ALKALOID BIOSYNTHESIS IN *PENICILLIUM CYCLOPIUM*—DOES IT REFLECT GENERAL FEATURES OF SECONDARY METABOLISM?[1]

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1. Introduction

Molds of the genus *Penicillium* synthesize several types of alkaloids. Compounds of the cyclopenin-viridicatin group are formed in species of the section Asymmetrica, subsection Fasciculata (table 1). As yet, these compounds have not been found in any other organisms. This restriction to a few species, the formation of the compounds by the producer strains during a certain developmental stage only (cf. section 6), and the fact that the substances are without use for the individual specialized cells synthesizing them but may be of importance for the producing organism as a whole (cf. section 10), demonstrate that the alkaloids are secondary products (Luckner, 1971, Luckner et al. 1977).

P. cyclopium was used in the investigation of different aspects of biosynthesis of the alkaloids of the cyclopenin-viridication group. In this review, we will discuss to what extent alkaloid synthesis in this mold may be considered as a model showing general characteristics of secondary product formation.

2. The path of alkaloid biosynthesis

The biosynthetic pathway (fig. 1) by which the alkaloids of the cyclopenin-viridication group are first formed was investigated *in vivo* by the tracer technique (Luckner and Mothes 1962, 1963, Luckner and Nover 1971, Nover and Luckner 1969a and b, Nover and Luckner 1971, Framm et al. 1973). All C- and N-atoms of the alkaloids are derived from L-phenylalanine, anthranilic acid, and L-methionine. Both the oxygen atom of the epoxide ring in cyclopenin and cyclopenol and the oxygen atoms of the hydroxyl groups in viridication, cyclopenol, and viridicatol come from molecular oxygen. Cyclopeptine and dehydrocyclopeptine are intermediates in cyclopenin biosynthesis, and cyclopenin is transformed into cyclopenol. In an additional step, the benzodiazepine alkaloids cyclopenin and cyclopenol are rearranged to form the quinoline alkaloids viridication and viridicatol. Further transformation or degradation of these alkaloids has not been observed.

This biosynthetic pathway shows that the alkaloids are derived from primary metabolic products by common reactions, i.e., peptide bond formation, methylation, dehydrogenation, hydroxylation, formation of epoxide groups on isolated double bonds and the subsequent rearrangement of the epoxides. Hence, as with other secondary products, it is the unusual combination, rather than the occurrence of peculiar reactions, which characterizes biosynthesis of the alkaloids.

3. The enzymes of alkaloid biosynthesis

All reactions of alkaloid formation in *P. cyclopium* are enzyme catalyzed.

[1]This paper was presented as a plenary lecture at the Twentieth Annual Meeting of the American Society of Pharmacognosy at Purdue University, West Lafayette, Indiana, July 30–August 3, 1979.

TABLE 1. Occurrence of the alkaloids of the cyclopenin-viridicatin group.

Compound	Producer	Structure elucidated by
Cyclopeptine	*P. cyclopium* Westling, *P. viridicatum* Westling (Framm et al., 1973)	Chemical-physical assay, degradation, synthesis (Framm et al., 1973, El Azzouny et al., 1977)
Dehydrocyclopeptine	*P. cyclopium* Westling, *P. viridicatum* Westling (Framm et al., 1973)	Chemical-physical assay, degradation, synthesis (Framm et al., 1973, El Azzouny et al., 1977)
Cyclopenin	*P. cyclopium* Westling, *P. viridicatum* Westling (Birkinshaw et al., 1963; cf. also Bracken et al., 1954)	Chemical-physical assay and degradation (Mohammed and Luckner, 1963), synthesis of the racemate (Smith et al., 1968, Martin et al., 1969, White et al., 1970, Richter et al., 1974), mass spectrometry (Luckner et al., 1969a, McCamish and White, 1970)
Cyclopenol	*P. cyclopium* Westling, *P. viridicatum* Westling (Birkinshaw et al., 1963)	Chemical-physical assay and degradation (Mohammed and Luckner, 1963), synthesis of the racemate (White et al., 1970), mass spectrometry (Luckner et al., 1969a)
Viridicatin[a]	*P. crustosum* Thom (Ciegler and Hou, 1970; Taniguchi and Satomura, 1970), *P. cyclopium* Westling (Bracken et al., 1954; Ciegler and Hou, 1970; Guseva et al., 1972), *P. granulatum* Bainier (Ciegler and Hou, 1970), *P. olivido-viride* Biourge (Ciegler and Hou, 1970), *P. palitans* Westling (Ciegler and Hou, 1970), *P. puberulum* Bainier (Austin and Meyers, 1964; Ciegler and Hou, 1970), *P. viridicatum* Westling (Cunningham and Freeman, 1953)	Degradation (Cunningham and Freeman, 1953), synthesis (Cunningham and Freeman, 1953; Eistert and Selzer, 1962), mass spectrometry (Luckner et al., 1969a, McCamish and White, 1970)
Viridicatol	*P. cyclopium* Westling (Birkinshaw et al., 1963), *P. viridicatum* Westling (Luckner and Mothes, 1962; Luckner and Mothes, 1963; Birkinshaw et al., 1963)	Degradation (Birkinshaw et al., 1963), synthesis (Luckner and Mohammed, 1964)
3-O-Methylviridicatin	*P. puberulum* Bainier (Austin and Meyers, 1964)	Chemical-physical assay, synthesis (Austin and Meyers, 1964)

