SUMMARY

1. The distribution of [^3H]valine in fibrinogen synthesized by the rabbit in a short time-interval *in vivo* was investigated. The valine located in peptide A was found to have a higher specific activity than that from the remainder of the molecule.

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Studies in the Biochemistry of Micro-organisms

110. PRODUCTION AND BIOSYNTHESIS OF ORSELLINIC ACID BY *PENICILLIUM MADRITI* G. SMITH*

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A study of the metabolism of *Penicillium madriti* G. Smith led to the isolation from the culture filtrate of two metabolic products, penicillic acid and orsellinic acid. Penicillic acid, a fairly common mould metabolite, was first isolated from *Penicillium puberulum* Bainier by Alsberg & Black (1913). Orsellinic acid had not previously been recognized as a fungal product, although Aghoramurthy & Seshadri (1954) had postulated it as an intermediate in mould metabolism. In view of the work of Birch, Massy-Westropp & Moye (1955) on 6-methylsalicylic acid, to which orsellinic acid is structurally closely related, acetate coupling seemed the most obvious pathway for biosynthesis, particularly since orsellinic acid, $\text{C}_8\text{H}_8\text{O}_4$, can

theoretically be derived from four acetate units without oxidation or reduction. We have now verified this hypothesis and investigated the possibility of a C_4 intermediate such as acetoacetic acid in the metabolic pathway between acetate and orsellinic acid. A fuller account of this work is given by Gowlland (1961).

While this investigation was in progress, Mosbach (1959) reported the isolation of orsellinic acid from the culture filtrates of *Chaetomium cochliodes* Palt. and showed that when supplied with $\text{CH}_3\cdot^{14}\text{CO}\cdot^{18}\text{ONa}$ the fungus produced orsellinic acid in which the labelling of both carbon and oxygen atoms conformed to the requirements for a direct condensation of activated acetate units to orsellinic acid (Gatenbeck & Mosbach, 1959). Later work by Mosbach (1960) has demonstrated

* Part 109: Birkinshaw & Gourlay (1961).

the sequence acetate $\rightarrow$ orsellinic acid $\rightarrow$ penicillic acid in *Penicillium barnense*. Bentley & Keil (1961), studying the biosynthesis of penicillic acid by *Penicillium cyclopium* and assuming that the same sequence operates, have indicated that orsellinic acid is produced by condensation of one molecule of acetyl-CoA and three of malonyl-CoA. Bu'Lock & Smalley (1961) have shown that the biosynthesis of 6-methylsalicylic acid by *Penicillium urticae* follows a similar course.

In the present study the biosynthetic pathway to orsellinic acid was investigated by incorporation of precursors labelled with ^{14}C and subsequent determination of the distribution of the radioactivity of the metabolite by degradative methods. Sodium $[1-^{14}\text{C}]$ acetate was added to the static culture on the fourth day and the culture filtrate was examined on the ninth day, since this period coincided with the most rapid production of orsellinic acid (see Table 1). Extraction with ether and separation of orsellinic acid and penicillic acid by crystallization gave samples of high activity.

The radioactive orsellinic acid was degraded according to the plan shown in Scheme 1. The radioactivities of the various fragments were compared with the theoretical values based on the incorporation of four acetate units, placed head-to-tail, into orsellinic acid. The results (Table 2) were in accordance with the acetate hypothesis.

The bromopicrin obtained from the degradation of trinitro-orscinol, when oxidized to carbon dioxide, gave only 50% of the theoretical yield. This may be due to incomplete conversion of trinitro-orscinol into bromopicrin. However, since yields of 85% of the theoretical have been obtained in this reaction (Thomas, 1961) it is clear that all the carbon atoms bearing nitro groups contribute to the yield of bromopicrin obtained. It may therefore be inferred that, as the bromopicrin is inactive, C-2, C-4 and C-6 are all inactive.

