2. Application of these techniques has resulted in the isolation of a new acid characterized by physical and chemical means as hexadeca-6:9:12:15-tetraenoic acid.

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Studies in the Biochemistry of Micro-organisms

93. CYCLOPENIN, A NITROGEN-CONTAINING METABOLIC PRODUCT OF *PENICILLIUM CYCLOPIUM* WESTLING

BY A. BRACKEN, ANNA POCKER AND H. RAISTRICK
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

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The *Penicillium cyclopium* series of species of *Penicillium* contains four accepted species and one variety, i.e. *P. cyclopium* Westling, *P. cyclopium* Westling var. *echinulatum* Raper & Thom, *P. puberulum* Bainier, *P. martensii* Biourge and *P. aurantio-virens* Biourge. The series has proved to be a fruitful source of mould metabolic products, the following having been described: penicillic acid from *P. puberulum* (Alsberg & Black, 1913) and *P. cyclopium* (Birkinshaw, Oxford & Raistrick, 1936); ergosterol in the mycelium of *P. puberulum* (Birkinshaw, Callow & Fischmann, 1931); the tropolones, puberulic and puberulonic acids, from *P. puberulum* and *P. aurantio-virens* (Birkinshaw & Raistrick, 1932) and *P. johannioli* Zaleski = *P. martensii* Biourge (Oxford, Raistrick & Smith, 1942); *i*-erythritol from *P. cyclopium* (Oxford & Raistrick, 1935); the anthraquinones, ω-hydroxyemodin and emodic acid, from *P. cyclopium* (Anslow, Breen & Raistrick, 1940); cyclopolic and cyclopaldic acids, two fully substituted benzoic acids, from *P. cyclopium* (Birkinshaw, Raistrick, Ross & Stickings, 1952) and an unnamed photosensitive nitrogenous compound, $C_{17}H_{12}O_2N_2$, m.p. 220° (decomp.), from *P. puberulum*, which gave phenol on heating and *p*-hydroxybenzoic acid on oxidation (Campbell, Foss, Hirst & Jones, 1945).

We now wish to report the isolation from culture filtrates of an authentic strain of *P. cyclopium* of a hitherto undescribed metabolic product for which we propose the name cyclopenin.

CYCLOPENIN

Cyclopenin was isolated, in roughly equal amounts, from culture filtrates of *P. cyclopium*, L.S.H.T.M. no. 72, grown on either Czapek-Dox or Raulin-Thom solutions, by adsorption on charcoal and elution with methanol. Cyclopenin, $C_{17}H_{14}O_3N_2$, crystallizes from methanol in colourless needles containing two molecules of water of crystallization. On heating, these crystals melt at 110–120° with frothing and loss of water, reset on cooling and remelt at 207°. Cyclopenin separates from a variety of other solvents, especially ethyl acetate, in crystals containing half a molecule of water of crystallization which melt sharply at 207°. It

contains one nitrogen-methyl group and has 1·5 atoms of active hydrogen, but has no methoxyl or carbon-methyl groups. It is strongly laevorotatory in ethanol solution. It dissolves only sparingly in cold water, does not dissolve readily in aqueous sodium bicarbonate but is easily soluble in dilute aqueous sodium hydroxide. Addition of bromine water to its saturated aqueous solution gives an immediate thick turbidity which readily flocks to a heavy precipitate under acid conditions. It shows slight antibacterial activity towards *Micrococcus pyogenes* var. *aureus* and *Escherichia coli*. It does not give a ferric colour either in aqueous or ethanolic solution, except on long standing.

It is readily decomposed, even in the cold, by dilute mineral acids with the formation of one molecule each of carbon dioxide, methylamine and viridicatin according to the equation

$$\underset{\text{cyclopenin}}{C_{17}H_{14}O_3N_2} + H_2O \rightarrow CO_2 + CH_3NH_2 + \underset{\text{viridicatin}}{C_{15}H_{11}O_2N}$$

Viridicatin

Viridicatin was first described by Cunningham & Freeman (1953), who isolated it from the dried mycelium of a strain of *Penicillium viridicatum* Westling by chloroform extraction. *P. viridicatum*, although not included in the *P. cyclopium* series is closely related to this species, so that strains of the two species are often difficult to distinguish. Viridicatin, $C_{15}H_{11}O_2N$, forms colourless needles, m.p. 268°, which are optically inactive in ethanol and give an intense green ferric colour in ethanol solution. Cunningham & Freeman described seven crystalline derivatives of viridicatin but lacked sufficient material for degradative work, since the mould ceased to produce viridicatin after being in cultivation for some time.

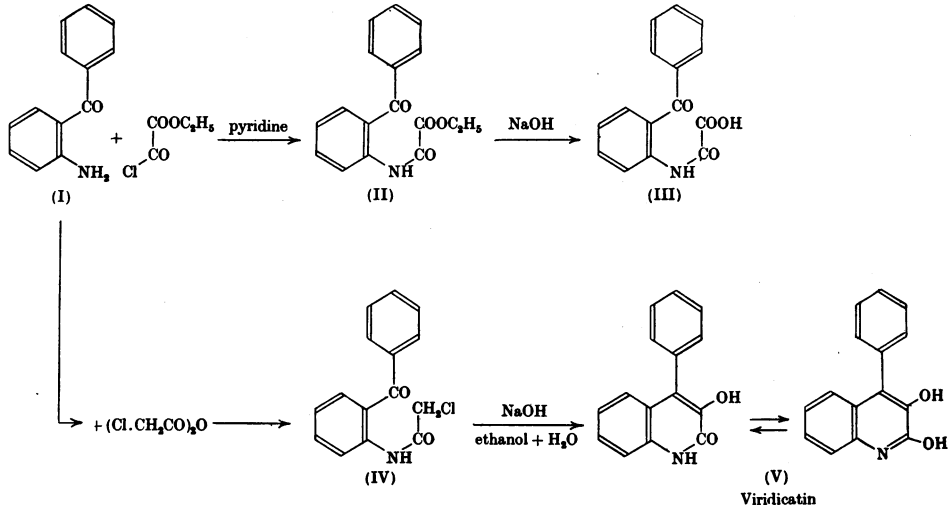
We have now found that, although we were unable to detect viridicatin in the mycelium of strain no. 72 of *P. cyclopium*, which produces cyclopenin, five other strains of this species give considerable amounts of viridicatin. Such strains can be readily detected by growing them on Czapek-Dox 5% glucose solution for 21–24 days. Portions of the fresh mycelium are pressed as dry as possible between sheets of filter paper and are then extracted for a short time with boiling ethanol. Production of a strong green colour on addition of ferric chloride to the filtered ethanolic extract is strong presumptive evidence of the presence of viridicatin.