[a]It is shown in section 2 that viridicatin is formed via cyclopeptine, dehydrocyclopeptine and cyclopenin. Hence these alkaloids should occur in all species in which viridicatin has been found.

This is true also for the transformation of cyclopenin/cyclopenol into viridicatin/viridicatol, which proceeds easily in acidic or alkaline solution without enzyme catalyzation (Bracken et al. 1954, Birkinshaw et al. 1963, White and Dimsdale 1969). This corresponds with results demonstrating that in secondary metabolism spontaneous, i. e., non-enzyme catalyzed reactions, are of minor importance (cf. Luckner 1972).

The results of the *in vivo* experiments suggest that five enzymes and enzyme systems are probably involved in the formation of the alkaloids (fig. 1):

a) CYCLOPEPTINE SYNTHETASE.—This enzyme-complex is still hypothetical. It is assumed to catalyze the activation of anthranilic acid and phenylalanine,

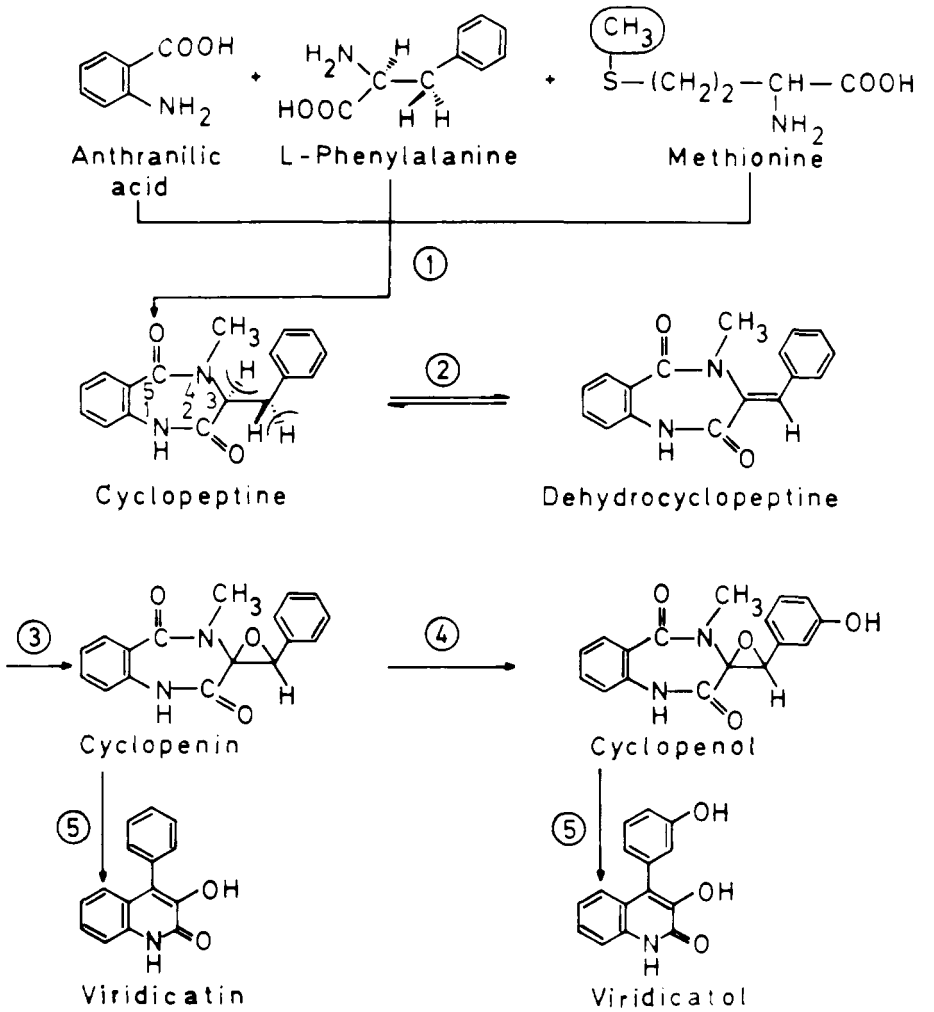

FIG. 1. Formation of the alkaloids of the cyclopenin-viridicatin-group in *P. cyclopium*. (1) Cyclopeptine synthetase complex: hypothetic; presumably catalyzing activation of anthranilic acid and phenylalanine, formation of peptide bonds, and methylation; intermediates evidently are covalently bound to the enzyme complex; (2) cyclopeptine dehydrogenase; (3) dehydrocyclopeptine epoxidase; (4) cyclopenin *m*-hydroxylase; (5) cyclopenase.

the synthesis of the two peptide bonds, and the methylation which are necessary to form cyclopeptine (Framm et al. 1973). As yet, however, the activation of the precursor anthranilic acid is the only reaction measurable *in vitro*. It is catalyzed by anthranilate adenylyltransferase (ATP: anthranilate adenylyltransferase) (N. Schwelle, S. Voigt, unpublished results).

Natural substrates: Anthranilate (K_m $139 \cdot 10^{-3}$ M), ATP (K_m $3.7 \cdot 10^{-3}$ M); Inhibitors: PP, AMP, *p*-Chloromercuribenzoate; pH optimum: 6.7 (Tris-HCl).