The possibility that orsellinic acid could arise by condensation of two C_4 units was examined by incorporation experiments with sodium $[1-^{14}\text{C}]$ -butyrate. In view of the pathway of fatty acid

oxidation by way of butyryl-CoA to acetoacetyl-CoA and acetyl-CoA, the mould should be able to convert sodium [$1-^{14}\text{C}$]butyrate into acetoacetyl-CoA for use in the formation of orsellinic acid. If orsellinic acid arises by condensation of two such acetoacetate units the carboxyl group should be labelled and its activity should be half the total activity of the molecule.

Labelled orsellinic acid, biosynthetically derived from sodium [$1-^{14}\text{C}$]butyrate, was subjected to acid hydrolysis to determine the proportion of activity in the carboxyl carbon atom. Table 4 shows that one-quarter of the total activity of the orsellinic acid was present in the carboxyl group. This is consistent with cleavage of the C_4 unit to two C_2 units before incorporation, but not with the utilization of the intact C_4 unit. In order to suppress the cleavage of acetoacetate, iodoacetamide, shown by Ferguson & Rudney (1959) to be a specific inhibitor of acetoacetyl thiolase, was then tried at a concentration of 0.5 mM. The results (Table 4) again showed that only one-quarter of the total activity of the orsellinic acid was present in the carboxyl group, as in the absence of iodoacetamide. There was thus no evidence of any utilization of acetoacetate as precursor of orsellinic acid.

The incorporation of labelled orsellinic acid into penicillic acid was examined by feeding to the mould (a) orsellinic acid labelled on C-1, (b) orsellinic acid labelled on C-1, C-3, C-5 and C-7, and assaying the penicillic acid produced in each case. In (a) the penicillic acid was inactive; in (b) it was strongly labelled, although the activity was less than that of the original orsellinic acid (Table 5). This is consistent with the direct conversion by *P. madriti* of orsellinic acid into penicillic acid with loss of the carboxyl carbon, as occurs with other fungi.

EXPERIMENTAL

All melting points are uncorrected.

Cultures and medium. In a preliminary survey, two strains of *Penicillium madriti* G. Smith were examined. These had the L.S.H.T.M. catalogue numbers BB 389 (type strain) and BB 389A (Smith, 1961). Both were isolated by G. Agosti in this Department from garden soil from Madrid. On Czapek-Dox medium at first each culture produced both orsellinic acid and penicillic acid, but on continued subcultivation strain BB 389 lost the power to accumulate orsellinic acid and produced only penicillic acid, whereas BB 389A retained this ability. In all subsequent work, therefore, only the strain BB 389A was used. The mould was grown as a surface culture on the usual Czapek-Dox medium containing 5% (w/v) of glucose. The medium was distributed in 1 l. flasks (350 ml./flask) which were sterilized by steaming on three successive days, inoculated, and incubated at 24°.

Growth characteristics. The mycelium of strain BB 389A covered the surface of the Czapek-Dox medium 2 days after inoculation. At first colourless, the mycelium usually turned green (with some white patches) after 3 or 4 days, but in some cases remained white until harvested at 9 days. The reverse was pale mushroom in colour. The culture medium gradually deepened in colour to a dark orange-brown.

Isolation and identification of metabolites. The culture medium was separated from the mycelium by filtration and acidified to pH 2 with HCl. It was then extracted twice with ether (0.5 vol.). On concentration of the ether solution, brownish yellow crystalline material was deposited. Recrystallization of the product from water with addition of charcoal gave colourless needles, m.p. 186–189°, with effervescence after shrinking at 180°—metabolite I. After being chilled, the mother liquors deposited colourless diamond-shaped crystals of m.p. 70°—metabolite II.