We have confirmed Cunningham & Freeman's proposed formula, $C_{15}H_{11}O_2N$, for viridicatin and have prepared and described the following new derivatives: 3:5-dinitrobenzoic acid salt, $C_{15}H_{11}O_2N.C_7H_4O_6N_2$, yellow prisms, m.p. 238°; monoacetylviridicatin, $C_{17}H_{13}O_3N$, short colourless prisms, m.p. 200–201°; *OO*-dimethylviridicatin, $C_{15}H_9N.(OCH_3)_2$, colourless plates, m.p. 86–87°; *ON*-dimethylviridicatin, $C_{15}H_9O.(OCH_3)(NCH_3)$, colourless plates, m.p. 197–198°.

Work carried out in this laboratory on the degradation of viridicatin has led to the establishment of structure (V) for it, and this structure has been confirmed by synthesis. It has been shown that when oxygen is bubbled at room temperature through a solution of viridicatin in ethanolic potassium hydroxide, oxidative decomposition of viridicatin takes place with the formation in high yield of *o*-aminobenzophenone (I) and oxalic acid according to the equation

$$C_{15}H_{11}O_2N + H_2O + O_2 \rightarrow C_{13}H_{11}ON + (COOH)_2.$$

Further, when viridicatin was oxidized with potassium permanganate in boiling acetone solution, or with hydrogen peroxide in glacial acetic acid solution



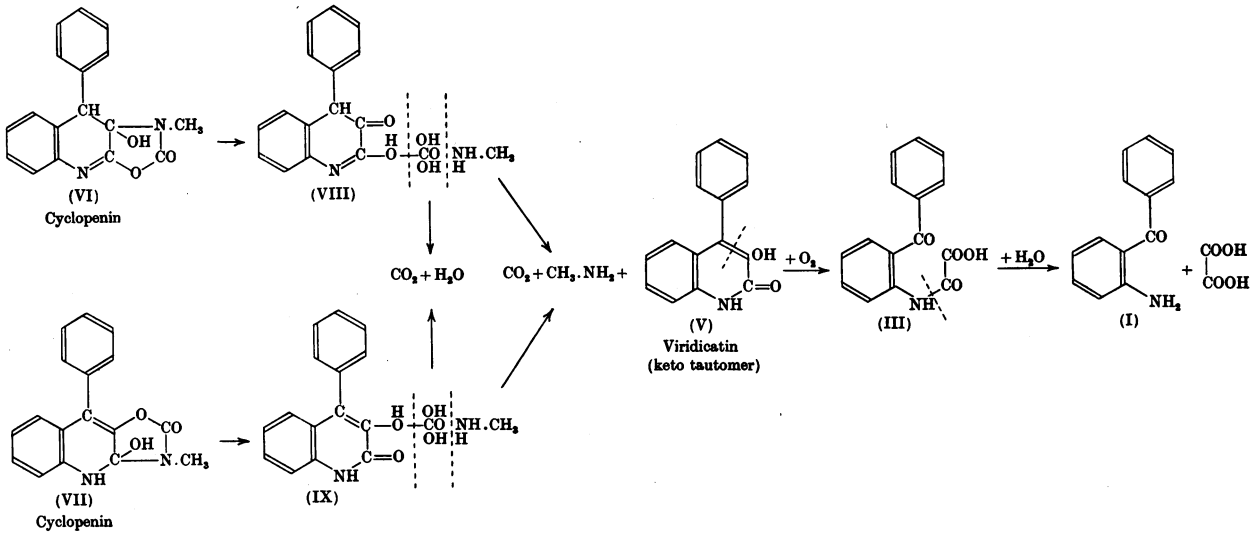
at 100°, a good yield of the hitherto unknown oxalyl-*o*-aminobenzophenone (III) was obtained. This compound, which crystallizes in colourless needles, m.p. 184° (decomp.), was synthesized as follows: *o*-aminobenzophenone (I), in pyridine solution, was condensed with ethoxalyl chloride. The resulting ester, ethoxalyl-*o*-aminobenzophenone (II), colourless irregular plates, m.p. 135–136°, was hydrolysed at room temperature with aqueous N sodium hydroxide giving an 81 % overall yield of oxalyl-*o*-aminobenzophenone (III) identical with that obtained by permanganate oxidation of viridicatin.

It is therefore very probable that the oxidation of viridicatin in alkaline solution with gaseous oxygen proceeds, through the formation and action of peroxides, to give oxalyl-*o*-aminobenzophenone (III) which is then hydrolysed by the alkali to give oxalic acid and *o*-aminobenzophenone (I).

Viridicatin was synthesized by a modification of the method of Camps (1899, 1902) for the synthesis of carbostyrils (2-hydroxyquinolines). *o*-Aminobenzophenone was chloroacetylated by treatment with chloroacetic anhydride. The resulting chloroacetyl *o*-aminobenzophenone (IV), colourless needles, m.p. 107–108°, was heated in aqueous ethanol with sodium hydroxide. The hot solution was then acidified with hydrochloric acid. The cyclization product (V) separated in 72 % overall yield as colourless needles, which, after crystallization from ethanol, melted at 268° not depressed on admixture with natural viridicatin of the same melting point. The *ON*-dimethyl derivative of synthetic viridicatin, that of natural viridicatin and a mixture of the two all melted at 197°.

Viridicatin is thus 2:3-dihydroxy-4-phenylquinoline or its keto tautomer.

