ISOLATION OF ORSELLINIC ACID SYNTHASE

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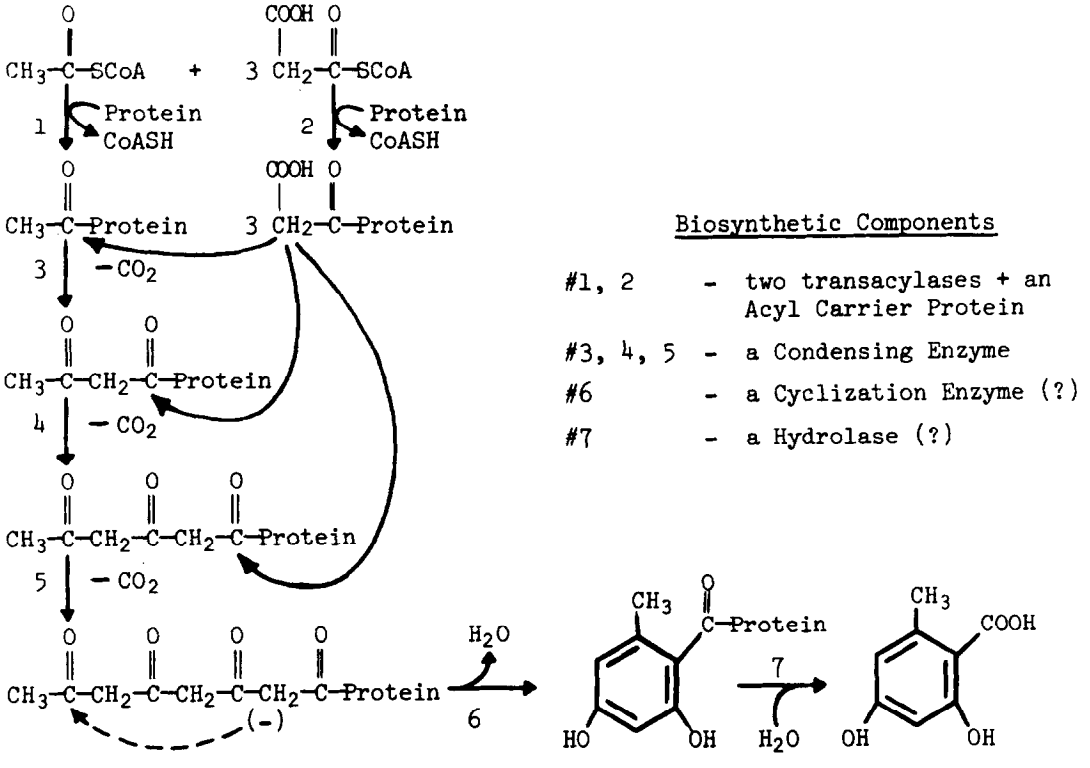
The phenol, orsellinic acid, was one of the first "secondary metabolites" shown to be derived from a linear condensation of acetate units according to the "acetate hypothesis" (Birch, 1967). Of all the acetate derived phenols, orsellinic acid is biogenetically the simplest. Only three

FIGURE 1

ORSELLINIC ACID BIOSYNTHESIS



condensations are required to yield the C_8 polyacetate chain which cyclizes to orsellinic acid without further modification. This is illustrated in the hypothetical scheme, Figure 1, which is based on current concepts of fatty acid biosynthesis. In this communication the in vitro biosynthesis of orsellinic acid from acetyl and malonyl-CoA is reported for the first time using cell free fungal extracts as a source of orsellinic acid synthase. This enzyme is shown to be associated with a particulate fraction by differential centrifugation which results in a greater than five fold purification over the first crude extract.

Organism: An orsellinic acid producing strain of Penicillium madriti, I.M.I. strain 96,506 (Birkinshaw and Gowlland, 1962) was obtained from the Commonwealth Mycological Institute, Surrey, England. In order to minimize strain variation, aliquots of a detergent (4 ppm Aerosol OT) spore suspension of P. madriti, prepared from Czapek-Dox agar slants, were lyophilized on filter paper strips, sealed under vacuum and stored at approximately 5° C.

