* (Steward & Packter, 1965).

Results from this Laboratory have indicated that gliorosein (I) is the actual metabolite that is secreted; compounds (II), (III) and (IV) are derived from it in the medium. Preliminary experiments have also shown that ^{14}C-labelled aurantiogliocladin is effectively incorporated into gliorosein, denoting that this substance or the quinol (II) is probably the immediate precursor of gliorosein within the mycelium (Steward & Packter, 1965) [quinol, quinhydrone and quinone refer to compounds (II), (III) and (IV) respectively].

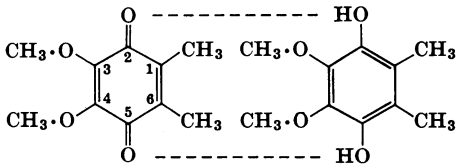
Birch, Fryer & Smith (1958) have shown that gliorosein, rubrogliocladin and aurantiogliocladin are synthesized through condensation of C_2 units derived from acetate. Bentley (1962) suggested that fungal products synthesized from acetyl-CoA may, in fact, be derived from condensations of the type acetyl-CoA + *n* malonyl-CoA. This view has since been confirmed for many different types of compound (for references see Packter, 1966*a*) including aurantiogliocladin (Bentley & Lavate, 1965). Moreover, Birch (1961) and Bentley (1962) proposed that 6-methylsalicylic acid (VII) and orsellinic acid (VIII), themselves formed in this



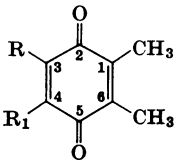
(I)



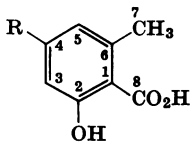
(II) R = O·CH$_3$
(VI) R = OH



(III)



(IV) R = R$_1$ = O·CH$_3$
(V) R = OH; R$_1$ = O·CH$_3$



(VII) R = H
(VIII) R = OH

manner, may be intermediates in the biosynthesis of other aromatic substances. Indeed, it has now been shown that orsellinic acid is rapidly and almost completely converted into fumigatol (3-hydroxy-4-methoxytoluquinol) in *Aspergillus fumigatus*. 6-Methylsalicylic acid itself appears to be inactive as a precursor but it may be hydroxylated to some extent at C-4 to form orsellinic acid and as such is incorporated into fumigatol (Packter, 1965, 1966*a*).

The present work was undertaken in an attempt to determine some of the stages involved in the biosynthesis of gliorosein in *G. roseum*. The incorporation of sodium [2-^{14}C]acetate into gliorosein and related products was measured at different time-intervals. Further, 6-methyl[^{14}C]salicylic acid and [^{14}C]orsellinic acid, and their methyl esters, were prepared and tested for precursor activity. Toluquinol derivatives, structurally related to aurantiogliocladin, were also synthesized and tested.

METHODS

Organisms and culture conditions. The fungi used in this work were obtained from the Commonwealth Mycological Institute (Kew, Surrey) and were *Gliocladium roseum* (I.M.I. 93065), *Penicillium griseofulvum* (I.M.I. 75832) and *Penicillium baarnense* (I.M.I. 40590). Stock cultures of these organisms were maintained on potato–dextrose agar (Difco) slopes at 4° and subcultured every 4–6 weeks. *G. roseum* was incubated as surface cultures in Roux bottles containing 200ml. of Raulin–Thom medium (half volume tap water), at 24–25° for various time-intervals. Previous studies were performed at 28–29° (Steward & Packter, 1965) but experience showed that the lower temperature gave a more uniform growth and an increased yield of metabolites (approx. 150–200mg./200ml. of medium after 15 days of incubation). Shake cultures of *G. roseum* produced much lower yields (approx. 40–50mg./200ml. after 21 days' growth). Again, gliorosein was the principal metabolite. *P. griseofulvum* and *P. baarnense* were cultured as described previously (Packter, 1965).

Solutions of ^{14}C-labelled and supplementary substrates were added by pipette beneath the surface of the mycelium.

Chromatography. Fractions containing phenolic substances were resolved by one-dimensional descending chromatography on Whatman no. 1 paper. The solvent systems used were benzene–acetic acid–water (20:5:4, by vol.) and butan-2-one–water–diethylamine (921:77:2, by vol.). Phenols were detected by examining the chromatograms under ultraviolet light (Hanovia Chromatolite). The presence of these compounds was confirmed by spraying the paper with a solution of diazotized *o*-dianisidine in methanol–water (1:1, v/v) or an aqueous solution of $FeCl_3$ (Curtis, Harries, Hassall & Levi, 1964).

The adsorbents used for column chromatography of substances isolated from the medium were silicic acid (Mallinckrodt A.R.; 100 mesh)–Celite 535 (Johns–Manville Ltd.) (2:1, w/w). Aurantiogliocladin (prepared from gliorosein) was occasionally purified on thin-layer plates of Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with benzene–methanol (99:1, v/v) as the solvent system. It had R_F 0·35. Ergosterol was isolated after chromatography on magnesia–Celite (2:1, w/w) (Packter, 1965).

Spectrophotometry. Ultraviolet-absorption measurements were carried out in cyclohexane ('special for spectroscopy'; British Drug Houses Ltd., Poole, Dorset) or ethanol in a Unicam SP.500 spectrophotometer or a Hilger and Watts

Ultrascan instrument. All substances were assayed spectrophotometrically. Infrared-absorption spectra were recorded by a Unicam SP.200 spectrophotometer as a Nujol mull between NaCl cells.

Assay of radioactivity. Radioactivity was measured with a Nuclear–Chicago Corp. gas-flow counter at 20% efficiency (Packter, 1965). Samples were always 'infinitely thin'. All radioactive substances were crystallized to constant m.p. and specific activity.

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

Analyses. C and H analyses were kindly performed by the Micro-analytical Laboratory, Department of Organic Chemistry, University of Leeds.