b) CYCLOPEPTINE DEHYDROGENASE (Cyclopeptine: NAD(P) oxidoreductase) (Aboutabl and Luckner 1975, Aboutal et al. 1976).—The enzyme is an NAD(P)-

dependent flavoprotein which catalyzes the reversible transformation of cyclopeptine into dehydrocyclopeptine. It reacts with the 3S-isomer of cyclopeptine, only. During the dehydrogenation of cyclopeptine, hydrogen atoms are displaced from positions 3 and 10 of the benzodiazepine nucleus, almost certainly by a synperiplanar elimination. The hydrid ion split off is transferred to the 4-proR position of NAD (cf. also Kirby and Narayanaswami 1976, who failed to demonstrate this in *in vivo* experiments).

Natural substrates: Cyclopeptine (K_m $1.6 \cdot 10^{-3}$ M), NAD (K_m $2.8 \cdot 10^{-4}$ M), NADP; dehydrocyclopeptine, NAD(P)H; pH optimum: 9.1 (Tris-HCl)

c) DEHYDROCYCLOPEPTINE EPOXIDASE (Dehydrocyclopeptine, NAD(P)H: O_2 oxidoreductase (Voigt and Luckner 1977, S. Voigt, unpublished results).—Tracer experiments performed on living cells demonstrated that the epoxide oxygen present in the molecule of cyclopenin and cyclopenol is derived from molecular oxygen (Nover and Luckner 1969a and b) indicating that it is introduced by a mixed function oxygenase. *In vitro* experiments showed that this enzyme uses NAD(P)H or other reducing compounds directly as cosubstrates. Inhibitor experiments indicated that the enzyme is an Fe^{2+} activated FAD-containing flavoprotein.

Natural substrates: Dehydrocyclopeptine (K_m $1.7 \cdot 10^{-4}$ M), NADH (K_m $1.4 \cdot 10^{-4}$ M), NADPH (K_m $1.2 \cdot 10^{-4}$ M), O_2; Unnatural substrates: Ascorbic acid (K_m $7.5 \cdot 10^{-3}$ M), D,L-6-methyl-5,6,7,8-tetrahydropteridine (K_m $3.1 \cdot 10^{-3}$ M); Inhibitors: Cyclopenin (K_i $1.4 \cdot 10^{-4}$ M), cyclopenol (K_i $1.1 \cdot 10^{-3}$M), viridicatin (K_i $2.2 \cdot 10^{-5}$ M), viridicatol (K_i $1.1 \cdot 10^{-4}$ M) KCN, 1,10-phenanthroline, dicoumarol, *p*-chloromercuribenzoic acid; pH optimum: 7.5 (Tris-HCl).

