6. The results suggest a selective incorporation of saturated fatty acids and aldehydes of 16 carbon atoms into lecithin and choline plasmalogen and of 18 carbon atoms into kephalin and kephalin plasmalogen.

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Studies in the Biosynthesis of Fungal Metabolites

THE BIOSYNTHESIS OF PALITANTIN

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Palitantin, $C_{14}H_{22}O_4$, a metabolite of *Penicillium palitans* Westling and other species, was first isolated and characterized by Birkinshaw & Raistrick (1936). It was further investigated by Birkinshaw (1952) and by Bowden, Lythgoe & Marsden (1959), who proposed the structure (I) (Fig. 1).

The biosynthesis of this C_{14}-alicylic compound is of interest in its relation to auroglaucin (II) and flavoglaucin, phenolic products of *Aspergillus glaucus* (Gould & Raistrick, 1934; Quilico, Cardani & Lucattelli, 1953), in that they each contain a C_1 and a linear C_7 side chain attached to adjacent carbon atoms of a C_6 ring. Also auroglaucin has been shown by Birch, Schofield & Smith (1958) to be derived by the condensation of acetate units. Since the formation of aromatic rings by this mechanism may well involve alicyclic intermediates the possible relevance of palitantin biosynthesis is readily apparent.

Although acetate coupling is the pathway most readily visualized (Bowden *et al.* 1959), the presence of three vicinal oxygen substituents is reminiscent of 5-dehydroshikimic acid (III), which is a known precursor of the aromatic amino acids and consequently an alternative source of benzenoid rings. Although the necessary additional modifications render this latter mechanism less probable, the possible operation of such a pathway added interest to the problem of palitantin biosynthesis.

The approach used in this investigation was based on the incorporation of labelled precursors into palitantin and subsequent determinations of the resulting distribution of radioactive carbon atoms. Thus a palitantin-yielding strain of *Penicillium cyclopium* Westling was grown in surface culture on Czapek-Dox medium and its rate of production determined (Fig. 2). The peak yield occurred at about 16 days and an attempt was

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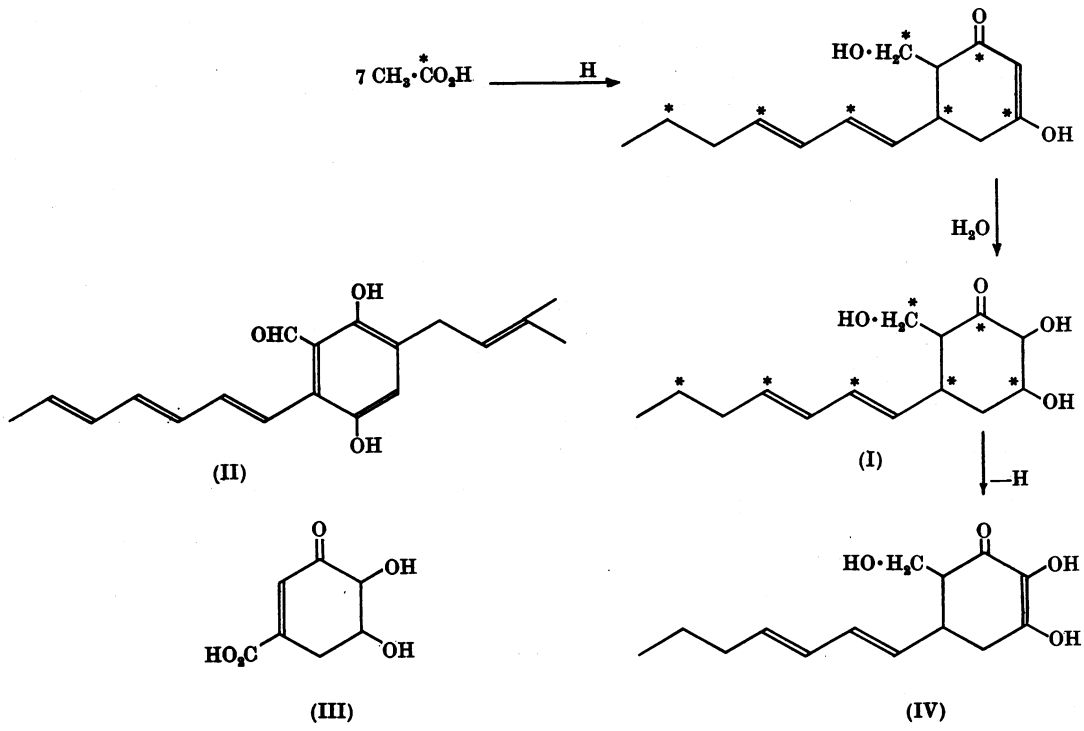

Fig. 1. Suggested pathway of palitantin biosynthesis. Asterisks indicate carbon atoms derived from acetate carboxyl groups.