The solution of the problem of the structural formula for cyclopenin therefore resolves itself into that of piecing together one molecule each of carbon dioxide, methylamine and 2:3-dihydroxy-4-phenylquinoline (V), with the loss of one molecule of water, in such a way as best to illustrate the known properties of cyclopenin. In particular, it must account for the optical activity of cyclopenin, the ready formation of the three hydrolytic products of cyclopenin mentioned above and the formation of the oxidative hydrolytic products of viridicatin, i.e. oxalic acid, oxalyl-*o*-aminobenzophenone (III) and *o*-aminobenzophenone (I). To this end we suggest for cyclopenin either structure (VI) or (VII) with a slight preference for (VI), although we have no evidence which conclusively supports one structure against the other. Both structures satisfactorily explain the optical activity of cyclopenin and the ready formation of its hydrolytic products through the intermediate formation either of the 2-methylurethane (VIII from VI) or the 3-methylurethane (IX from VII) of 2:3-dihydroxy-4-phenylquinoline (V), either of which, on acid or alkaline hydrolysis, would be expected to yield carbon dioxide, methylamine and viridicatin (V). When viridicatin (and therefore almost certainly cyclopenin) is submitted to oxidative hydrolysis, e.g. by gaseous oxygen, in alkaline solution, potassium permanganate in acetone or hydrogen peroxide in glacial acetic acid, the initial oxidation product is oxalyl-*o*-aminobenzophenone (III) which is then readily hydrolysed to give oxalic acid and *o*-aminobenzophenone (I). All these changes are illustrated in the following scheme:



EXPERIMENTAL

CYCLOPENIN

History of culture

The culture used for the preparation of cyclopenin was isolated by Mr R. L. Hughes from a catch plate exposed by him in a laboratory in Croydon, Surrey. The culture, which bears Mr Hughes's catalogue no. 26, was received by one of us (A. B.) in March 1947 and was given the London School of Hygiene catalogue no. 72. The following note on the culture was kindly supplied by Mr George Smith of this Department. 'I have examined the *Penicillium* no. 72 and find that it is a typical strain of *P. cyclopium* Westling. Colonies on Czapek agar are bright blue-green, with little colour in reverse, spreading, granular and showing well-marked fasciculation, particularly in the younger areas. Conidiophores are slightly rough, and the conidia round, smooth, about 3 μ. in diameter. These characteristics alone are sufficient to place the mould with certainty as *P. cyclopium*. This species is probably the commonest member of the genus in this country, growing on a very wide range of substrata, so that it is hardly surprising that different isolates vary in their biochemical characteristics.'

Sand cultures of *P. cyclopium* strain no. 72 were prepared, for preservation, as follows. Acid-treated, washed and dried sand was sterilized by heating at 140–150° for 4–6 hr. Portions of this sterile sand were added to each of a number of 11-day-old cultures of *P. cyclopium* no. 72 grown at 24° on malt-agar slopes. The cultures were thoroughly shaken and the sand acquired a greenish tint from adhering spores. The sand and spores were transferred aseptically to sterile test tubes and dried for some days *in vacuo* over P_2O_5. Each tube, still plugged with sterile cotton wool, was drawn out in a flame to a capillary some distance below the cotton-wool plug. After cooling, each tube was evacuated for 5–10 min. on an oil pump, hermetically sealed at the capillary while still under vacuum, and stored in the dark at room temperature. Sand cultures so prepared in February 1948 were still highly viable in October 1953 and, after cultivation on malt-agar slopes at 24°, furnished cultures which gave good yields of cyclopenin.

Cultural conditions and cultural characteristics of Penicillium cyclopium *strain no.* 72

(*a*) *On Raulin-Thom solution.* Glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4.7H_2O$, 0·07 g.; $ZnSO_4.7H_2O$, 0·07 g.; distilled water, 1500 ml. This solution was distributed into fifty 1 l. conical flasks (350 ml./flask), which were plugged with cotton wool and sterilized. Each flask was inoculated with a portion of a spore suspension, in sterile distilled water, of *P. cyclopium* strain no. 72 which had been cultivated on 12 malt-agar slopes at 24° for 10–12 days. The inoculated flasks were incubated at 24° in the dark. The mould was somewhat slow to germinate, but after 4 days the surface of the medium in most of the flasks was practically covered with a thin, white growth showing occasional green patches of sporing growth. After 6 days the medium in almost all flasks was covered with a somewhat fragile bright parrot-green felt which had an orange or pinkish orange reverse. The culture solution was now bright orange in colour. With continued cultivation the felts became somewhat bluer in tone and from 14 days onwards became much duller in appearance and wrinkled and buckled in texture.

(*b*) *On Czapek-Dox solution.* Glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1000 ml. The cultural conditions were the same as for the cultures on Raulin-Thom solution in (*a*) above. Growth in the early stages was similar to that on Raulin-Thom solution except that after 4 days, when the mould was beginning to spore in patches, there was much yellow growth, and after 8 and 15 days the mycelium was a much duller green in shade than the bright parrot-green of the Raulin-Thom cultures. There was also no marked orange colour in the mycelial reverse and the culture solution was only pale yellow.

At the end of the incubation period the culture filtrates obtained from culture solutions (*a*) and (*b*) gave very similar reactions which differed chiefly in intensity. Addition of aqueous $FeCl_3$ solution did not give an immediate colour reaction, although an emerald-green colour slowly developed in the course of 3–4 days. An intense emerald-green colour was however immediately produced on the addition of aqueous $FeCl_3$ to a portion of the culture filtrate which had been acidified with HCl, heated at 100° for 20 min., cooled and neutralized with aqueous NaOH. As will be seen later this is due to the hydrolysis of the metabolic product (cyclopenin), which itself gives no ferric colour, with the formation of a product (viridicatin) which immediately gives the characteristic ferric colour. Addition of saturated Br_2 water to either culture filtrate leads to absorption of Br_2 and the formation of a thick turbidity which slowly coagulates to a heavy, pale yellow, amorphous precipitate. If the culture filtrate is acidified with dilute HCl before the addition of Br_2 water a heavy precipitate is formed almost immediately.

Isolation of cyclopenin from culture filtrates of Penicillium cyclopium *strain no.* 72

Cultures of *P. cyclopium* strain no. 72, grown on either Raulin-Thom solution or on Czapek-Dox solution, were harvested after 14–17 days and 21–22 days respectively. The culture fluid from fifty flasks was decanted from the mycelium and filtered through a thin layer of kieselguhr on a large Büchner funnel. The mycelium was pressed as thoroughly as possible in a tincture press, dried in a vacuum oven at 40–45° and weighed. The filtered pressings were added to the main filtrate and the solution was adjusted to about pH 2·0 with conc. HCl. Activated charcoal (50–60 g.) (British Drug Houses Ltd., London) was then added. The mixture was stirred vigorously for 1–2 hr. when a sample, freed from charcoal by filtration, should be quite colourless, give no turbidity with Br_2 water and no colour with aqueous $FeCl_3$ after hydrolysis with HCl. The charcoal was separated by filtration through a thin layer of kieselguhr (10 g.) on a 24 cm. Büchner filter and washed with distilled water (1–1·5 l.) till the washings were free from chlorides. It was drained as thoroughly as possible, dried in the open laboratory overnight and then thoroughly dried in a vacuum desiccator over conc. H_2SO_4. The charcoal was suspended in methanol (500 ml.) and poured on to a preformed kieselguhr pad on a filter disk at the foot of a long, wide, glass column. A total of 3–3·5 l. of methanol was then passed through the column under a very slight vacuum, thus eluting all the desired product. The methanol solution was concentrated *in vacuo* to about 40 ml., when cyclopenin began to separate

Table 1. *Cultural details and yields of metabolic products from* Penicillium cyclopium *strain no.* 72

Each batch contains fifty flasks. For other details see text.