Culture Conditions and Preparation of Crude Cell-free Extract: Typical changes in dry weight, glucose concentration and orsellinic acid concentration for submerged cultures of P. madriti are illustrated in Figure 2 with culture conditions and assays detailed in the accompanying legend. At 42 hours the bulk of a 6-liter fermentor culture, grown as described in the legend of Figure 2, was harvested by filtration, the cells were washed twice with distilled water and dried by packing between filter papers. The cells were then slurried with distilled water and lyophilized overnight. A suspension of 3 g of lyophilized cells[1] in 80 ml of cold 0.2 M TES[2] buffer (pH 7.5) was sonicated for 20 minutes at the top setting of a Branson model S-110 Sonifier. The supernatant from a 20 minute centrifugation (48,000 x g)

[1]In the cold, lyophilized cells retain their activity for 5-10 days, while fresh cells are devoid of activity within 3-4 hours.

[2]N-tris(Hydroxymethyl)methyl-2-amino-ethane sulfonic acid from Calbiochem.

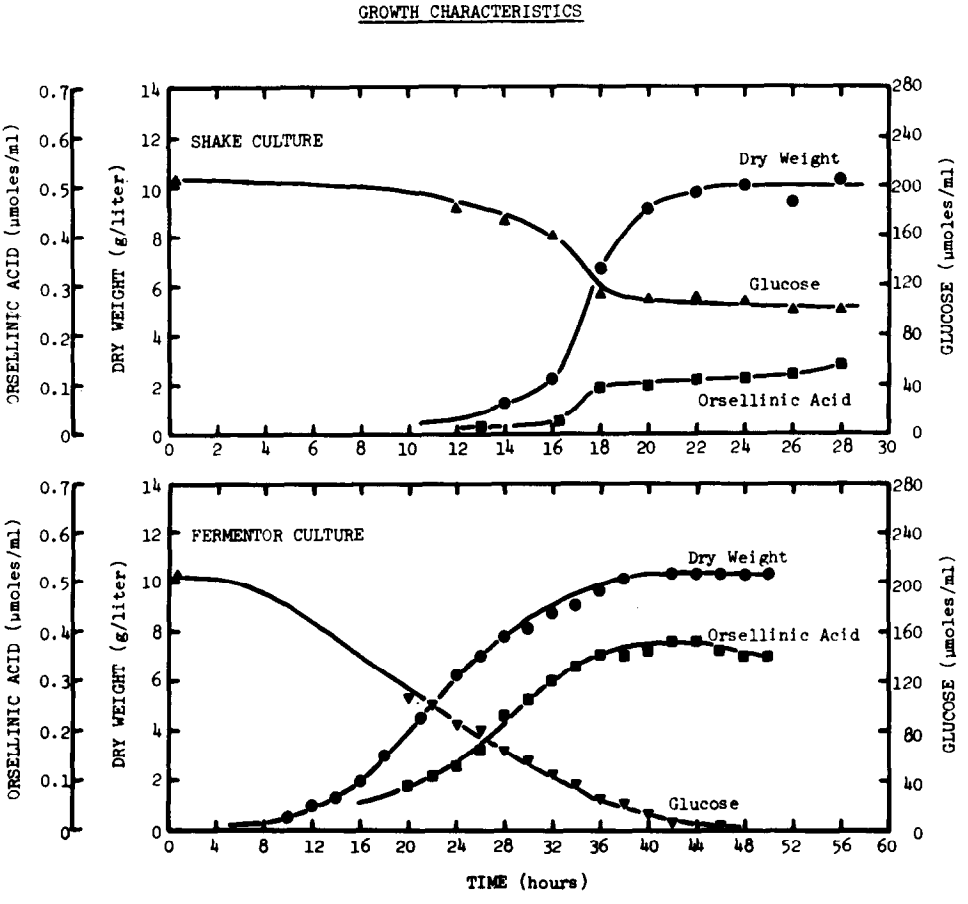


Fig. 2. Lyophilized P. madriti samples were regenerated by placing the filter paper strip upside down on a Czapek-Dox agar slant in an 8 dram vial, followed by incubation at 28° C for approximately 5 days. From each regenerated agar vial slant a further 6-10 vial slants were prepared by a sterile cotton swab spore transfer, followed by the usual 28° C incubation. A 3 ml detergent spore suspension from one such vial slant was then used to inoculate a 300 ml prescription bottle slant of Malt Extract Agar. When a uniform green conidial coat had formed after 5-7 days incubation at 28° C, a 25 ml detergent spore suspension prepared from the bottle slant was transferred to a 2-liter erlenmeyer containing 200 mls of the following modified Czapek-Dox liquid medium: $NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; $ZnSO_4 \cdot 7H_2O$, 0.0003 g; Glucose, 40.0 g; Yeast Extract (Difco), 3.0 g, and double distilled water, 1.0 liter. This shake culture was grown at 28° C on a reciprocal shaker (3 cm stroke, 150 displacements/minute) and at 18 hours, a point which corresponds to approximately the mid-point of the linear phase of growth, was used to inoculate a 14-liter New Brunswick Microferm fermentor jar containing 6-liters of the modified Czapek-Dox liquid medium described above. This culture was then grown at 28° C with an aeration rate of 4 liters/minute and a stirring rate of 500 rpm. The pH was maintained at 6.0 ± 0.1 by the automatic addition of N NaOH and N HCl using a Beckman combination electrode in conjunction with a Radiometer automatic titrator. Foaming was controlled by the automatic addition of small aliquots of Antifoam C (Dow Corning) as necessary. In