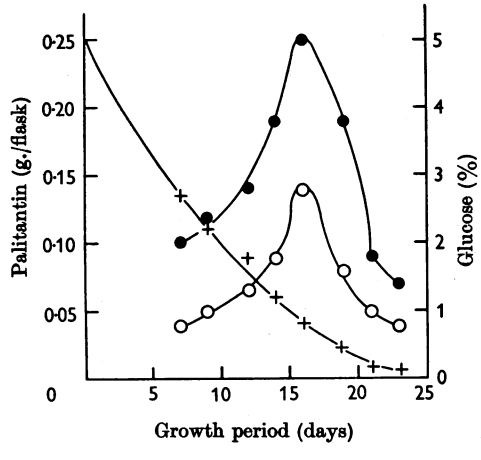

Fig. 2. Palitantin production. Each flask contains 350 ml. of culture medium. +, Glucose; ●, crude palitantin; ○, crude frequentin.

made to prepare labelled palitantin by adding sodium [1-^{14}C]acetate to the medium after 8 days' growth and subsequent ether extraction of the metabolism solutions of the individual flasks after 11, 13 and 15 days' growth.

The resulting extract was fractionated with sodium carbonate solution into crude palitantin and frequentin, $C_{14}H_{20}O_4$, a related product of possible structure (IV) (Bowden *et al.* 1959). Both fractions were highly labelled, as shown in Table 1.

As only single flasks were tested it is not possible to make any significant observations about the influence of incubation time on the incorporation of acetate. However, it is apparent in each case that the activity of the palitantin fraction appreciably exceeded that of the frequentin fraction. This result suggests that palitantin may be a precursor of frequentin, which in terms of structures (I) and (IV) would involve a single dehydrogenation step. However, the lower activity of the crude frequentin fraction could also result from the presence of unlabelled impurities.

The crude palitantin fractions were combined, purified and subsequently diluted with inactive material in order to produce sufficient product for the degradations outlined in Fig. 3.

The results of these degradations are given in Table 2 and compared with the theoretical values based on the incorporation of seven acetate units into palitantin according to the scheme shown in Fig. 1.

Table 1. *Incorporation of labelled acetate and mevalonic acid lactone when added to single flasks containing 8-day cultures of* Penicillium cyclopium

Each flask contained 350 ml. of culture fluid (for details see text).

Labelled precursor	Sodium acetate 40 μc of [1-^{14}C]-labelled			Mevalonic acid lactone 10 μc of [2-^{14}C]-labelled	Mevalonic acid lactone 25 μc of [1-^{14}C]-labelled
Growth (days)	11	13	15	11	11
Glucose (%)	1·32	1·00	0·95	1·51	1·44
Mycelial weight (g.)	2·11	2·10	2·02	2·24	2·40
Activity of mycelium powder (counts/min.)					
Before extraction	764	693	957	22	4
After extraction	419	485	482	—	—
Light petroleum extract					
Weight (g.)	0·137	0·054	0·081	0·108	0·112
Activity (counts/min.)	5991	3563	8863	147	26
Ether extract					
Weight (g.)	0·098	0·062	0·064	0·076	0·078
Activity (counts/min.)	1255	1384	1932	140	29
Crude palitantin					
Weight (g.)	0·207	0·094	0·151	0·142	0·149
Activity (counts/min.)	7722	4986	5227	0	0
Incorporation of added acetate into palitantin (%)	15	4·4	5·7	—	—
Crude frequentin					
Weight (g.)	0·081	0·071	0·102	—	—
Activity (counts/min.)	5539	3735	3640	—	—