	Raulin-Thom solution				Czapek-Dox solution			
Batch no.	*R*1	*R*2	*R*3	*R*4	*C*1	*C*2	*C*3	*C*4
Incubation period (days)	17	14	14	14	22	21	22	22
Mycelial dry weight (g.)	154	195	193	192	134	138	—	154
Glucose (% by polarimeter)	0·17	0·45	0·60	0·44	0·82	0·76	0·63	0·52
pH	4·4	3·9	3·6	3·9	4·9	4·6	4·6	4·6
Crystalline cyclopenin (g.)	3·50	4·42	4·50	2·15	3·39	3·36	2·71	2·80
Residual gum (g.)	6·45	6·64	6·96	7·00	6·64	7·00	10·10	6·69
Total extract (g.)	9·95	11·06	11·46	9·15	10·03	10·36	12·81	9·49

and was collected by filtration. The cyclopenin so obtained melted at 106–110° with evolution of water of crystallization, then reset and remelted at 207°. By evaporation of the filtrate other crops of crystals were obtained. The final mother liquors, on evaporation to dryness, gave a light orange residual gum.

Cyclopenin is produced on both Raulin-Thom and Czapek-Dox media in comparable amounts, but we prefer to use Raulin-Thom medium, since the incubation period is about 7 days shorter, the yield appears to be more constant and cyclopenin is obtained as beautiful snow-white needles from Raulin-Thom medium, whereas they are just off colourless with Czapek-Dox medium. The yield of cyclopenin and a number of cultural details are given in Table 1.

Purification of cyclopenin

Most of the specimens of cyclopenin listed in Table 1 as crystalline are already sufficiently pure for preparative work. Cyclopenin may, however, be further purified by recrystallization from a variety of solvents. Thus 3·2 g. cyclopenin, dissolved in 25 ml. of boiling methanol, yielded 1·90 g. of colourless needles containing 2 molecules of water of crystallization (see below), which melt with frothing and loss of water at 110–120°. The melt resets on cooling in well formed crystals which, on reheating, melt at 207°. Cyclopenin also crystallizes well from ethyl acetate (0·55 g. in 15 ml.) and ethanol (1·2 g. in 10 ml.). It separates from both these solvents in colourless tablets, m.p. 207° with little decomposition, which are less soluble in most solvents than the crystals from methanol.

General properties of cyclopenin

Cyclopenin contains C, H, O and N, but not S, Cl or P. (Found (*a*) on sample crystallized from methanol and air dried: C, 62·2, 62·4; H, 5·5, 5·4; N, 8·4, 8·5. $C_{17}H_{14}O_3N_2$, $2H_2O$ requires C, 61·8; H, 5·5; N, 8·5%. (*b*) On sample crystallized from methanol and dried to constant weight *in vacuo* at room temp.: C, 66·9; H, 4·9; N, 9·2; OMe, nil. $C_{17}H_{14}O_3N_2$, $\frac{1}{2}H_2O$ requires C, 67·3; H, 5·0; N, 9·2%. (*c*) On different samples crystallized from ethyl acetate and dried in high vacuum, without loss in weight, either at room temp. or at 60–65°: C, 66·9, 67·1; H, 4·9, 4·65; N, 9·1 (Dumas), 9·2, 9·3 (Kjeldahl); OMe, nil; *C*-Me, nil; *N*-Me, 4·0; active H, 0·54%; mol.wt., cryoscopic in camphor, 300. $C_{17}H_{14}O_3N_2$, $\frac{1}{2}H_2O$ requires C, 67·3; H, 5·0; N, 9·2; *N*-Me, 5·0; 1·5 active H, 0·50%; mol.wt. 303.)

It is laevo-rotatory in ethanol solution and shows no sign of mutarotation, the rotation remaining unchanged overnight. (Found (*a*) on sample crystallized from methanol and containing $2H_2O$ of crystallization: $[\alpha]^{18}_{5461}$ − 280°, $[\alpha]^{18}_{5790}$ − 240° in ethanol (*c*, 2·000). (*b*) On sample crystallized from ethyl acetate and containing $\frac{1}{2}H_2O$ of crystallization: $[\alpha]^{20}_{5461}$ − 306°, $[\alpha]^{20}_{5790}$ − 263° in ethanol (*c*, 1·000). A solution of cyclopenin (0·50 g. of the dihydrate) in ethanol (15 ml.) and aqueous N-HCl (75 ml.), which showed an initial rotation in a 1 dm. tube of − 1·52° in the Hg green (5461 Å) light, slowly became optically inactive at room temp., the following rotations being observed at the stated times: 1·75 hr., − 1·40°; 3·25 hr., − 1·20°; 5·5 hr., − 1·05°; 23 hr., − 0·44°; 96 hr., − 0·10°; 102 hr., 0·00°. Viridicatin separated as colourless crystals (0·27 g., m.p. 248–252°, raised to 268° on recrystallization from ethanol).

Cyclopenin does not dissolve in aqueous $NaHCO_3$. It is readily soluble in N-NaOH, but is precipitated on neutralizing with HCl and redissolves in excess of HCl. Cyclopenin does not reduce Fehling's solution, even on boiling. Addition of Br_2 water to a saturated aqueous solution of cyclopenin gives an immediate thick turbidity which does not flocculate rapidly. If, however, Br_2 water is added to a cyclopenin solution acidified with HCl an immediate heavy precipitate is formed. Aqueous $FeCl_3$ gives no immediate colour with an ethanolic cyclopenin solution. After standing for 24 hr. a pale emerald-green colour develops, becoming intense emerald green after some days and red on dilution with much tap water. The development of an intense green ferric colour is almost certainly due to hydrolysis, since viridicatin, which is one of the hydrolytic products of cyclopenin, gives an immediate intense emerald-green ferric colour. Cyclopenin is readily hydrolysed, with the formation of viridicatin, by treatment at room temperature with conc. H_2SO_4 or conc. HCl, or by boiling with aqueous N-NaOH or 2N-H_2SO_4.