MATERIALS

Identification and properties of metabolites of G. roseum. Gliorosein (I), the related quinol (II), quinhydrone (rubrogliocladin, III) and quinone (aurantiogliocladin, IV) were isolated from the medium of *G. roseum* and resolved from each other after chromatography on silicic acid–Celite (Steward & Packter, 1965). The appropriate fractions were always rechromatographed and were crystallized from light petroleum. Occasionally, small amounts of quinone were eluted with quinhydrone and the two substances were then separated by fractional crystallization from light petroleum at 4°. The quinhydrone is considerably less soluble at this temperature.

Gliorosein (I). Gliorosein gave colourless needles, m.p. 48–49° and $\lambda_{max.}$ in cyclohexane (and ethanol) 290mμ ($E^{1\%}_{1\,cm.}$ 540) (Found: C, 60·7; H, 7·2. Calc. for $C_{10}H_{14}O_4$: C, 60·5; H, 7·1%). Its nuclear-magnetic-resonance spectrum was determined with a Varian Associates (Palo Alto, Calif., U.S.A.) A-60 spectrometer to establish the position of the two lone H atoms. The sample (50mg.) was dissolved in 0·5ml. of carbon tetrachloride. Tetramethylsilane (TMS) was used as the internal reference and the data are expressed in p.p.m. referred to 10·00 (τ values). The signal due to the methyl protons (attached to C-1) was observed as a doublet (J 7 cyc./sec.) at 1·18 and 1·28 p.p.m. (8·82 and 8·72τ), denoting that a H atom was also attached to C-1 (Fig. 1). In addition, the peak due to this H at 2·23–2·63 p.p.m. was split eightfold owing to the effect of (*a*) the adjacent methyl protons and (*b*) the H on the adjacent C atom (C-6). Thus the grouping $CH(CH_3)\cdot CH$ was present. Further, the methoxyl protons gave rise to a single peak at 3·88 p.p.m. (6·12τ). The conjugated system of double bonds to which the methoxyl is attached caused a displacement of this peak from the 6·70τ value for $CH_3\cdot O\cdot X$, confirming that the grouping $CH_3\cdot O\cdot C(:C)\cdot C:O$ occurred. Similar signals would be obtained from the methyl H and H attached to C-6 and the methoxyl H attached to C-4, resulting in a doubling of the intensity of these three regions. If the H atoms had been attached to C-3 and C-4, splitting of the methyl and H signals would not have occurred. Moreover, the τ value for the methoxyl protons would not have been displaced to the same extent. The proton ratio calculated from the integrating curve was 3:1:3 for the methyl H, H and methoxyl H respectively. Accordingly therefore gliorosein is the 1,6-dihydro form (I).

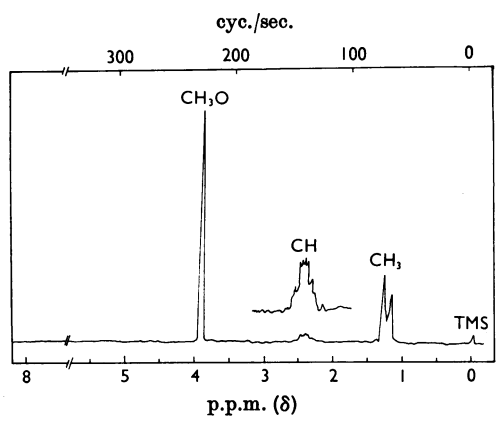


Fig. 1. Nuclear-magnetic-resonance spectrum of *trans*-(+)-gliorosein (I). The sample (50mg.) was dissolved in 0·5ml. of CCl_4; tetramethylsilane (TMS) was used as the internal reference.

The optical activity of gliorosein was also measured (Bellingham and Stanley Ltd., London, polarimeter). It gave $[\alpha]^{22}_D + 91 \pm 2°$ (*c* 2·0 in chloroform) and $+145 \pm 2°$ (*c* 1·2 in cyclohexane). Thus gliorosein must possess a *trans*-configuration around the asymmetrical C atoms, C-1 and C-6. The *cis*-isomer would be mesomeric and hence optically inactive. As the keto groups are conjugated with the double bond in the cyclohexene ring, all these six carbon atoms and the two methoxyl groups are co-planar. Thus *trans*-(+)-gliorosein has the structure (I) in which one of the CH_3 groups and H atoms lie above this plane while the others are below it.

Related metabolites. The quinol (II), when freshly crystallized, formed white plates, m.p. 84° and $\lambda_{max.}$ in cyclohexane 291mμ (in ethanol 290mμ) ($E^{1\%}_{1\,cm.}$ 175) (Found: C, 60·3; H, 7·1. Calc. for $C_{10}H_{14}O_4$: C, 60·5; H, 7·1%). The dry solid was fairly stable at 4° but oxidized in aqueous or ethanolic solution to rubrogliocladin (III) and then to aurantiogliocladin (IV). Rubrogliocladin (quinhydrone) was the least soluble of the four related compounds. It crystallized as deep-red plates and had m.p. 74° and $\lambda_{max.}$ in cyclohexane 275 and 403mμ ($E^{1\%}_{1\,cm.}$ 430 and 10 respectively) (Found: C, 61·2; H, 6·5. Calc. for $C_{20}H_{26}O_8$: C, 60·9; H, 6·6%). Aurantiogliocladin (quinone) gave orange needles and exhibited m.p. 63–64° and $\lambda_{max.}$ in cyclohexane 273 and 403mμ ($E^{1\%}_{1\,cm.}$ 735 and 25 respectively) (Found: C, 61·0; H, 6·0. Calc. for $C_{10}H_{12}O_4$: C, 61·2; H, 6·1%). This substance was stable in acidic and neutral solutions.