Metabolite I, when sublimed, had m.p. 174–176° with effervescence after shrinking at 160°, but recrystallization from water restored the original m.p. The crystalline material contained 1 mol.prop. of water of crystallization, which it lost on drying at 100° but rapidly regained when left exposed to the air (Found on hydrated material: C, 51.9; H, 5.4. Calc. for $\text{C}_8\text{H}_8\text{O}_4 \cdot \text{H}_2\text{O}$: C, 51.6; H, 5.4%). Found on material dried at 100° immediately before analysis: C, 57.2; H, 4.9. Calc. for $\text{C}_8\text{H}_8\text{O}_4$: C, 57.15; H, 4.8%). The product gave a purple colour with aq. FeCl_3 and when subjected to hydrolysis by boiling N-HCl , 1 mol.prop. of CO_2 was evolved and the residue was shown to consist of orcinol. Metabolite I corresponded in all its properties with orsellinic acid and did not depress the m.p. of an authentic sample. Similarly, the methyl ester and diacetate of the natural product agreed with the corresponding derivatives of orsellinic acid and there was no m.p. depression on mixing the respective samples.

Metabolite II, when recrystallized from light petroleum, had m.p. 82–83°; it agreed in properties with, and did not depress the m.p. of, authentic penicillic acid.

Relative production of orsellinic acid and penicillic acid. Three flasks from a batch of 60 sown with *P. madriti* were harvested each day from the fourth day onwards. The culture filtrates were combined, assayed for residual glucose, acidified, and extracted with ether. The ether was removed and the residual solid was weighed. A known weight of the extract was then fractionally sublimed *in vacuo*. The penicillic acid sublimed at 80–90° and the orsellinic acid at 110–130°, leaving an orange gum. Although a trace of orsellinic acid sublimed at the lower range of temperature, a colorimetric check showed that the amount was negligible. The results (Table 1) indicate a peak production of orsellinic acid at 12 days, whereas penicillic acid increases throughout (up to 15 days).

Radioactive assay. All counts were made with a Panax scaler (type 100C) coupled with an end-window Geiger-Müller tube. A poly(methyl methacrylate) sample with ^{14}C activity of $1 \mu\text{C}/\text{mole}$, in a planchet of diameter 2.5 cm., was used as standard.

Sodium [$1-^{14}\text{C}$]acetate and sodium [$1-^{14}\text{C}$]butyrate were purchased from The Radiochemical Centre, Amersham, Bucks. Samples were counted at infinite thickness where possible, otherwise they were corrected by using a calibration curve. Nickel planchets of diameter 1.5 cm. were used for the samples. Activities given in the Tables are cor-

rected for background, except where otherwise stated, and are expressed as $\mu\text{C}/\text{mole}$ or $\mu\text{C}/\text{g}$.

Incorporation of sodium $[1-^{14}\text{C}]$ acetate. Four days after inoculation, two flasks received additions of sterile solutions containing $20\mu\text{C}$ (0.82 mg.) and $10\mu\text{C}$ (0.41 mg.) of sodium $[1-^{14}\text{C}]$ acetate respectively (batch I). Solutions containing $200\mu\text{C}$ (8.2 mg.) of sodium $[1-^{14}\text{C}]$ acetate were added to each of two further flasks (batch II). The orsellinic acid was isolated as described above and the activities of the samples were measured. The mycelium from one of the flasks of batch II, and the fat extracted from it by light petroleum, were also assayed. The orsellinic acid from batch I, together with 10 mg. of the highly active acid from batch II, was diluted with inactive material and the mixture was sublimed twice *in vacuo* at $110-120^\circ$. This gave 1 g. of orsellinic acid, m.p. $174-176^\circ$, with a constant activity of 671 counts/min. ($475\mu\text{C}/\text{mole}$). The extent of incorporation of ^{14}C into the fats and into orsellinic acid is recorded in Table 3.