A sterile solution of cyclopenin (40·0 mg.) in ethanol (0·5 ml.) was added to sterile nutrient broth (9·5 ml.) thus giving a concentration of cyclopenin in the broth of 1:250. Serial dilutions with sterile nutrient broth were then made and inoculated with either *Micrococcus pyogenes* var. *aureus* (Oxford H strain) or *Escherichia coli* (National Collection of Type Cultures R. 6). The tubes were incubated at 37° for 24 hr. when there was complete inhibition of the growth of *E. coli* at a concentration of cyclopenin of 1:500, partial inhibition at 1:1000 and no inhibition at 1:2000; partial inhibition of *M. pyogenes* at 1:500 and no inhibition at 1:1000.

Attempts to prepare a crystalline acetyl derivative of cyclopenin failed, either with acetic anhydride and pyridine at room temp. or with acetic anhydride and anhydrous sodium acetate at 100°.

Quantitative hydrolysis of cyclopenin with 2N-HCl, *giving* CO_2, *methylamine and viridicatin* (V)

A suspension in 2N-HCl (100 ml.) of cyclopenin hydrate (1·981 g.; m.p. 207°; recryst. from ethyl acetate), contained in a two-necked flask fitted with an air condenser, was gently boiled in an oil bath in a stream of CO_2- and O_2-free N_2. The effluent N_2 was scrubbed first through a bubbler containing Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl) and then through two bubblers, each containing 50·00 ml. of 0·2N-$Ba(OH)_2$. Evolution of CO_2 began quickly and was completed in 2·5 hr. when the hydrolysis was stopped. The contents of the baryta bubblers were then titrated against 0·1N-HCl. The observed titre was 120·2 ml. 0·1N-HCl, equivalent to 92·0% of 1 mole CO_2/mole cyclopenin.

No trace of precipitate was obtained in the Brady bubbler indicating the absence, as hydrolytic products, of any volatile aldehyde or ketone.

The hydrolysis solution was held at 0° overnight and the colourless solid (crude viridicatin) which separated was collected quantitatively, washed with water and dried (1·431 g., m.p. 246–252°, corresponding to 92·3% of 1 mole of viridicatin/mole of cyclopenin). A portion of the crude viridicatin was carefully purified by sublimation in high vacuum at 160–170° followed by repeated crystallization from ethanol. It was finally obtained as colourless, fine, shining needles, m.p. 268° not depressed on admixture with a specimen of viridicatin isolated from *P. cyclopium* strain no. 578. (Found: C, 76·0; H, 4·7; N, 6·0. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.)

The combined acid filtrate and washings from the crude viridicatin were now distilled *in vacuo*, distilled water being run in at about the same rate as the distillate came over. Titration of the distillate showed that no volatile organic acids had been formed during the hydrolysis.

The combined acid filtrate and washings remaining after the vacuum distillation described in the previous paragraph were now evaporated to dryness *in vacuo*. The residue was dissolved in aqueous Na_2CO_3 (2·0 g. of Na_2CO_3, $10H_2O$) and the alkaline solution was distilled in a gentle stream of N_2. The effluent vapours were scrubbed through aqueous 2N-HCl and on evaporating the distillate to dryness there remained 0·399 g. of colourless methylamine hydrochloride, m.p. 225°, corresponding to 90·4% of 1 mole/mole of cyclopenin. The methylamine hydrochloride was purified for analysis by crystallization from ethanol and was obtained as colourless, glistening plates, m.p. 229° alone or in admixture with an authentic specimen. (Found: C, 18·0; H, 8·9; N, 21·0; Cl, 52·6. Calc. for CH_6NCl: C, 17·8; H, 9·0; N, 20·7; Cl, 52·5%.) The identity of the methylamine was confirmed by benzoylating it with benzoyl chloride in a Schotten Baumann reaction. The crude product so obtained was purified by sublimation in high vacuum at 60° followed by crystallization from light petroleum (b.p. 40–60°), giving *N*-methylbenzamide as long, fine, colourless needles, m.p. 81° not depressed on admixture with an authentic specimen, m.p. 82°. (Found: C, 71·4; H, 6·55. Calc. for C_8H_9ON: C, 71·1; H, 6·7%.)

Viridicatin

Five different strains of *P. cyclopium* isolated in this Department, were used for the preparation of viridicatin. Their history is summarized in Table 2.

Table 2. *History of cultures of* Penicillium cyclopium *used*

L.S.H.T.M. catalogue no.	Date of isolation	Source
525	October 1951	Garden soil ex Carron, Co. Clare, Eire
578	October 1951	Woodland soil ex Forest of Dean, Glos.
716	June 1952	Soil ex sand dunes, Perranporth, Cornwall
747	July 1952	Woodland soil ex copse, Watton at Stone, near Hertford
754	July 1952	Cultivated soil ex potato field, Watton at Stone, near Hertford

We are indebted to Mr G. Smith for the following description of these cultures. 'In culture on Czapek-agar all five strains are very similar being indeed virtually indistinguishable. Colonies are thick velvety, granular in the younger areas, blue-green turning gradually to grey-green with a broad white edge and regular outline; drops colourless; reverse pale peach colour to pale tan. Conidiophores delicately roughened, fasciculate; penicilli compact, mostly 3-verticillate; conidia globose or subglobose, smooth, mostly 3·5 μ. in diameter. The colony colours and the rough conidiophores united in fascicles leave no doubt that all the five isolates belong to *P. cyclopium* Westling.'

Isolation and purification of viridicatin

Batches, usually of one hundred 1 l. conical flasks, each containing 350 ml. of Czapek-Dox solution, were sterilized and sown with a spore suspension of the chosen strain of *P. cyclopium* grown on Czapek-agar slopes. The flasks were incubated at 24° for the period of time shown in Table 3. After 4–5 days the surface of the liquid was covered with a blue-green mycelial mat which began to curl slightly after 2 weeks. The reverse of the mycelium was very often pitted with long white needles of palitantin and had brown to dark brown patches. The pigment partly diffused into the culture fluid giving it a brownish colour when the flasks were harvested.