3-Hydroxy-4-methoxy-6-methyltoluquinone (V). This was prepared from gliorosein after its initial conversion into quinol and subsequent oxidation with $FeCl_3$ (Vischer, 1953). The oxidation products were extracted with ether and chromatographed on silicic acid–Celite before crystallization. Aurantiogliocladin (100mg.) was then treated with 0·1N-NaOH (50ml.) at 27° for 60min. The solution rapidly became purple owing to the formation of the anion of the hydroxyquinone. Unchanged aurantiogliocladin (approx. 10%) was removed by extraction of the alkaline solution with ether. The residual aqueous material was acidified and extracted threefold with ether. Compound (V) was purified by chromatography on silicic acid–Celite and

eluted with ether–light petroleum (1:4, v/v). It was crystallized from light petroleum and gave m.p. 70° and $\lambda_{max.}$ in ethanol 279 and 445 mμ ($E^{1\%}_{1cm.}$ 890 and 35 respectively) and in ethanolic 0·01N-NaOH 281 and 525 mμ ($E^{1\%}_{1cm.}$ 580 and 80 respectively). Isosbestic points occurred at 254 and 287 mμ (Found: C, 59·4; H, 5·5. Calc. for $C_9H_{10}O_4$: C, 59·2; H, 5·5%). This quinone was fairly stable in aqueous solution and over 80% remained after incubation with Raulin-Thom medium for 24 hr. The related quinone, fumigatin, decomposed at a considerably faster rate (Packter, 1965) and only 40% remained under these conditions.

The corresponding quinol (3-hydroxy-4-methoxy-6-methyltoluquinol, VI) was prepared after mixing the hydroxyquinone with an aqueous solution of sodium dithionite. The resulting solution was extracted with ether and the hydroxyquinol was crystallized from cyclohexane–benzene (2:1, v/v). Compound (VI) had m.p. 119–122° (decomp.) and $\lambda_{max.}$ in ethanol (and cyclohexane) 285 mμ ($E^{1\%}_{1cm.}$ 170). Its oxidation product was identical with 3-hydroxy-4-methoxy-6-methyltoluquinone (Found: C, 59·3; H, 6·6. Calc. for $C_9H_{12}O_4$: C, 58·7; H, 6·5%).

The infrared-absorption spectrum of gliorosein (I) has not been recorded previously and is given in Fig. 2. Gliorosein showed strong bands at 1600, 1675 cm.$^{-1}$ (ene-dione); rubrogliocladin possessed similar bands at 1620, 1650 cm.$^{-1}$ (quinone) and, in addition, at 3500 cm.$^{-1}$ (OH). The infrared-absorption spectra of aurantiogliocladin (IV), quinol (II) and the corresponding monohydroxy derivatives [compounds (V) and (VI) respectively] are shown in Fig. 3. Aurantiogliocladin and the monohydroxyquinone exhibited strong bands around 1620, 1640, 1670 cm.$^{-1}$ (quinone). In addition, the hydroxyquinone had bands at 1205, 1290 cm.$^{-1}$ (phenolic OH deformation) and 3400 cm.$^{-1}$ (OH stretching). The quinols absorbed around 1505, 1620 cm.$^{-1}$ (aromatic C=C), 1195, 1300 cm.$^{-1}$ (phenolic OH) and had a strong band at 3460 cm.$^{-1}$(OH) (Bellamy, 1954).

Methyl esters of 6-methylsalicylic acid and orsellinic acid. It was found that the mycelium of *G. roseum* was not permeable to 6-methylsalicylic acid (VII), and that orsellinic acid (VIII) was readily decarboxylated to orcinol. This was then secreted into the medium. The methyl esters were synthesized in an attempt to overcome these effects.

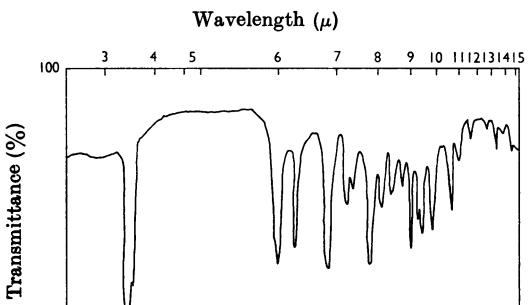


Fig. 2. Infrared-absorption spectrum of gliorosein (I). The spectrum was taken as a mull in Nujol.

They were prepared by mixing the parent acid with an ethereal solution of diazomethane at 0° for 30 min. Diazomethane was formed *in situ* by the addition of alkali to *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide (Diazald; Aldrich Chemical Co., Milwaukee, Wis., U.S.A.). The phenolic groups of orcinol were not affected under these conditions. The esters were resolved from traces of unchanged starting material by solution in warm cyclohexane. They were purified by chromatography on silicic acid–Celite and were eluted with ether–light petroleum (1:19, v/v). Finally, the methyl esters of 6-methylsalicylic acid and orsellinic acid were crystallized from light petroleum and chloroform respectively. Both possessed a typically sweet smell, reminiscent of oil of wintergreen. The methyl ester of 6-methylsalicylic acid was appreciably volatile at temperatures above 30°. It had m.p. 30° and $\lambda_{max.}$ in cyclohexane 245 and 315 mμ ($E^{1\%}_{1cm.}$ 500 and 260 respectively) (Found: C, 64·2; H, 5·9. Calc. for $C_9H_{10}O_3$: C, 65·0; H, 6·0%). Methylorsellinate gave m.p. 138–139° and $\lambda_{max.}$ in ethanol 262 and 298 mμ ($E^{1\%}_{1cm.}$ 765 and 285 respectively) (Found: C, 59·2; H, 5·4. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5%). The infrared-absorption spectra bore many similarities to those of the original acids (Packter, 1965) but the bands around 2550 cm.$^{-1}$ (OH from CO_2H stretching) and 900 cm.$^{-1}$ (OH from CO_2H deformation) were absent.

Both esters were stable in autoclaved Raulin-Thom medium at 26° for at least 5 days and could be recovered unchanged after extraction with ether.