Degradation of labelled orsellinic acid (Table 2)

Acid hydrolysis. Orsellinic acid (186 mg.) was heated with 10 ml. of N-HCl in a stream of N_2 . The CO_2 evolved was collected in $0.2\text{M-Ba}(\text{OH})_2$. The active BaCO_3 (206 mg.) was then assayed. The residual orcinol was extracted with

ether, dried and sublimed, giving colourless crystals (77 mg.), m.p. $97-98^\circ$. The activity of the orcinol was also determined.

Nitration of orcinol. Orcinol (70 mg.) was added slowly to 1 ml. of conc. H_2SO_4 with continuous stirring, and the solution was heated on a water bath for 15 min. The temperature was reduced and maintained below -10° while conc. HNO_3 (6 ml.) was added. The reaction mixture after 18 hr. was poured into water and the solid was collected and recrystallized from water giving yellow needles of trinitro-orcinol (82 mg.), m.p. 168° [Merz & Zetter (1879) found 163.5°].

Conversion of trinitro-orcinol into bromopicrin. Trinitro-orcinol (49 mg.) in water (10 ml.) was converted by addition of $0.2\text{M-Ba}(\text{OH})_2$ (8 ml.) into the barium salt which separated as yellow crystals. The mixture at 5° was treated with barium hypobromite prepared from bromine (2.5 ml.) in aq. $\text{Ba}(\text{OH})_2$ (17 g./74 ml. of water). The BaCO_3 (75.5 mg.) was washed with hot water and acetone and dried for assay. The bromopicrin in the filtrate was isolated by steam-distillation, collected in ice and separated from the water by centrifuging and decantation.

Oxidation of bromopicrin. The bromopicrin was transferred to the reaction flask by freezing it to a globule which was heated with Van Slyke & Folch (1940) oxidation mixture in a stream of N_2 . The bromine vapour produced on

Table 1. *Production of orsellinic acid and penicillic acid by Penicillium madriti*

The mould was grown in flasks containing 350 ml. of medium. Three flasks were pooled for each sample; the products were extracted with ether and separated by sublimation.

Time of growth (days)	Residual glucose (%)	Wt. of crude ether extract (mg./flask)	Wt. of orsellinic acid (mg./flask)	Wt. of penicillic acid (mg./flask)	Wt. of residual gum (mg./flask)
4	4.2	78	40	31	4.8
5	3.8	135	64	61	9
6	3.7	160	73	81	6
7	3.6	205	97	101	6
8	3.4	150	82*	62*	5.5
9	3.1	157	84*	66*	6.9
11	2.4	249	115	126	7.2
12	2.2	345	150	182	9
13	1.6	350	148	190	8
15	1.5	330	108	206	8.5

* The apparent decrease in acid production at 8 and 9 days is attributed to irregularities in growth.

Table 2. *Degradation of orsellinic acid derived from sodium $[1-^{14}\text{C}]$ acetate*

P. madriti was grown in the presence of $[1-^{14}\text{C}]$ acetate (see text). Orsellinic acid was extracted, degraded according to Scheme 1 and the specific activity of the carbon atoms was measured.

Carbon atom no.	Activity ($\mu\text{C}/\text{mole}$)	^{14}C equivalent	
		Found	Required
1-8 (as orsellinic acid)	475	—	4*
1 (as BaCO_3)	124	1.05	1
2-8 (as orcinol)	325	2.74	3
2-8 (assuming orcinol monohydrate)	374	3.13	3
2-8 (as trinitro-orcinol)	359	3.02	3
2, 4, 6 (as BaCO_3 from bromopicrin)	0	0	0
3, 5 (as BaCO_3)	105	1.77	2
7 (as BaCO_3)	84	0.71	1
8 (as iodoform)	0	0	0
7, 8 (as <i>p</i> -bromophenacyl acetate)	107	0.90	1

* Based on the theoretical incorporation of four acetate units.

Table 3. *Incorporation of labelled acetate and butyrate into fats and orsellinic acid by Penicillium madriti*

Cultures were treated with [^{14}C]acetate or [^{14}C]butyrate 4 days after incubation (see text). After further incubation for 5 days the fats and orsellinic acid were isolated for measurement of specific activity.