d) CYCLOPENIN *m*-HYDROXYLASE (Cyclopenin, NAD(P)H: O_2 oxidoreductase, 3'-hydroxylating) (Richter and Luckner 1976, P. Breitung, unpublished results).—Incorporation of oxygen from $^{18}O_2$ into the hydroxyl group revealed that the hydroxylation is catalyzed by a mixed-function oxygenase (Nover and Luckner 1969a and b). The hydroxylation is accompanied by an NIH-shift. Cyclopenin *m*-hydroxylase may use directly different reduced cosubstrates. Inhibition by dicoumarol (but not by CO) indicates that it is a flavoprotein.

Natural substrates: Cyclopenin (K_m $0.80 \cdot 10^{-3}$ M), NADH ($0.76 \cdot 10^{-4}$ M), NADPH ($0.62 \cdot 10^{-4}$ M), O_2; Unnatural substrates: *N*(1)-Methylcyclopenin (K_m $0.76 \cdot 10^{-3}$ M), cyclopeptine (K_m $3.4 \cdot 10^{-3}$ M), dehydrocyclopeptine (K_m $2.5 \cdot 10^{-3}$ M), ascorbic acid ($0.40 \cdot 10^{-3}$ M), D,L-6-methyl-5,6,7,8-tetrahydropteridine; Inhibitors: Cyclopenol (K_i $2.1 \cdot 10^{-4}$ M), viridicatin (K_i $3.6 \cdot 10^{-5}$ M), viridicatol (K_i $3.0 \cdot 10^{-4}$ M) KCN, dicoumarol, *p*-chloromercuribenzoate; pH optimum: 7.5 (Tris-HCl).

e) CYCLOPENASE (Cyclopenin methylisocyanate lyase) (Luckner 1967, Luckner et al. 1969(b), Luckner and Nover 1971, Wilson et al. 1974, Wilson and Luckner 1975, S. Wilson, unpublished results).—In contrast to the above-mentioned enzymes, which are found in hyphae and conidiospores, cyclopenase is a constituent only of the conidia. Cyclopenase uses cyclopenin and cyclopenol as substrates. From these benzodiazepine derivatives, the corresponding quinoline alkaloids are formed by the splitting-off of one mole each of CO_2 and CH_3NH_2. The CO_2 derives from the carbonyl group in position 5, the CH_3NH_2 from the N–CH_3 group in position 4 of the benzodiazepine nucleus (fig. 1). Model experiments indicate that the two carbon atoms and the nitrogen atom are presumably extruded as methylisocyanate, which is also evolved in the thermal degradation of cyclopenin and cyclopenol (Luckner et al. 1969a). Hence the methylamine and CO_2 found are the result of subsequent hydrolysis. There is no incorporation of isotopes from ^2H_2O and $H_2{}^{18}O$ into viridicatin/viridicatol during the course of the reaction. This result demonstrates that closure of the heterocyclic ring of

the quinoline alkaloids involves C-atoms 5a and 10 of the benzodiazepines and that the hydroxyl oxygen in position 3 of the quinoline is derived directly from the epoxide oxygen.

The transformation of the benzodiazepine to the quinoline alkaloids is started by the attack either of an electron acceptor on the epoxide oxygen or by an electron donor on the nitrogen atom in position 1 (White and Dimsdale 1969). Probably a tricyclic intermediate is formed, although attempts to detect a compound of this type have so far proved unsuccessful (White and Dimsdale 1969). Other mechanisms of transformation have also been formulated (Luckner and Nover 1971).

Natural substrates of cyclopenase: Cyclopenin (K_m $3.3 \cdot 10^{-4}$ M), cyclopenol (K_m $4.7 \cdot 10^{-4}$ M); Inhibitors: Cyclopeptine, dehydrocyclopeptine, viridicatin, viridicatol, N(1)-methylcyclopenin, *N,O*-dimethylcyclopenol; pH optimum: 5.0–5.6 (Succinate/NaOH).