Orcinol. When *G. roseum* was incubated with medium supplemented with [^{14}C]orsellinic acid or its methyl ester, a considerable amount of orcinol was secreted. This was resolved from gliorosein and other metabolites by removal of the latter substances with light petroleum and was purified after chromatography on thin-layer plates of Kieselgel G. It was identified by its R_F value in different solvent systems and by its ultraviolet- and infrared-absorption spectra. It was crystallized from benzene to constant specific activity. Its identity was confirmed after the addition of carrier material and subsequent crystallization.

It has been established that orsellinic acid is readily decarboxylated at 100° under acidic conditions (Birkinshaw & Gowlland, 1962). It was therefore possible that the orcinol might have been formed spontaneously in the medium over a period of time. This was checked by incubating orsellinic acid with autoclaved Raulin-Thom medium at 27° for 5 days. The medium was acidified, extracted with ether and chromatographed. Orsellinic acid was eluted with ether–light petroleum (1:4 and 3:10, v/v) and orcinol was eluted with ether–light petroleum (7:13 and 2:3, v/v). Approx. 20% of the orsellinic acid was converted into orcinol under these conditions.

Lipid material. Total lipid, unsaponifiable matter and ergosterol were isolated and purified as described by Packter & Glover (1965) and Packter (1965). Ergosterol was estimated spectrophotometrically by using its *E* value at 282 mμ ($E^{1\%}_{1cm.}$ in cyclohexane 270).

Radioactive compounds. [U-^{14}C]Tyrosine, sodium [1-^{14}C]- and [2-^{14}C]-acetate were obtained from The Radiochemical Centre (Amersham, Bucks.). The [^{14}C]acetate was diluted with inert material before use. ^{14}C-labelled specimens of compounds (V) and (VI) were prepared from [^{14}C]aurantiogliocladin, which was formed biosynthetically from sodium [1-^{14}C]acetate by cultures of *G. roseum*. Equal amounts of

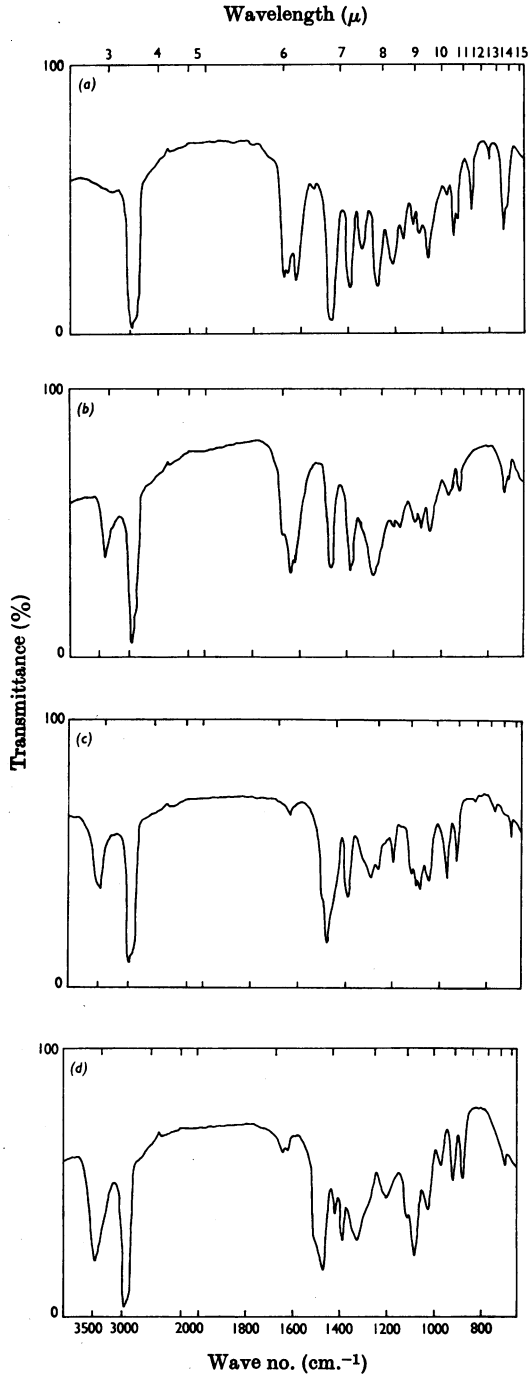


Fig. 3. Infrared-absorption spectra of (*a*) aurantiogliocladin (quinone, IV), (*b*) 3-hydroxy-4-methoxy-6-methyltoluquinone (V), (*c*) 3,4-dimethoxy-6-methyltoluquinol (quinol, II) and (*d*) 3-hydroxy-4-methoxy-6-methyltoluquinol (VI). The spectra were all performed as a mull in Nujol.

radioactivity are distributed among C-1, C-3 and C-5 respectively (Birch *et al.* 1958). [^{14}C]Fumigatin (3-hydroxy-4-methoxytoluquinone) was obtained from experiments with *A. fumigatus* by using [^{14}C]orsellinic acid as labelled substrate (Packter, 1966*a*). 6-Methyl[^{14}C]salicylic acid and [^{14}C]orsellinic acid were prepared biosynthetically from sodium [1-^{14}C]acetate by cultures of *P. griseofulvum* (Birch, Massy-Westropp & Moye, 1955) and *P. baarnense* (Mosbach, 1960) respectively. These acids are labelled equally in alternate C atoms, C-2, C-4, C-6 and C-8 (Birch *et al.* 1955; Birkinshaw & Gowland, 1962). ^{14}C-labelled methyl esters were synthesized as described above. Paper chromatograms of the purified products showed no trace of unchanged acid and gave one discrete spot in each case.

Solvents. Ether, light petroleum (b.p. 40–60°) and ethanol for spectroscopy were purified as described by Packter (1965).