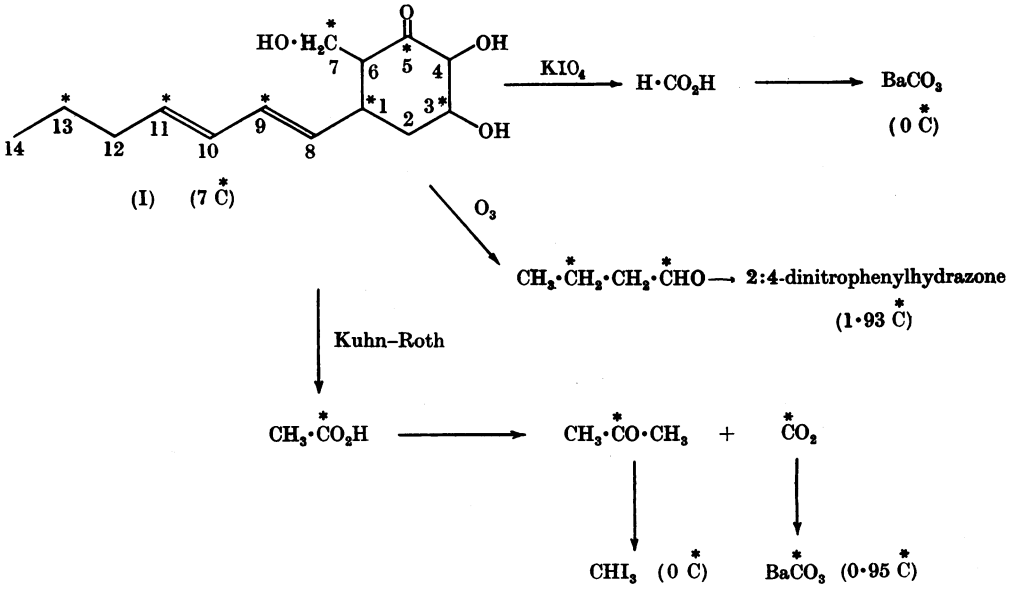

Fig. 3. Palitantin degradation scheme. The activities of the degradation products (shown in parentheses) are calculated on the basis of a theoretical incorporation of seven acetate unite into palitantin.

Table 2. *Distribution of labelling in palitantin derived from* [1-^{14}C]*acetate*

Carbon atom in palitantin	Radioactivity (counts/min.)	Radioactivity (μc/mole)	Active carbon equivalent Found	Active carbon equivalent Required
1–14 as palitantin	641	611	—	7*
14 (as CHI_3)	0	0	0	0
13 (as $BaCO_3$)	112	83	0·95	1
11, 12, 13, 14 (as *n*-butyraldehyde 2:4-dinitrophenylhydrazone)	178	168	1·93	2
11, 12 (by difference)	—	—	0·98	1
4 (as $BaCO_3$)	0	0	0	0

* Based on theoretical incorporation of seven acetate units.

Thus a Kuhn–Roth oxidation isolated C-13 and C-14 as acetic acid. This was converted by pyrolysis of its lithium salt into acetone and subsequently iodoform, which corresponded to C-14 of palitantin and was inactive. The residual lithium carbonate (from C-13) contained an activity of 0·95 unit when assayed as barium carbonate.

A periodate oxidation isolated C-4 as formic acid, which on oxidation to carbon dioxide and assay as barium carbonate or on direct assay as lead formate was inactive.

Ozonolysis of palitantin yielded *n*-butyraldehyde, which was assayed as its 2:4-dinitrophenylhydrazone. The activity (1·93 units) corresponded to the aggregate of C-11, C-12, C-13 and C-14 from which it followed, by difference, that C-11 and C-12 shared an activity of 0·98 unit.

These results are in good agreement with the values required by the acetate head-to-tail condensation mechanism. Further experiments to confirm the presence of alternate labelling throughout the molecule were discontinued at this stage, when it was learnt from Professor A. J. Birch, F.R.S., that an investigation of the biosynthesis of palitantin had recently been completed in his Department at the University of Manchester. He kindly informed us of his findings, which agreed with those described above and consequently confirmed the validity of the postulated pathway from acetate.

The hydroxymethyl side chain is apparently derived from a reduced acetate carboxyl and this also occurs in the biosynthesis of citromycetin (Birch, Fitton *et al.* 1958). In auroglaucin, a parallel reduction of the acetate carboxyl-derived C_1 side chain also takes place but proceeds only as far as the aldehyde. No evidence is available to indicate the stage at which this reduction takes place but one possibility involves the acetate-derived, isoprenoid precursor, mevalonic acid (V). In this case the incorporation would not involve the usual decarboxylation stage (Fig. 4).

Accordingly, [2-^{14}C]mevalonic acid lactone (10 μc) and also the [1-^{14}C]-labelled lactone (25 μc) were fed in separate experiments to *P. cyclopium* under conditions shown to be conducive to acetate uptake. However, in neither case was the resulting palitantin significantly labelled. The utilization by the fungus of the added mevalonic acid was tested by determining the activity of the crude lipoid fraction obtained from extraction of the mycelium with light petroleum. Such extracts normally contain steroidal material, e.g. ergosterol (Thomas, 1955).