handling all lyophilized, agar and liquid cultures aseptic techniques were used throughout and all transfers were made in a sterile transfer hood. Fermentor cultures in particular were checked for bacterial contamination.

With care being taken to prevent any significant change in the total volume of the cultures being sampled, the following culture assays were used:

1. Dry Weight - Samples were suction filtered through a pre-weighed filter paper and the mycelial mats were washed twice with distilled water and dried overnight in an oven at 110° C, cooled to room temperature and weighed.
2. Glucose - The filtered medium was assayed for glucose using Worthington Biochemical's Glucostat assay.
3. Orsellinic Acid - To 3 mls of $FeCl_3$ reagent (0.04 M $FeCl_3 \cdot 6H_2O$ in 0.02 M HCl), 3 mls of medium (diluted if necessary) are added, mixed and read in 30 minutes at 540 mμ against a reagent blank. The standard plot is linear up to 1.75 μ moles of orsellinic acid.

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was filtered through glass wool to yield a "crude extract".[3]

Enzyme Assay and Cofactor Requirements: In the standard assay, as detailed in the legend of Figure 3, the incorporation of radioactivity into orsellinic acid from labeled malonyl-coenzyme A was on the average 2500 dpm. Because of the general susceptibility of fungi to strain variation, we have found that cultures of P. madriti which are actively producing orsellinic acid will yield the average activity indicated here, but that occasionally cultures are obtained which lack the ability to produce orsellinic acid and are therefore devoid of enzymatic activity. As illustrated in Figure 3, if NADPH was added to the crude enzyme extract in addition to the usual acetyl and malonyl-coenzyme A, both orsellinic acid and fatty acids were synthesized. Omission of NADPH resulted in the loss of the fatty acid peak and retention of the other two peaks. Omission of both NADPH and acetyl-CoA resulted in the loss of both fatty acid and orsellinic acid peaks leaving only the peak at the origin. Controls in which the crude enzyme extract was boiled or replaced with buffer yielded only the peak at the origin. To further support the chromatographic evidence that radiolabeled orsellinic acid was being synthesized, silica gel corresponding to the orsellinic acid peak from a number

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[3]At 0-4° C this extract retains approximately 80% of the original activity after 6 hours, while in 24 hours it is devoid of activity.