RESULTS

Incorporation of sodium [2-^{14}C]*acetate into gliorosein.* The values for the specific activities of gliorosein, quinol, quinhydrone and quinone, at different time-intervals, are given in Table 1. The [^{14}C]acetate was rapidly incorporated into gliorosein and related metabolites (3·3% conversion) within 2 days of supplementation of the medium (group 1). The total radioactivity did not increase after this time although synthesis of gliorosein continued. The yield of mycelium in groups 2 and 4 was considerably less than that found in the other two groups. The apparent decrease in the total radioactivity determined for these groups was presumably related to variations in growth. The specific activity of gliorosein isolated from group 1 was lower than that of the quinol, which, in turn, was considerably lower than that of quinhydrone. A similar but less pronounced pattern was found in group 2. In the remaining groups, however, the specific activities of these metabolites was approximately the same as each other.

Production of gliorosein, quinol, quinhydrone and (in group 4) quinone followed a similar pattern to that described previously by Steward & Packter (1965).

Incorporation of sodium [2-^{14}C]*acetate into unsaponifiable lipid and ergosterol.* Sodium [2-^{14}C]-acetate was also rapidly incorporated into unsaponifiable lipid and ergosterol in the mycelium (2·8 and 3·1% for ergosterol in groups 1 and 2 respectively). The radioactivity reached a maximum after 11–13 days' growth (as was the case with gliorosein) but subsequently decreased (Table 2).

Conversion of [^{14}C]*orsellinic acid and methyl-*[^{14}C]*orsellinate into orcinol by growing cultures of* G. roseum. Both [^{14}C]orsellinic acid and its methyl ester were readily taken up by the mycelium of *G. roseum* and an approximately equivalent amount of orcinol was secreted into the medium within 2 days (Table 3). In all cases, the specific activity of

Table 1. *Incorporation of sodium* [2-^{14}C]*acetate into gliorosein, quinol, quinhydrone and quinone by* Gliocladium roseum

Sodium [2-^{14}C]acetate (12·5 μc; specific activity 1·3 μc/μmole) was added to each of eight Roux bottles, each containing 200 ml. of Raulin-Thom medium, after 9 days' growth. Groups consisting of two Roux bottles were subsequently removed at intervals. Metabolites were isolated from the medium and purified as described in the text. Results are expressed as totals/400 ml. of medium. The specific activity of the quinhydrone is given as counts/min./0·5 μmole; this corresponds to four ^{14}C atoms (as for gliorosein, quinol and quinone). N.D., Not detected (less than 1 mg.).

Group	Total incubation time (days)	Final pH of medium	Wt. of mycelium (g.)	Ether extract (mg.)	Gliorosein		Quinol		Quinhydrone		Quinone		Total radioactivity in gliorosein, quinol, quinhydrone and quinone (counts/min./g. of mycelium)
					Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./0·5 μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	
1	11	5·4	15·0	195	138	412	8	457	23	565	N.D.	—	24700
2	13	4·1	12·5	310	186	149	27	162	45	191	N.D.	—	16500
3	15	3·8	13·5	545	395	139	49	143	40	151	N.D.	—	25300
4	21	2·6	11·0	475	372	101	N.D.	—	N.D.	—	31	122	19000

the purified orcinol was somewhat lower than the theoretical value. Some residual methylorsellinate (3·1 mg.) was also found. Moreover, the lipid extract of the mycelium possessed significant amounts of radioactivity (10% of the initial dose). No radioactivity was present in gliorosein, quinol or quinhydrone of any group.

Washed mycelium of *G. roseum* decarboxylated orsellinic acid (10 mg.) almost quantitatively within 18 hr.

Testing of other ^{14}C*-labelled phenolic compounds for possible precursor activity of gliorosein.* Similar experiments were conducted with 6-methyl[^{14}C]-salicylic acid and its methyl ester. Growing cultures of *G. roseum* were incubated with 6-methyl-[^{14}C]salicylic acid for 5 days but gliorosein did not become labelled. However, over 90% of the acid was recovered from the medium with no change in its specific activity. The organism was permeable to the methyl ester of 6-methyl[^{14}C]-salicylic acid as only 5% of the unchanged ester plus 5% as the acid were recovered from the medium. No *m*-cresol was detected. Again, no radioactivity was present in gliorosein or the related metabolites.

[U-^{14}C]Tyrosine was effectively incorporated into gliorosein (0·8% conversion). Unsaponifiable lipid and ergosterol were isolated from the mycelium and were also radioactive. About 0·3% of the initial radioactivity was present in ergosterol.

Production of gliorosein by washed mycelium of G. roseum. Aurantiogliocladin and its quinol were readily taken up by the mycelium. The quinol was oxidized to some extent, however, under these conditions before it penetrated the cells. The results presented in Table 4 clearly indicate that the substrates were converted almost quantitatively into gliorosein within 18 hr., at different stages of growth. No quinol was detected in any group but trace amounts of quinhydrone and quinone (less than 1 mg.) were present in the medium of groups A1, A2 and A3. Small but significant amounts of quinone were isolated from groups B1, B2 and B3.

Conversion of [^{14}C]*aurantiogliocladin and related compounds into gliorosein.* [^{14}C]Aurantiogliocladin and certain substances structurally related to it were tested for possible precursor activity of gliorosein. The results are given in Table 5. The two quinols [(II) and (VI)] were not appreciably oxidized before they were taken up by the mycelium. Aurantiogliocladin, quinol (II), the monohydroxy quinone (V) and its quinol (VI) were effectively converted into gliorosein (30, 57, 25 and 43% respectively). In the last-named two groups, the crystalline gliorosein was further purified by conversion into aurantiogliocladin and subsequent chromatography on thin-layer plates of Kieselgel

Table 2. *Incorporation of sodium* [2-^{14}C]*acetate into unsaponifiable lipid and ergosterol by* Gliocladium roseum

Experimental details are given in Table 1. Unsaponifiable lipid and ergosterol were isolated and purified as described by Packter (1965).