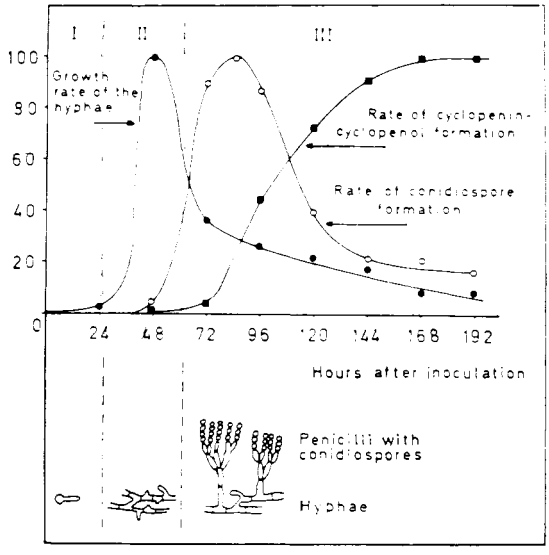

FIG. 2. Growth and cell specialization in emerged cultures of *P. cyclopium* (From Framm et al., 1973, redrawn).
Cultures were grown on a nutrient solution containing 5% glucose, 0.12% NH_4^+ and 0.025% phosphate (NL I). Synchronization of development with respect to the transition from trophophase to idiophase is brought about by removal of the surplus nutrients, i.e. by replacement of the original culture broth 48 h p.i. and then every 24 h by a nutrient solution containing only 20% of the original carbon and nitrogen amounts and 2% of the phosphate content, respectively (NL II, cf. Nover and Luckner 1974). All values are given in units/cm² culture area.

●——● Growth rate of the hyphae (100 = 79 μg);
○——○ Rate of conidiospore formation (100 = 400 000 conidia);
■——■ Rate of cyclopenin-cyclopenol formation by the hyphae (100 = 12 pmol/sec).

I Germination phase, II Trophophase, III Idiophase

The enzymes of alkaloid biosynthesis in *P. cyclopium*, like most other enzymes catalyzing reactions of secondary metabolism, do not participate in primary metabolism (cf. also section 4). However, as shown above, they possess biochemical and kinetic properties which resemble those of primary metabolic enzymes. Their *in vitro* measurable activities are relatively small. The only exception in this respect is cyclopenase (fig. 5).

4. THE CONTROL OF ENZYME AMOUNT AND ACTIVITY

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL CONTROL OF ENZYME SYNTHESIS.—A basic feature in the regulation of alkaloid biosynthesis in *P. cyclopium* is the phase-dependent formation of the alkaloids (cf. section 6 and fig. 2) and of the involved enzymes. The expression of alkaloid metabolism, like that of many other secondary pathways, is therefore the result of a differentiation process (cf. Luckner 1971, Luckner et al. 1977). Of the large number of methods used to investigate regulation of enzyme synthesis (cf. Luckner et al. 1977), the following have so far been applied to the alkaloid metabolism of *P. cyclopium:* (*a*) comparison of the time course of the synthesis of secondary products *in vivo* with the time course of the *in vitro* measurable activities of involved enzymes (Voigt et al., 1978, Luckner 1979); and (*b*) examination of the influence of inhibitors of gene expression on the expression of alkaloid biosynthesis (El Kousy et al. 1975, Nover and Müller 1975, Nover and Luckner 1976).

During the transition to the phase of alkaloid biosynthesis, the increase of the *in vitro* measurable activities of cyclopeptine dehydrogenase, dehydrocyclo-