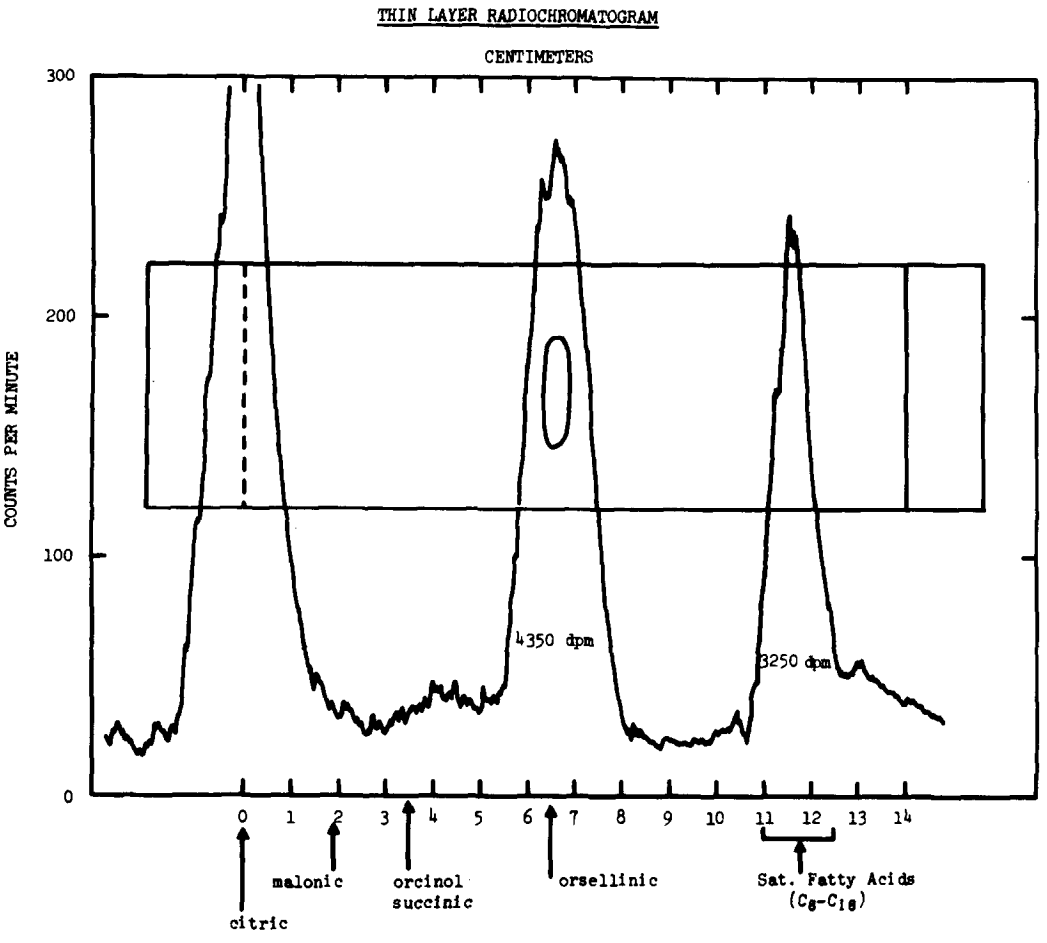

Fig. 3. In the standard enzyme assay, the crude extract at pH 7.5 (0.1 ml) was incubated in the following reaction mixture: 2-^{14}C-Malonyl-CoA,[1] 6.84 x 10^4 dpm in 14.8 mμ moles; acetyl-CoA,[2] 10 mμ moles; total volume 0.13 ml. After a 10 minute incubation at 21° C the reaction was terminated by addition of 1 ml of N H_2SO_4 and 0.5 ml of methanol, and extracted with three 4 ml portions of ether. Synthetic orsellinic acid[3] (0.25 μ moles) was added and the ether extract was evaporated to dryness and redissolved in a minimum of acetone. The entire extract was spotted on a 5 x 20 cm neutral silica gel plate and developed for 14 cm in chloroform-acetic acid (90:10, V/V). The plate was then scanned in a Packard Radiochromatoscanner.[4] The silica gel encompassed by the radioactive peak which corresponded to the average Rf of orsellinic acid (0.47) was scraped into a vial containing 10 ml of a scintillator solution[5] and counted for 10 minutes in a Chicago nuclear scintillation counter. The incorporation of radioactivity into the orsellinic acid peak ranged from 500 dpm to greater than 10,000 dpm per assay. In the particular assay illustrated by this radiochromatogram NADPH (40 mμ moles) was added to the crude enzyme extract in addition to the usual acetyl and malonyl-coenzyme A.