	Unsaponifiable lipid		Ergosterol		
Group	Wt. (mg.)	$10^{-5}\times$ Total radioactivity (counts/min.)	Wt. (mg.)	$10^{-5}\times$ Total radioactivity (counts/min.)	Sp. activity (counts/min./ μmole)
1	120	11·4	16·4	3·10	7500
2	85	11·6	17·7	3·45	7720
3	98	6·4	16·2	2·28	5550
4	83	6·0	9·7	1·27	5240

Table 3. *Decarboxylation of orsellinic acid and methylorsellinate by* Gliocladium roseum

The test substrates were added to cultures of *G. roseum* after 8 days' growth. Each group consisted of two Roux bottles and all the results are expressed as totals/400 ml. of medium. Experimental details are given in the text.

					Recovered from medium			
					Orcinol		Gliorosein	
Test substrate	Wt. (mg.)	Sp. activity (counts/ min./ μmole)	Sp. activity (counts/ min./ μg.atom of ^{14}C)	Total incubation time (days)	Wt. (mg.)	Sp. activity (counts/ min./ μg.atom of ^{14}C)	Wt. (mg.)	Sp. activity (counts/ min./ μmole)
Orsellinic acid	12	4284	1071	10	8·1	990	18	0
Orsellinic acid	12	4284	1071	12	7·5	850	95	0
Methylorsellinate	20	2293	573	15	5·9	530	245	0

Table 4. *Conversion of aurantiogliocladin* (IV) *and* 3,4-*dimethoxy*-6-*methyltoluquinol* (II) *into gliorosein* (I) *by washed mycelium of* Gliocladium roseum

G. roseum was incubated with Raulin-Thom medium. Groups of three Roux bottles were taken after 10 and 14 days' growth (groups A and B respectively) and the medium was removed by pipette. The mycelium was washed with sterile water and 75 ml. of autoclaved medium was then added beneath the surface. Quinone (10 mg.) and quinol (10 mg.) were added to one bottle in each group, the third acting as control. The media were extracted 18 hr. later. Experimental details for the isolation and estimation of metabolites are described in the text and by Steward & Packter (1965). T, trace (less than 1 mg.).

						Gliorosein	
Group	Supplement	Time of addition (days)	Wt. of mycelium (g.)	Ether extract (mg.)	(mg.)	(mg./g. of mycelium)	Quinone (mg.)
A1	None	10	5·0	5·0	2·9	0·6	T
2	Quinone	10	5·0	15·3	11·1	2·2	T
3	Quinol	10	5·2	16·5	12·2	2·4	T
B1	None	14	7·5	35·5	23·2	3·1	3·5
2	Quinone	14	7·5	42·0	34·5	4·6	2·1
3	Quinol	14	6·0	27·5	22·1	3·7	2·5

(see the Methods section). Moreover, the data given in Table 4 indicated that virtually all quinone and quinol was converted into gliorosein; any quinol, quinhydrone or quinone subsequently found in the ether-extractable material must have come from gliorosein in the medium. Thus a more accurate measure of conversion into gliorosein and related metabolites would be 57, 68, 30 and 49% respec-

Table 5. *Testing of possible precursor activity of toluquinol derivatives on gliorosein biosynthesis*

^{14}C-labelled substrates were obtained and purified as described in the text. Each group consisted of two Roux bottles and the test substrates were added after 10 days' growth. Incubation proceeded for a further 7 days. Results are expressed as totals/400 ml. of medium. The groups containing aurantiogliocladin and fumigatin were incubated at 28°. T, trace (less than 1 mg.).

	Gliorosein		Quinol		Quinhydrone		Quinone		
Test substrate	Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./0·5 μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	Incorporation into gliorosein and related products (%)
Aurantiogliocladin (IV)	20	805	—	—	50	66·5	10·0	65	57
Quinol (II)	20	228	—	—	41	11·2	5·5	11	68
3-Hydroxy-4-methoxy-6-methyltoluquinone (V)	20	746	T	—	34·8	14·6	—	—	30
3-Hydroxy-4-methoxy-6-methyltoluquinol (VI)	12	736	T	—	32·4	15·8	—	—	49
Fumigatin	25	185	5·0	—	38	—	—	—	0
	Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	Wt. (mg.)	Sp. activity (counts/min./0·5 μmole)	Wt. (mg.)	Sp. activity (counts/min./μmole)	Incorporation into gliorosein and related products (%)
	Gliorosein		Quinol		Quinhydrone		Quinone		

tively (Table 5). In both groups of compounds the incorporation of the quinones was somewhat lower than that determined for the quinols. Fumigatin was not incorporated into gliorosein. [^{14}C]Aurantiogliocladin was also incubated with a solution of gliorosein in autoclaved medium for 5 days. No radioactivity was subsequently found in gliorosein.

DISCUSSION

Although gliorosein continued to be synthesized during the tenth to fifteenth days of growth, the amount of radioactivity in this substance and related products remained constant (within the limits of biological variation) (Table 1). The apparent discrepancies in the total radioactivity between the groups are probably caused by irregularities in growth. Production of mycelium in groups 2 and 4 was considerably less than that for the other two groups. Presumably, synthesis of gliorosein did not commence until a later period, at which time the [^{14}C]acetyl-CoA had been diluted by endogenously produced material. These results indicate that all the [^{14}C]acetyl-CoA has been utilized within 2 days of supplementation of the medium. The labelled C atom is eventually lost as $^{14}CO_2$ during reactions of the tricarboxylic acid cycle, after randomization of the label at the level of succinate.

The specific activities determined always increased in the order: gliorosein, quinol, quinhydrone and quinone. Sodium [2-^{14}C]acetate was added to the medium 9 days after inoculation. At this time, the organism just commences to secrete gliorosein and production of quinol and quinhydrone is minimal (Steward & Packter, 1965; see also Table 4). Thus the gliorosein that is synthesized initially from [^{14}C]acetyl-CoA would be relatively highly radioactive. Quinol, formed by isomerization, and its subsequent oxidation products would have very similar specific activities. Gliorosein produced at a later stage of growth from unlabelled acetyl-CoA would therefore give rise to material with a lower specific activity. The slow rate of conversion of gliorosein into quinol at low pH values enables the differences in specific activity of the various metabolites to be noted (groups 1 and 2 in Table 1).