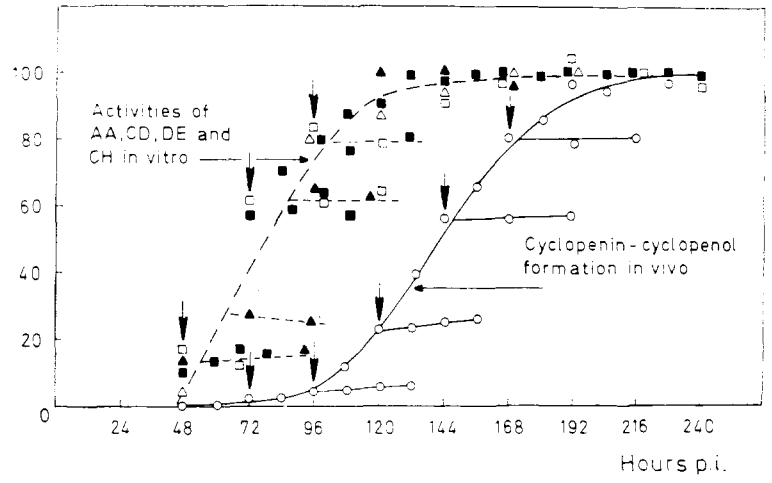

FIG. 3. *In vitro* activities of the enzymes of alkaloid biosynthesis and rates of cyclopenin-cyclopenol formation *in vivo* in hyphae of *P. cyclopium*. *P. cyclopium* was cultivated emerged by discontinuous replacement of the nutrient solution (cf. fig. 2). At time indicated by arrows cycloheximide (100 μg/ml) was added to the culture medium. All values are given in units/cm² culture area. △———△ Anthranilate adenylyltransferase activity (AA) (100=5.6 pkat); ■———■ Cyclopeptine dehydrogenase activity (CD) (100=40 pkat); □———□ Dehydrocyclopeptine epoxidase activity (DE) (100=0.42 pkat); ▲———▲ Cyclopenin *m*-hydroxylase activity (CH) (100=12 pkat); ○———○ Cyclopenin-cyclopenol formation (100=9 pmol/sec). Similar results were obtained by use of 5-fluorouracil instead of cycloheximide.

peptine epoxidase and cyclopenin *m*-hydroxylase, as well as the increase of the rates of alkaloid formation *in vivo*, are inhibited by 5-fluorouracil or cycloheximide. This inhibition indicates that the presence of RNA- and protein-biosynthesis is necessary (fig. 3). Hence regulation at the level of transcription obviously is an important feature in the expression of alkaloid formation although the de novo synthesis of the proteins involved in the alkaloid metabolism still has to be demonstrated directly, e. g., by labelling experiments. This confirms the results obtained in many other organisms where transcription was also shown to be the main regulatory principle in the expression of secondary metabolism (cf. Luckner et al. 1977, Luckner, 1979b).

In some instances, however, control of transcription is superimposed by translational and posttranslational regulation (cf. Luckner et al. 1977, Luckner 1979b). Examples in the alkaloid metabolism of *P. cyclopium* are the appearance and increase of anthranilate adenylyltransferase (fig. 4) and cyclopenase activities (cf. section 6), which cannot be prevented by cycloheximide in concentrations suppressing protein biosynthesis (El Kousy et al. 1975, N. Schwelle, unpublished results). The mechanism by which the appearance of the activities of those two enzymes is regulated, however, has so far remained unclear.

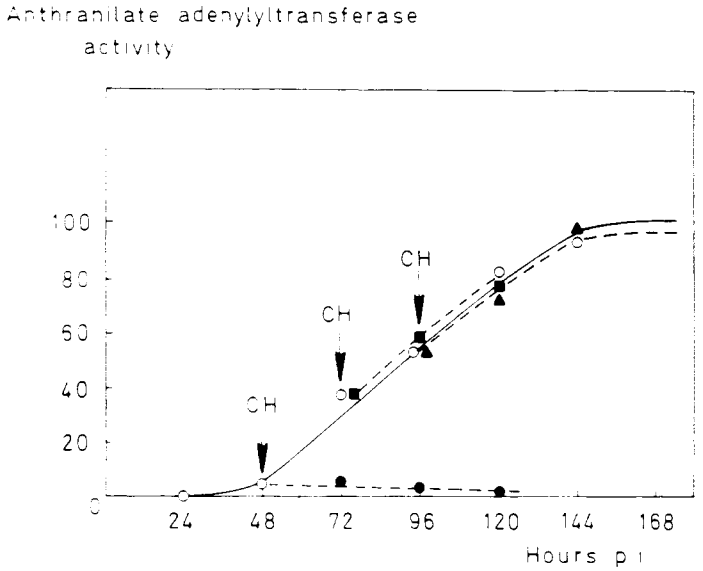

FIG. 4. *In vitro* measurable activity of anthranilate adenylyltransferase in hyphae of *P. cyclopium* after addition of cycloheximide (N. Schwelle, unpublished results).
Experimental details cf. fig. 2. Control ○———○; Cycloheximide added for 48 h: ●———● 48 h p.i., ■———■ 72 h p.i., ▲———▲ 96 h p.i.; 100 = 16.0 pkat/cm² culture area.