Antifungal tests on the culture filtrates from strains 525 and 578 were carried out by the method of Brian & Hemming (1945) using spores of *Botrytis allii* Munn. as the test organism and adjusting the pH of the germination medium to 4·0 with H_3PO_4. Both strains showed antifungal activity, and the details for strain 525 are as follows: after 7 days, no germination of spores at a dilution of the culture filtrate of 1:32; after 9, 16, 18 and 22 days, no germination at a dilution of 1:64.

After 22 days' incubation, forty-three flasks of strain 525 were harvested and the culture filtrate was extracted twice with half its volume of ether. Removal of the solvent gave 12·5 g. of a brownish, largely crystalline solid consisting mainly of palitantin (Birkinshaw & Raistrick, 1936; Birkinshaw, 1952) but giving a purplish red ferric reaction owing to the presence of frequentin (Curtis, Hemming & Smith, 1951; Curtis & Duncanson, 1952). The brown solid was fractionated as follows. 5 g. were triturated with ether (150 ml.) to remove readily soluble impurities. The residual

Table 3. *Cultural characteristics and yields of metabolic products of strains of* Penicillium cyclopium

For details see text.

L.S.H.T.M. catalogue no.	Incubation period (days)	pH of culture filtrate	Residual glucose (by polarimeter) (%)	Weight of mycelium (g.)	Weight of viridicatin (g.)	Weight of palitantin (g.)
525*	22	5·5	0·26	107	0·6	10·2
578	24	6·4	0·10	200	3·1	1·2
716	24	6·2	0·07	212	3·2	4·1
747	24	5·2	0·64	207	6·5	9·2
747	26	5·6	0·30	225	3·7	9·1
747	16	4·8	0·87	222	3·8	14·1
747	21	5·5	0·35	225	1·0	7·7
747	21	5·2	0·50	230	1·7	9·4
747	21	5·4	0·60	247	2·0	9·0
754	21	6·0	0·48	230	1·9	11·5
754	18	5·2	1·01	225	3·6	22·9

* This batch consisted of only forty-three flasks, all other batches of 100 flasks.

solid was dissolved in $CHCl_3$ (200 ml.) and the $CHCl_3$ solution was shaken with 2 N-Na_2CO_3 (50 ml.) to extract frequentin.

Removal of the $CHCl_3$ gave palitantin (4·08 g. ≡ 10·2 g. for forty-three flasks) which was purified by crystallization from ethyl acetate giving colourless needles m.p. 164–165°, not depressed on admixture with an authentic specimen from *P. palitans*. (Found: C, 66·2; H, 8·6. Calc. for $C_{14}H_{22}O_4$: C, 66·1; H, 8·7%.)

The Na_2CO_3 extract was acidified with HCl. A brown oil separated and crystallized overnight at 0°. It was purified by crystallization first from water (charcoal) (0·63 g. ≡ 1·6 g. for forty-three flasks, m.p. 128–132°) and then from benzene giving frequentin as colourless plates, m.p. 134° not depressed on admixture with an authentic specimen from *P. frequentans* and giving the same purple-red ferric colour. (Found: C, 66·7; H, 8·1. Calc. for $C_{14}H_{20}O_4$: C, 66·6; H, 8·0%.)

On similar treatment, the culture filtrate from forty-three flasks of strain 578, grown for 22 days, gave 11·2 g. of crude ether extract from which palitantin and frequentin were isolated.

The culture filtrates from strains 578, 716, 747 and 754 were extracted with $CHCl_3$ for the recovery of palitantin. We are indebted to Dr A. Kamal for the experimental data concerning this substance given in Table 3, column 7.

The mycelium was washed with a little water, drained, pressed and dried *in vacuo* at 40–50°. The dried mycelium was ground and extracted in a Soxhlet with light petroleum (b.p. 40–60°) for 8 hr. Removal of the solvent from this extract gave an oily, partly crystalline residue containing fat, palitantin and traces of viridicatin. The light-petroleum-extracted mycelium was dried and re-extracted with ether until the ether extract no longer gave a green ferric reaction. Removal of the ether gave a brownish crystalline residue of crude viridicatin, melting usually in the range 250–258°, which was purified by repeated fractional crystallization from methanol. Viridicatin was thus obtained as colourless needles, m.p. 268°. (Found: C, 75·5; H, 4·7; N, 6·4. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.)

General properties of viridicatin

Viridicatin crystallizes in colourless, shining needles which show a definite violet fluorescence on exposure to ultraviolet light. No fluorescence is shown by cyclopenin under the same conditions. Viridicatin melts at 268° without decomposition and the melt resets on cooling at 250° and remelts at 268°. A sublimate of colourless well defined and characteristic prisms is formed in the m.p. tube. It readily sublimes unchanged in high vacuum at 160–170°. It crystallizes readily from ethanol (0·78 g. in 60 ml. ethanol). Its ethanolic solution (0·25%) showed no optical rotation in a 4 dm. tube. It does not dissolve in aqueous $NaHCO_3$ but dissolves readily in cold aqueous 2 N-KOH and is precipitated in crystalline clusters on saturating this solution with CO_2. It dissolves with some difficulty in cold conc. HCl but readily in glacial acetic acid and is precipitated in fine, colourless needles on diluting either solution with water. It gives no colour with either hot or cold conc. H_2SO_4, but on addition of $NaNO_2$ to the latter solution, a deep brownish red colour is produced. Addition of Br_2 water to a solution of viridicatin in dilute aqueous acetic acid gives no precipitate (cf. cyclopenin). The most characteristic colour reaction of viridicatin is the intense emerald-green colour given immediately (cf. cyclopenin) on addition of small amounts of $FeCl_3$ to its ethanolic solution. This colour is completely discharged on addition of conc. HCl and changes to a deep orange-red on addition of a few drops of aqueous N-NaOH, the original green colour being restored in each case on adjustment to a slightly acid pH.

Derivatives of viridicatin

Monoacetylviridicatin. A solution of viridicatin (0·1 g.) in acetic anhydride (0·6 ml.) was refluxed for 15 min., cooled, poured into water (4 ml.) and boiled for 1 min. The colourless crystals which separated on cooling were collected and recrystallized from aqueous ethanol giving *monoacetylviridicatin* (0·05 g.) as short colourless prisms, m.p. 200–201°. (Found: C, 72·6; H, 4·7; N, 5·0, 5·3. $C_{17}H_{13}O_3N$ requires C, 73·1; H, 4·7; N, 5·0%.) The acetate decomposes slowly on standing, but a freshly prepared specimen gives no green ferric colour in ethanol solution nor is it soluble in aqueous Na_2CO_3.