The incorporation of radioactivity from sodium [2-^{14}C]acetate into gliorosein and ergosterol follows a similar pattern (Tables 1 and 2). The decrease in specific activity of the ergosterol in groups 3 and 4 (15 and 21 days' growth respectively) may be accounted for by the catabolism of the radioactive material and subsequent synthesis of non-labelled ergosterol. Thus gliorosein is synthesized from acetyl-CoA and secreted into the medium in large amounts while the organism is still continuing to

form cell constituents. The production of fumigatol by *A. fumigatus* also occurs at this stage of growth (Packter & Glover, 1962; Packter, 1965).

[U-^{14}C]Tyrosine was incorporated into gliorosein (0·8% conversion). However, an appreciable amount of radioactivity was also present in the unsaponifiable lipids; ergosterol contained 0·3% of the initial radioactivity. These results are consistent with incorporation of tyrosine after initial breakdown into homogentisic acid and subsequent ring-opening to form acetoacetate (and hence acetyl-CoA) and fumarate, at a time when synthesis of gliorosein would be maximal. This pathway of tyrosine and phenylalanine metabolism has been established in liver preparations (Ravdin & Crandall, 1951; Hager, Gregerman & Knox, 1957; Knox & Edwards, 1955) and in bacteria (Suda & Takeda, 1950; Dagley, Fewster & Happold, 1953).

Orsellinic acid is not incorporated into gliorosein but is converted quantitatively into orcinol by *G. roseum*. Presumably therefore orcinol is also inactive as a precursor. These results are in marked contrast with those determined in *A. fumigatus*. Orsellinic acid and orcinol are both readily converted into fumigatol (90 and 60% respectively) within 2 days of supplementation of the medium. Orsellinic acid is also incorporated into 3,4-dihydroxytoluquinol in this organism (Packter, 1966*a*).

A preliminary report by Birch (1961) has indicated that 6-methyl[^{14}C]salicylic acid is incorporated into aurantiogliocladin. No experimental details were given. We have shown that 6-methylsalicylic acid and its methyl ester are not converted into gliorosein or any of the related products. The acid can be recovered almost quantitatively from the medium after 5 days of incubation with growing cultures of *G. roseum*. These results suggest that the aurantiogliocladin isolated by Birch (1961) was probably contaminated with some unchanged 6-methyl[^{14}C]salicylic acid.

The methyl esters of 6-methylsalicylic acid and orsellinic acid are both metabolized by *G. roseum*. The former is partially hydrolysed by an esterase in the mycelium into the parent acid while methylorsellinate is converted into orcinol after initial hydrolysis to orsellinic acid.

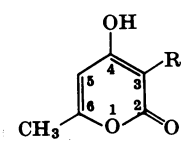
Birch *et al.* (1958) and Bentley & Lavate (1965) have established by degradative studies that aurantiogliocladin is synthesized via head-to-tail condensations of C_2 units derived from acetate. The order of incorporation of [^{14}C]acetate determined by Birch *et al.* (1958) and by the authors (Table 1; see also Steward & Packter, 1965) appears to confirm that this direct route of synthesis occurs. Accordingly, it would seem that the 'acetate-plus-malonate' pathway is operative despite the apparent lack of precursor activity of 6-methylsalicylic acid and orsellinic acid. Another phenolic acid may therefore be involved in the biosynthesis of 3,4-dimethoxy-6-methyltoluquinol and gliorosein.

These results may readily be explained by suggesting that 5-methylorsellinic acid is the first aromatic compound formed in *G. roseum*; that is, the extra methyl group is added at the β-polyketide level and not after cyclization. The enolizable CH_2 groups in the aliphatic C_8 polyketide would display the appropriate reactivity for this electrophilic attack and this might not be present in the stabilized cyclic (aromatic) form (Birch, 1957; Bu'Lock, 1965). Indeed, experimental verification of this hypothesis has been obtained by McCormick (1965) during mutation studies of organisms which produce tetracyclines. He has shown that the methylation step occurs before ring closure; one particular mutant synthesizes 6-nor-pretetramide (the parent naphthacene) and hence tetracyclines lacking the methyl group at the C-6 position (McCormick, Sjölander, Hirsch, Jensen & Doerschuk, 1957).

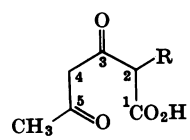
The isolation of the lactones, 4-hydroxy-6-methyl-2-pyrone (triacetic acid lactone; IX) from the medium of *P. patulum* (Harris, Harris & Light, 1966) and 4-hydroxy-3,6-dimethyl-2-pyrone (X), from cultures of *P. stipitatum* (Brenneisen, Acker & Tanenbaum, 1964) also supports the suggestion that methylation may occur at the β-polyketide level. These substances are probably derived from 3,5-dioxocaproic acid (XI) and 2-methyl-3,5-dioxocaproic acid (XII) respectively, by dehydration.

It must be remembered, however, that *C*-methylation of the aromatic ring may occur in other types of compound derived from shikimic acid or related substances, e.g. the flavonoids (Geissman, 1963) and novobiocin in *Streptomyces niveus* (Birch, Cameron, Holloway & Rickards, 1960).