With [2-^{14}C]mevalonic acid lactone the count rate was approximately 16-fold in excess of the background count, thus demonstrating significant incorporation. On the other hand, [1-^{14}C]mevalonic acid lactone, which would not be expected to produce appreciable labelling in normal steroidal components because of the decarboxylation step accompanying condensation of these units, in fact gave rise to only comparatively slight activity in the lipoid fraction (Table 1). It follows from these findings that although mevalonic acid is metabolized by *P. cyclopium*, it is apparently not a normal intermediate in the biosynthesis of palitantin.

EXPERIMENTAL

All melting points are uncorrected.

Culture and medium. The culture used to obtain palitantin was a strain of *Penicillium cyclopium* Westling (London School of Hygiene Catalogue no. 525) grown on Czapek-Dox medium [cf. Bracken, Pocker & Raistrick (1954)].

Radioactive assay. All measurements were carried out with a Panax scaler (type 100C) coupled to an end-window Geiger–Müller tube and compared with a polymethyl methacrylate standard of known ^{14}C content (1 μc/g.).

Isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. Samples were counted at infinite thickness with planchets 1·5 cm. in diameter, the standard error not exceeding $\pm 3\%$. All the activities quoted in Tables 1 and 2 are corrected for background (approx. 9 counts/min.).

Determination of palitantin production. Ninety-six 1 l. flasks containing Czapek-Dox medium (350 ml./flask) were inoculated with a suspension of *P. cyclopium* in sterile water and incubated in the dark at 24°. At regular intervals batches of eight flasks were selected at random and examined for pH and glucose content (determined polarimetrically). The culture filtrate was extracted with ether

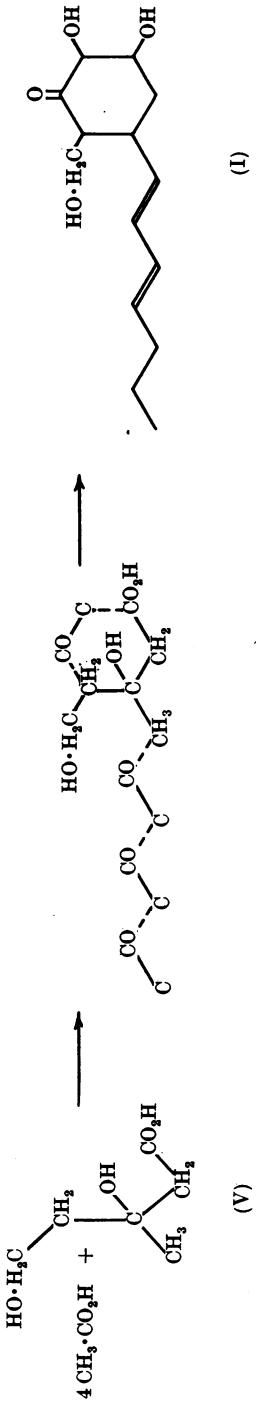

Fig. 4. Suggested incorporation of mevalonic acid into palitantin.

(4 × 0·25 vol.) which was then partially evaporated before shaking with 2N-Na_2CO_3 and subsequent separation into carbonate-soluble (A) and carbonate-insoluble (B) fractions. Fraction (A) yielded crude frequentin accompanied by a yellow syrupy contaminant; concentration of fraction (B) yielded palitantin as a crude crystalline deposit. The results are shown graphically in Fig. 2.

Incorporation of [1-^{14}C]*acetate.* *P. cyclopium* was cultured as before and after 8 days 40 μc of sodium [1-^{14}C]acetate in sterile water (5 ml.) was added to each of three flasks. These were harvested at 11, 13 and 15 days and examined as described above. In addition the mycelium was washed, dried and Soxhlet-extracted, initially with light petroleum (b.p. 40–60°) for 18 hr. and subsequently with ether for 30 hr. The activities of the crude palitantin and frequentin fractions and of the crude mycelial extracts were determined (Table 1).

Purification and dilution of labelled palitantin. The combined palitantin fractions of the 11-, 13- and 15-day flasks were purified by three sublimations in high vacuum at 130–135°, giving colourless crystals of palitantin (208 mg.) of constant m.p. (164–165°) and radioactivity (6370 counts/min., 24 μc/g.). This sample was mixed with pure inactive palitantin (1·792 g.) and dissolved in warm acetone (30 ml.), which was then evaporated to yield diluted palitantin (2 g.) with activity, 641 counts/min. (unchanged on vacuum sublimation).

Degradation of labelled palitantin

Kuhn–Roth oxidation. A Kuhn–Roth oxidation of palitantin (0·508 g.) (cf. Birkinshaw, 1952), gave a quantitative yield of acetic acid which was separated by steam-distillation and neutralized with 0·1N-LiOH. The dried lithium acetate was pyrolysed in a current of N_2 at 390° for 10 min. and the effluent acetone was absorbed in water and converted into iodoform by the addition of aqueous alkaline iodine. The resulting iodoform was inactive.