3:5-*Dinitrobenzoic acid salt.* Pure 3:5-dinitrobenzoic acid (0·2 g.) was added to a hot saturated solution of viridicatin (0·1 g.) in glacial acetic acid. The bright yellow solution, on cooling, deposited rosettes of yellow prisms of the 3:5-*dinitrobenzoic acid salt of viridicatin* (0·2 g.), m.p. 228–230° raised to a constant m.p. of 238° by recrystallization from glacial acetic acid. (Found: C, 58·4; H, 3·4; N, 9·75. $C_{15}H_{11}O_2N.C_7H_4O_6N_2$ requires C, 58·8; H, 3·4; N, 9·4%.)

OO-*Dimethylviridicatin*. A mixture of viridicatin (1 g.), methanol (25 ml.) and a large excess of Ag_2O and methyl iodide was refluxed for 4 hr., cooled, filtered and the residue washed with methanol. The clear yellow filtrate which no longer gave a ferric reaction, was decolorized with a little charcoal and filtered. On removal of the solvents from the filtrate there remained a white residue (1·0 g.), m.p. 60–140°, consisting of a mixture of *OO*-dimethyl and *ON*-dimethylviridicatin which was fractionated from light petroleum (b.p. 40–60°). Two crude fractions were obtained. (*a*) 0·15 g., m.p. 160–180°, insoluble in light petroleum, contained the *ON*-dimethyl ether which is more conveniently prepared as described below. (*b*) 0·7 g., m.p. 70–110°, soluble in light petroleum, was purified by fractional crystallization from ethanol, giving OO-*dimethylviridicatin* as colourless glistening plates, m.p. 86–87°. (Found: C, 76·5; H, 5·7; N, 5·5; OMe, 23·7. $C_{17}H_{15}O_2N$ requires C, 76·95; H, 5·7; N, 5·3; 2OMe, 23·4%.)

ON-*Dimethylviridicatin*. A mixture of viridicatin (1 g.), methanol (50 ml.) and dimethyl sulphate (3 ml.) was refluxed, and NaOH pellets (total, 2 g.) were added from time to time to keep the reaction alkaline. After 1·5 hr. a green ferric reaction could no longer be detected. Water (70 ml.) was added to the hot solution from which, on cooling, the crude methyl ether separated (0·95 g., m.p. 184–190°). Recrystallization from ethanol gave ON-*dimethylviridicatin* as colourless glossy plates, m.p. 197–198°. (Found: C, 76·65; H, 5·7; N, 5·2; OMe, 11·7, 11·25. $C_{17}H_{15}O_2N$ requires C, 76·95; H, 5·7; N, 5·3; 1 OMe, 11·7%. In the Zeisel methoxyl estimation the temperature was not allowed to rise above 130–135° to avoid demethylation of the N.Me group.)

Degradation of viridicatin

(*a*) *By alkaline oxidation: Isolation of* o-*aminobenzophenone* (I) *and oxalic acid*. CO_2-free O_2 was bubbled through a solution, held at room temp., of viridicatin (0·5 g.) in 10% ethanolic KOH (50 ml.). After a few minutes the clear colourless solution became yellow and the depth of colour increased with time. The reaction was stopped after 7–8 hr., when the solution no longer gave any green ferric colour, and the pH was adjusted to about 5 with aqueous 2N-HCl. Ethanol was removed *in vacuo*. On cooling, yellow crystals of *o*-aminobenzophenone (I) separated and were collected together with a little more extracted with ether from the mother liquors. Combined yield, 0·42 g., m.p. 95–100°. The product was purified by repeated sublimation in high vacuum at 70–80° giving pure *o*-aminobenzophenone as yellow plates, m.p. 106–107°, not depressed on admixture with an authentic synthetic specimen of the same m.p. (Scheifele & DeTar, 1952). (Found: C, 78·6; H, 5·6; N, 7·3. Calc. for $C_{13}H_{11}ON$: C, 79·2; H, 5·6; N, 7·1%.) The monoacetyl derivative of the degradation product, prepared in the usual way, crystallized from aqueous methanol as colourless tablets, m.p. 88–89° alone or in admixture with an authentic synthetic specimen of the same m.p. (Found: C, 75·2; H, 5·5; N, 5·9. Calc. for $C_{15}H_{13}O_2N$: C, 75·3; H, 5·5; N, 5·9%.) The hydrochloride, long colourless needles from conc. HCl, m.p. 175° (decomp.), and the mono-2:4-dinitrophenylhydrazone, dark red, diamond-shaped crystals from glacial acetic acid, m.p. 261–262° (decomp.), were also prepared.

In a second experiment viridicatin (0·48 g.) was oxidized as described above. The oxidation solution was adjusted to pH 6·5 with N acetic acid (140 ml.). Ethanol was removed *in vacuo* and the *o*-aminobenzophenone which separated was collected by filtration and washed well with water. The combined filtrate and washings were acidified with glacial acetic acid (1 ml.), and 0·5N-$CaCl_2$ (22 ml.) was added. Calcium oxalate which separated as a white ppt. was collected next day and dried at 110° (0·264 g.; calc. for 1 mole $(COO)_2Ca$, H_2O = 0·297 g.). The ppt. was then suspended in 2N-H_2SO_4 (100 ml.) and extracted continuously with ether for 3 days. The ether extract was dried (Na_2SO_4) and the solvent removed. The residue was twice sublimed in high vacuum at 80–90° and the sublimate (0·1 g.) was equilibrated with water vapour to give oxalic acid dihydrate, m.p. 101°. (Found: C, 19·5; H, 5·1. Calc. for $(COOH)_2$, $2H_2O$: C, 19·1; H, 4·8%.)