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[1]Prepared by the method of Trams and Brady (1960) from 2-^{14}C-Malonic acid.

[2]Prepared by the method of Simon and Shemin (1953).

[3]Prepared according to Gaucher and Shepherd (in press).

[4]Apart from scanning for radioactivity the orsellinic acid spot is readily detected by the coloration of the spot which occurs upon standing or by spraying the plate with diazotized p-nitroaniline.

[5]According to Snyder (1964).

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of thin layer plates was extracted with ether. Synthetic orsellinic acid (200 mg) was added to the ether extract and the extract was evaporated to dryness. The resulting solid was recrystallized to constant specific activity from acetic acid-water. The specific activity of the first crystals (113 mg) was 266 dpm per 10 mg, while subsequent recrystallizations yielded 264 dpm per 10 mg (47 mg) and 277 dpm per 10 mg (13 mg).

Differential Centrifugation: Orsellinic acid synthase activity in the crude enzyme extract (48,000 x g supernatant) was found to be retained in the supernatant after centrifugation at 90,000 x g for 30 minutes. Upon subsequent centrifugation at 210,000 x g for 60 minutes this 90,000 x g supernatant yielded a pellet which contained the enzymatic activity. Using the method of Trautman (1963) it was calculated that this pellet was composed of components with S-rates greater than 60 S. As indicated in Table 1, this pellet represented a five-fold purification of orsellinic acid synthase.

TABLE 1

PRELIMINARY PURIFICATION

FRACTION	TOTAL PROTEIN (mg)	INCORPORATION (dpm/assay)	SPECIFIC ACTIVITY (dpm/assay/ mg protein)	FOLD PURIFICATION
SUPERNATANT (90,000 xg; 30 min.)	192	1040	4333	1
PELLET (210,000 xg; 60 min.)	54	1530	22,500	5

Discussion: The whole cell incorporation of acetate and malonate into secondary metabolites such as orsellinic acid (Gatenbeck and Mosbach, 1959) has uniformly supported the close similarity between the biosynthesis of these metabolites and the biosynthesis of the fatty acids. Thus as expected only acetyl-CoA and malonyl-CoA are required for the cell-free synthesis of orsellinic acid. Identical requirements have been reported for the cell-free synthesis of the C_{14} metabolite alternariol (Gatenbeck and Hermodsson, 1965) while the cell-free synthesis of the C_8 phenol 6-methylsalicylic acid has the additional requirement of NADPH (Lynen and Tada, 1961; Light, 1967). The postulated role of an enzyme bound β-polyketoacyl intermediate in these systems is supported by the observations that either triacetic acid (3,5-diketohexanoic acid) or its pyrone derivative, triacetic acid lactone is synthesized by the pigeon liver and yeast fatty acid synthetases in the absence of NADPH (Brodie, et al, 1964; Lynen, 1967) and by the E. Coli fatty acid synthetase in the presence of NADPH and sulfhydryl compounds (Brock and Bloch, 1966). The nonenzymatic conversion of appropriate pyrones to secondary metabolites such as orsellinic acid (Money et al, 1967) further supports this hypothesis. Thus the scheme in Figure 1 indicates a growing, enzyme bound, polyketoacyl intermediate whose synthesis is mediated by four components one of which is an acyl carrier protein (Vagelos, et al, 1965). Not indicated here is the potentially key role of metal ion chelation in stabilizing this polyketo intermediate and in specifying its subsequent mode of cyclization (Bu'Lock, 1967; Douglas and Money, 1967). Also not indicated is the degree of aggregation of the protein components concerned and hence the applicability of the term "multi-enzyme complex" (Reed and Cox, 1966). In clarification of this latter point, a recent determination of the molecular weight of 6-methylsalicyclic acid synthetase (Light and Hager, in press) suggests that at least some of these systems may be multi-enzyme complexes. In summary it is obvious that further work with purified enzymes will be necessary to produce the desired transition from hypothesis to fact in this area.

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