5-Methylorsellinic acid and 5-methylorcinol have not been detected in the medium of *G. roseum* but they have been found in the medium of mutant strains of *A. terreus* that normally synthesize geodoxin (Curtis *et al.* 1964) and sulochrin (Curtis, Harries, Hassall, Levi & Phillips, 1966). Curtis *et al.* (1964) have demonstrated that the extra methyl group is derived from methionine, pre-



(IX) R = H
(X) R = CH_3



(XI) R = H
(XII) R = CH_3

sumably via the intermediate formation of *S*-adenosylmethionine (Greenberg, 1963). Birch *et al.* (1958) have established that one of the *C*-methyl groups and the two *O*-methyl groups of aurantiogliocladin may be synthesized from formate, probably after intermediate production of methionine.

The results outlined in Tables 4 and 5 demonstrate that 3-hydroxy-4-methoxy-6-methyltoluquinol (VI), 3,4-dimethoxy-6-methyltoluquinol (II) and their corresponding quinones are readily converted into gliorosein. Presumably the toluquinone derivatives are incorporated after initial reduction to the toluquinol form. These findings confirm that both *C*-methyl groups are present at an early stage in the biosynthetic pathway. Moreover, the lack of incorporation of fumigatin (Table 5) supports this view. Thus the toluquinols (VI) and (II) appear to be the immediate precursors of gliorosein; this indicates that the final stage in its formation is the isomerization of the toluquinol (II). Accordingly, we propose the following sequence of reactions for its biosynthesis in *G. roseum*: acetyl-CoA + 3 malonyl-CoA + *S*-adenosylmethionine → 5-methylorsellinic acid → 3-hydroxy-4-methoxy-6-methyltoluquinol → 3,4-dimethoxy-6-methyltoluquinol → gliorosein. It is evident that a decarboxylation must occur at some stage. Pettersson (1965) has partially purified a decarboxylase from cell-free extracts of *G. roseum* which converts orsellinic acid and 5-methylorsellinic acid into orcinol and 5-methylorcinol (2,4-dihydroxy-5,6-dimethylbenzene) respectively. 6-Methylsalicylic acid is not affected by this enzyme.

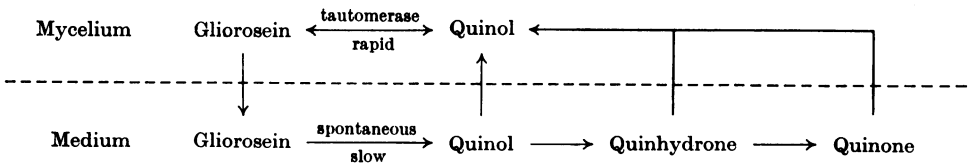
The aromatic ring of ubiquinone, which is closely related structurally to aurantiogliocladin (IV), is not synthesized from acetate. Parson & Rudney (1964, 1965) and Burton & Glover (1965) have established that *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid are precursors of ubiquinone in micro-organisms and animal tissues. Olson *et al.* (1963) have also shown that the methyl group attached to C-1 of the toluquinone ring is derived from formate, and Parson & Rudney (1965) confirmed that [*methyl*-^{14}C]methionine is highly incorporated into this group. Further, it has been established that the fungal metabolite 4-methoxytoluquinol (secreted into the medium by *Lentinus degener*) is not formed via the 'acetate-plus-malonate' pathway. The results show that acetate and 6-methylsalicylic acid are not incorporated (less than 0·05%) into 4-methoxytoluquinol (Packter, 1966*b*). Preliminary experiments also indicate that tyrosine or a substance more closely related to shikimic acid is involved in its biosynthesis (N. M. Packter, unpublished work). Thus the mere inspection of the formulae of these phenolic or quinonoid compounds may prove an unreliable guide to their biosynthetic origin.

Gliorosein (I) is optically active (dextrorotatory) and therefore possesses a *trans*-configuration around C-1 and C-6 (see the Materials section). Moreover, the fact that gliorosein exists as one optical isomer denotes conclusively that it has been produced enzymically. Formation by spontaneous rearrangement of the quinol (II) by keto–enol tautomerism (even if this could occur within the cell) would result in a racemic mixture. In general, the phenolic structure is more stable than the corresponding ketone form. However, if the energy difference between the keto and enol forms is small (e.g. in polyhydric phenols), the ketonic isomer may also be stable especially in the solid state and in non-polar solvents (Thomson, 1964). Thus gliorosein is stable in light petroleum and dry ether but slowly tautomerizes into the quinol (enol) form after it has been secreted into the medium. This conversion is catalysed by OH^- ions (Vischer, 1953).

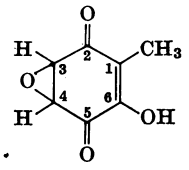
Some quinol, quinhydrone or quinone is always present in the culture medium despite the fact that these substances are readily converted into gliorosein by *G. roseum*. Presumably therefore an equilibrium exists between them as shown in Scheme 1.

Experiments are now in progress in an attempt to isolate the tautomerase from cell-free extracts of *G. roseum*.

This is the first occasion that a keto–enol tautomerase has been shown to be involved in the metabolism of a 'secondary metabolite'. However, another instance of a naturally occurring ene-dione has been reported. Kaplan, Hooper & Heinemann (1954) isolated terreic acid from a strain of *A. terreus*; Sheehan, Lawson & Gaul (1958) later



Scheme 1.



(XIII)

characterized it as 3,4-dihydro-3,4-epoxy-6-hydroxytoluquinone (XIII). No experimental evidence is available regarding its biosynthesis but the final stage in its formation could be tautomerization of the corresponding quinol. Alternatively, the epoxide oxygen may be inserted across C-3 and C-4 of 6-hydroxytoluquinone, giving rise to an enedione structure. A similar tautomerase enzyme (EC 5.3.2.1), which catalyses the conversion of phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid into their respective enol forms, has been purified from a variety of animal tissues and microorganisms (Knox & Pitt, 1957). Preliminary details concerning the isolation of an enzyme from ox heart which catalyses the enolization of oxaloacetate have also been described (Annet & Kosicki, 1965).

M. W. S. acknowledges the support of an S.R.C. Research Studentship. We thank Mr E. Bellion for assistance in interpreting the n.m.r. spectrum.

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