Substrate	Activity of substrate (μC)	Wt. of mycelium (g.)	Wt. of extracted fats (g.)	Wt. of orsellinic acid (mg.)	Incorporation of activity (%)	
					Into fats	Into orsellinic acid
[^{14}C]Acetate	200	2.35	0.434	62	5.4	4.25*
[^{14}C]Butyrate	20	2.41	0.406	64	3.8	1.6*

* These represent minimum values since losses inevitably occur on purification.

Table 4. *Effect of iodoacetamide on butyrate-derived orsellinic acid*

Cultures (4 days old) were treated with [^{14}C]butyrate in the presence and absence of iodoacetamide (0.5 mM). After further incubation for 5 days orsellinic acid was extracted and the specific activity of the carbon atoms was measured.

Carbon atom no.	Iodoacetamide	Activity ($\mu\text{C}/\text{mole}$)	^{14}C equivalent	
			Found	Required
1-8 (as orsellinic acid)	-	674	.	2*
1 (as BaCO_3)	-	172	0.51	1
1-8 (as orsellinic acid)	+	162	.	2*
1 (as BaCO_3)	+	41.9	0.52	1

* Based on the incorporation of two butyrate units.

oxidation was removed by passing the dried gases over granular zinc and then through a scrubber containing aq. AgNO_3 . The CO_2 was precipitated as BaCO_3 (36% of the theoretical yield) which was found to be inactive. In a modification, the bromopierin transfer was avoided by performing the oxidation in the flask in which the bromopierin had been collected, oleum being used to remove the residual water. The BaCO_3 yield was then 50% of the theoretical.

Kuhn-Roth oxidation. Orsellinic acid (372 mg.) was oxidized with 4N-chromic acid- H_2SO_4 mixture (4:1, v/v) for 4 hr. and the acetic acid produced was separated by distillation. Titration with 0.1N-LiOH indicated a 96% yield of acetic acid. The dried lithium acetate was pyrolysed in N_2 at 390-400° for 30 min. The acetone was converted into iodoform by hypoiodite and was found to be inactive. The residual Li_2CO_3 in the pyrolysis flask, when acidified with lactic acid (10 ml.), yielded active CO_2 , assayed as BaCO_3 . The activity of the acetic acid was confirmed by conversion into the *p*-bromophenacyl ester, the activity of which was measured.

Incorporation of sodium [^{14}C]butyrate

The butyrate (0.74 mg.; 20 μC) was added in sterile solution to each of two flasks after 4 days' growth of the mould on Czapek-Dox medium. The culture fluid was collected after a further 5 days and the orsellinic acid was separated, assayed and diluted with inactive material before acid hydrolysis. The activity of the dried mycelium and of the dried fat fraction, extracted by light petroleum, was also assayed (see Table 3 for extent of incorporation of ^{14}C).

Incorporation of sodium [^{14}C]butyrate in the presence of iodoacetamide

A preliminary experiment showed that the most useful concentration of iodoacetamide was 0.5 mM, which pro-

Table 5. *Incorporation of labelled orsellinic acid into penicillic acid*

The orsellinic acid labelled on C-1 was prepared synthetically; that labelled on C-1, C-3, C-5 and C-7 was biosynthesized from sodium [^{14}C]acetate (see text).

Labelling of orsellinic acid on carbon atom	...	...	1	1, 3, 5, 7
Yield of orsellinic acid (mg.)			37	88
Activity of orsellinic acid ($\mu\text{mC}/\text{g.}$)	3	270		29 800
Yield of penicillic acid (mg.)			84	100
Activity of penicillic acid ($\mu\text{mC}/\text{g.}$)			2.2	6 150
Activity of mycelium ($\mu\text{mC}/\text{g.}$)			3.8	64.4

duced marked inhibition of growth but some incorporation of active acetate into orsellinic acid. After 4 days' growth on Czapek-Dox solution, a sterile solution containing sodium [^{14}C]butyrate was added to each of five flasks, together with iodoacetamide to produce a concentration of 0.5 mM in the medium.