The Li_2CO_3, remaining after pyrolysis was acidified with lactic acid, heated and the resulting CO_2 was passed, in a stream of N_2, into 0·2N-$Ba(OH)_2$, giving active $BaCO_3$ which was then assayed.

Ozonolysis. Ozonized oxygen was passed through an ice-cooled solution of palitantin (0·508 g.) in aldehyde-free $CHCl_3$ (75 ml.) for 3 hr. The solution was extracted [by shaking with an equal volume of water and, after separation, the aqueous phase was heated to 60°]. The effluent *n*-butyraldehyde was passed in a current of N_2 through bubblers containing Brady's reagent (2:4-dinitrophenylhydrazine in 2N-HCl). The deposited phenylhydrazone (0·131 g., m.p. 121–123°) was purified by recrystallization from aqueous 50% ethanol, giving lemon-yellow needles, m.p. 123°, which were then assayed directly.

Periodic acid oxidation. Palitantin (0·254 g.) in ethanol (25 ml.) was treated with a solution of potassium periodate (0·508 g.) in CO_2-free water (100 ml.). After 72 hr. mercuric oxide (3 g.) was added and the solution was refluxed in a stream of N_2 for 2 hr. in order to convert the formic acid formed initially into CO_2 (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The CO_2 was precipitated as $BaCO_3$ (yield 91% of the theoretical), which on assay proved to be inactive.

The periodate oxidation of palitantin was repeated but this time, after 72 hr. the solution was concentrated by distillation. The distillate was treated with excess of lead

carbonate, heated and filtered. The filtrate was then concentrated and cooled, when a precipitate (25 mg.), apparently identical with lead formate, separated. This also had zero activity.

The values of the radioactivities of the fragments obtained in the above degradations are summarized in Table 2.

Attempted incorporation of labelled mevalonic acid lactone

DL-[1-^{14}C]Mevalonic acid lactone (25 μc) was added in sterile water to a flask containing an 8-day culture of *P. cyclopium*. A second flask was similarly treated with DL-[2-^{14}C]mevalonic acid lactone (10 μc). After a further 3 days the flasks were harvested and the palitantin was extracted as before but in neither case did it show any significant activity. In addition the mycelium was Soxhlet-extracted with light petroleum (b.p. 40–60°) for 18 hr. and the extract was concentrated to dryness. On assay the crude product from [2-^{14}C]mevalonic acid lactone was significantly active (147 counts/min.), whereas the product from the [1-^{14}C]-lactone was only slightly active (26 counts/min.), cf. Table 1. The lipoid fraction was assumed to contain steroidal material, e.g. ergosterol (Thomas, 1955).

SUMMARY

1. The biosynthesis of palitantin, $C_{14}H_{22}O_4$, an alicyclic metabolite of *Penicillium palitans* and also of *Penicillium cyclopium*, has been studied.

2. Degradations of labelled palitantin derived from [1-^{14}C]acetate strongly support a biosynthetic mechanism involving the head-to-tail condensation of acetate units.

3. The biosynthetic pathway does not appear to involve mevalonic acid.

We are grateful to Imperial Chemical Industries Ltd. for a grant towards the purchase of tracer equipment and ^{14}C-labelled compounds. One of us (R.T.) participated in this work during the tenure of a Wellcome Research Fellowship. We wish to thank Professor J. H. Birkinshaw for much helpful discussion during the course of this work.

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A Further Genetic Variety of Glutamic Acid Dehydrogenase in *Neurospora crassa*

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The amination-deficient mutants of *Neurospora crassa* all appear to be deficient in glutamic dehydrogenase, and are all due to genetic change within a short segment (the *am* locus) of the chromosome corresponding to linkage group 5 (Pateman & Fincham, 1958; Fincham, 1959*a*; R. W. Barratt, personal communication). Treatment of conidia of several of these mutants with ultraviolet light has resulted in the induction of apparent reversions to the wild-type condition. Some of the 'revertants' were indistinguishable from the standard, wild-type organism, whereas in others there seemed to have been only partial repair of the original mutational damage to the genetic basis of glutamic-dehydrogenase formation (Pateman, 1957). Two of these imperfect revertants have now been investigated in detail. The first, derived from the enzyme-negative allele am^2 [the revised gene symbols adopted in Fincham (1959*a*) are used here], and designated am^{2l}, has been shown to produce an abnormal kind of glutamic dehydrogenase which is almost inactive as normally extracted, but

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