(*b*) *By* $KMnO_4$ *oxidation in acetone: Isolation and synthesis of oxalyl-o-aminobenzophenone* (III). $KMnO_4$ (6·0 g.) was added in two portions to a solution of viridicatin (1 g.) in acetone (200 ml.), and the mixture was refluxed for 4 hr. The acetone solution was then nearly colourless and was separated by filtration and discarded. The dark MnO_2 precipitate was suspended in 2N-H_2SO_4 (200 ml.) and SO_2 was passed until a clear solution was obtained. Pale yellow needles separated on cooling and were collected (0·95 g., m.p. 160–175°). This product was crystallized several times from $CHCl_3$ (charcoal) and *oxalyl-o-aminobenzophenone* (III) was thus obtained in clusters of fine colourless needles, m.p. 184° (decomp.). (Found: C, 66·3; H, 4·2; N, 5·0; equiv. by potentiometric titration, 270. $C_{15}H_{11}O_4N$ requires C, 66·9; H, 4·1; N, 5·2%; equiv. titrating as a monobasic acid, 269.) (III) (0·265 g.; m.p. and mixed m.p. 184°) was also obtained by oxidizing viridicatin (0·5 g.) at 100° with H_2O_2 (100 vol.; 5 ml.) in glacial acetic acid (20 ml.).

Freshly distilled ethoxalyl chloride (0·3 ml.; Fourneau & Sabetay, 1928) was added to a solution of *o*-aminobenzophenone (0·1 g.) in pyridine (0·5 ml.). When the initial vigorous reaction had subsided the mixture was warmed on the water bath for 15 min., cooled and poured into water (10 ml.). A colourless oil, which quickly solidified, separated and was collected (0·137 g., m.p. 70–115°). Crystallization from ethanol gave *ethoxalyl-o-aminobenzophenone* (II) as colourless irregular plates, m.p. 123–124°. (Found: C, 68·6; H, 5·2; N, 4·85. $C_{17}H_{15}O_4N$ requires C, 68·7; H, 5·1; N, 4·7%.)

The pure ester (II; 0·125 g.) was dissolved in aqueous N-NaOH (2 ml.) and held at room temp. for 20 min. A little of the ester was completely hydrolysed to *o*-aminobenzophenone (I), but, after acidification with 2N-HCl (1 ml.), oxalyl-*o*-aminobenzophenone (III) separated and was collected in good yield as a yellowish product (0·11 g.; overall yield, 81%), m.p. 172–179° (decomp.), raised to 184° (decomp.) on crystallization from $CHCl_3$ and not depressed on admixture with the $KMnO_4$ oxidation product from viridicatin. (Found: C, 66·7; H, 3·9; N, 5·65. $C_{15}H_{11}O_4N$ requires C, 66·9; H, 4·1; N, 5·2%.)

Synthesis of viridicatin

o-Aminobenzophenone (I; 5 g.), m.p. 105°, was dissolved in warm (temp. not above 100°) molten chloroacetic anhydride (6·5 g.). The orange solution was kept below 100° for 1 min. and was then cooled to room temp. The reaction mixture quickly crystallized and finally solidified. The product was ground in ice cold water (50 ml.), filtered, washed with water and dried (5·48 g., m.p. 92–96°). Two crystallizations of the product from methanol gave *chloro-*

acetyl-o-aminobenzophenone (IV) as colourless long needles, m.p. 107–108°. (Found: C, 65·8; H, 4·3; N, 5·8; Cl, 13·2. $C_{15}H_{12}O_2NCl$ requires C, 65·8; H, 4·4; N, 5·1; Cl, 13·0%.)

Cyclization of the chloroacetyl derivative was effected by dissolving it (5·36 g.) in hot ethanol (50 ml.) and diluting with water (140 ml.). When the solution became clear again, NaOH (1·5 g.) in water (10 ml.) was added, and a yellow colour developed owing to partial hydrolysis. The solution was refluxed for 30 min. and acidified with HCl while still hot. On cooling, colourless, chlorine-free, prismatic needles separated and were collected (4·3 g., 72% overall yield, m.p. 240–242°). Crystallization from ethanol gave synthetic viridicatin, m.p. 264–265°, not depressed on admixture with natural viridicatin from *P. cyclopium*. (Found: C, 75·7; H, 4·7; N, 6·1. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.) The two specimens also gave identical intense emerald-green ferric reactions. The *ON*-dimethyl derivative of synthetic viridicatin, prepared as described above, melted at 197–198° and gave no depression in m.p. on admixture with the *ON*-dimethyl derivative of natural viridicatin of the same m.p. (Found: C, 76·8; H, 5·7; N, 5·9. Calc. for $C_{17}H_{15}O_2N$: C, 76·95; H, 5·7; N, 5·3%.)

SUMMARY

1. A hitherto undescribed mould metabolic product, cyclopenin, has been isolated from culture filtrates of laboratory cultures of a strain of *Penicillium cyclopium* Westling.

2. Cyclopenin, $C_{17}H_{14}O_3N_2$, forms colourless tablets, m.p. 207°, $[\alpha]_{5461}^{20} - 306°$ in ethanol.

3. Cyclopenin is readily decomposed by dilute mineral acids giving one mole each of carbon dioxide, methylamine and viridicatin, $C_{15}H_{11}O_2N$, which is optically inactive.

4. Viridicatin, previously isolated from *P. viridicatum* Westling, by Cunningham & Freeman (1953), has now been prepared from five different strains of *P. cyclopium* but appears to be absent from the strain of this species which gives cyclopenin.

5. Viridicatin is oxidized in alkaline solution by gaseous oxygen to one mole each of *o*-aminobenzophenone and oxalic acid.

6. Viridicatin has been synthesized and has been shown to be 2:3-dihydroxy-4-phenylquinoline or its keto tautomer.

7. The five strains of *P. cyclopium* which gave viridicatin also gave good yields of palitantin and two of them gave the anti-fungal substance, frequentin.

8. Two possible, closely related, structures are suggested for cyclopenin.

We wish to thank Sir Robert Robinson, O.M., F.R.S., for his friendly advice on the structure of cyclopenin.

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The Purification of Alkaline Phosphatases of Animal Tissues

By R. K. MORTON*

Department of Biochemistry, University of Cambridge

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Despite the fact that much of the considerable literature concerning alkaline phosphatases is devoted to methods for purification, no procedure for obtaining a homogeneous preparation from animal tissues has so far been described. This may possibly be due to the fact that the cytoplasmic alkaline phosphatases are intimately associated with insoluble cellular granules. Special procedures must therefore be used to obtain these enzymes in true solution in order to permit of purification.

The difficulty of extracting alkaline phosphatases from tissue 'homogenates' was recognized by early investigators (see Ehrensvärd, 1933). It was found, however, that various forms of proteolysis, such as controlled autolysis (Albers & Albers, 1935) and

* Present address: Department of Biochemistry, University of Melbourne, Australia.