After 9 days in all, the contents of the five flasks were harvested. The recrystallized and sublimed orsellinic acid (51 mg.; activity 748 counts/min.) was diluted with unlabelled material and further purified to give 60 mg. with a constant activity of 297 counts/min. (162 $\mu\text{C}/\text{mole}$). This was subjected to acid hydrolysis; the active CO_2 produced was assayed as BaCO_3 . The results are given in Table 4.

Incorporation of labelled orsellinic acid into penicillic acid

(i) *With orsellinic acid labelled in the carboxyl group.* The [^{14}C]carboxy-orsellinic acid was synthesized by Thomas (1961). After 4 days' growth, the orsellinic acid (30 mg.; activity 7.2 $\mu\text{C}/100$ mg.), dissolved in a little warm water, was added to a culture flask. The culture was harvested

after 9 days and the purified orsellinic acid, penicillic acid and dried mycelium were assayed.

(ii) *With orsellinic acid derived from sodium* [$1-^{14}\text{C}$]-acetate. The orsellinic acid used was a sample prepared by addition of sodium [$1-^{14}\text{C}$]acetate (200 μC) to cultures of *P. madriti* and was thus labelled in the 1, 3, 5 and 7 positions. After 4 days' growth of the mould, the labelled orsellinic acid (58 mg.; 7.95 μC), dissolved in ethanol (1 ml.) plus water (9 ml.), was added to one flask and on the ninth day the products were extracted and assayed for activity. The results (Table 5) show no activity of the penicillic acid in (i), but considerable activity in (ii).

SUMMARY

1. *Penicillium madriti* G. Smith strain BB389A, when grown on Czapek-Dox glucose medium, produces orsellinic acid and penicillic acid.

2. When sodium [$1-^{14}\text{C}$]acetate was added to the culture medium the orsellinic acid produced was shown by degradation to be labelled equally on four of the eight carbon atoms, consistent with its production from four acetate units linked head-to-tail.

3. When sodium [$1-^{14}\text{C}$]butyrate was used as precursor, the resulting orsellinic acid was labelled. The carboxyl group had one-quarter of the total activity. This indicates breakdown of the butyrate to two C_2 units before incorporation and precludes utilization of the intact C_4 chain.

4. When labelled orsellinic acid was presented to the mould, the labelling of the penicillic acid was consistent with the direct conversion of orsellinic acid into penicillic acid with elimination of the carboxyl carbon.

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Distribution of Metabolic Activity, Phospholipid and Hyaluronidase between the Heads and Tails of Bull Spermatozoa

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Early investigations on the distribution of chemical components and metabolic activities between the heads and tails of spermatozoa were based on materials obtained by the mechanical disruption of spermatozoa by such means as ultrasonic disintegration, shaking with fine glass beads or grinding the frozen cells in a mortar (Henle, Henle & Chambers, 1938; Zittle & O'Dell, 1941*a, b*; Mann, 1949, 1951; Nelson, 1954). Procedures of this type are of limited value since motility is invariably abolished and some fragmentation of the sperm tails always occurs. However, a new approach to the problem was made possible by the

acquisition of an infertile bull of the Guernsey breed, the semen of which contains spermatozoa which are all in a 'decapitated' state, namely as free sperm heads and detached, yet perfectly motile, midpiece-tails. The production of decapitated spermatozoa is a congenital defect and is not of pathological origin. Using this semen Mann (1958*a, b*) separated the two cell components by differential centrifuging. He showed that rates of fructolysis and respiration were much higher in the midpiece-tails than in the heads, and that, though the midpiece-tails contained a high concentration of acid-soluble phosphorus compounds and phospholipid, nucleic acid was confined entirely to the tails.

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