Experiments with inhibitors of gene expression demonstrated a relative stability of the enzymes involved in the biosynthesis of the alkaloids in *P. cyclopium* (cf. fig. 3). This is in contrast to results with many other enzymes of secondary metabolism, e. g. phenylalanine ammonia-lyase, the amount of which is regulated by continuous synthesis and degradation (cf. Hahlbrock 1977). However, in the hyphae the rates of alkaloid biosynthesis and the *in vitro* measurable activities

of the enzymes involved begin to decrease after a cultivation period of about 10–14 days due to the general processes of senescense, even if the mold is grown with a constant supply of nutrients.

Cyclopenase activity disappears during spore germination within a short period (cf. section 6.1.), though in resting spores it is stable for a long time. This different behavior is an example that stability is not an absolute characteristic, but depends on the physiological state of the cell.

REGULATION OF ENZYME ACTIVITY.—In addition to regulation by enzyme amount, secondary metabolism is controlled by regulation of the activity of secondary metabolic enzymes (cf. Luckner 1979b). Evidence in this respect comes, for instance: (a) from the large discrepancies which exist in many organisms between the relatively high enzyme activities measurable *in vitro* and the much lower *in vivo* rates of product formation and (b) from the differences in the time-course between the *in vitro* and *in vivo* activities of enzymes during the development of the producer organisms.

In some instances, the reduced *in vivo* activity is due to limited substrate supply. Hence alkaloid production in conidiospores (fig. 5) and older batch cultures of *P. cyclopium* gradually ceases due to the exhaustion of substrates.

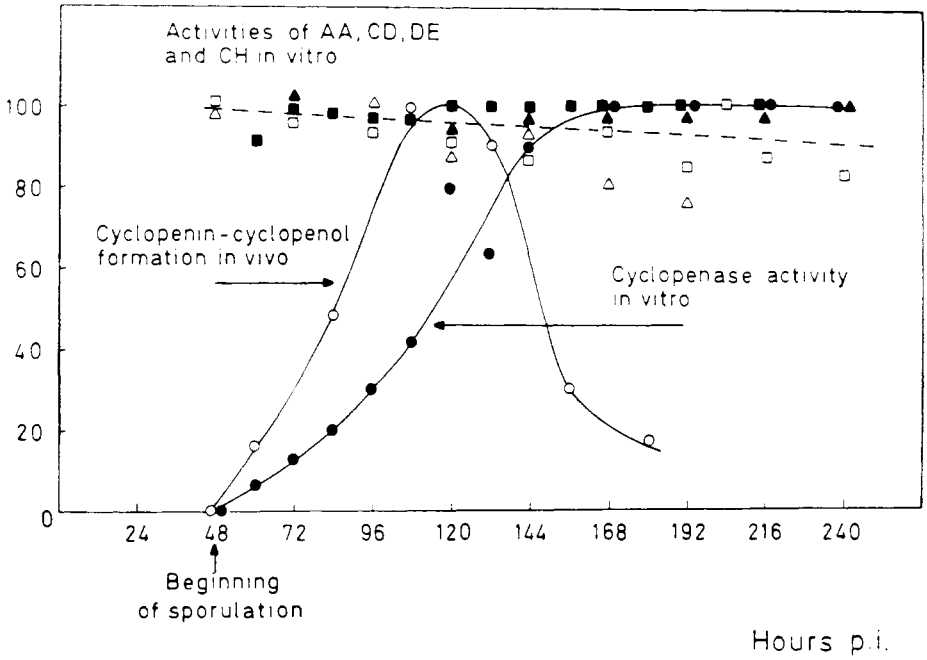

FIG. 5. *In vitro* activities of the enzymes of alkaloid biosynthesis and rates of cyclopenin-cyclopenol formation *in vivo* during ripening of the conidiospores of *P. cyclopium* (From Voigt *et al.* 1978, B. Sept and P. Breitung unpublished results).
P. cyclopium was cultivated emerged by discontinuous replacement of the nutrient solution (cf. fig. 2). At time indicated conidiospores were brushed off. All values are given per mg dry weight. △———△ Anthranilate adenylyltransferase activity (AA) (100=1.15 pkat); ■———■ Cyclopeptine dehydrogenase activity (CD) (100=16 pkat); □———□ Dehydrocyclopeptine epoxidase activity (DE) (100=0.007 pkat); ▲———▲ Cyclopenin *m*-hydroxylase activity (CH) (100=0.17 pkat); ●———● Cyclopenase activity (100=250 pkat (!)); ○———○ Cyclopenin-cyclopenol formation (100=0.5 pmol/sec).

In other cases the low *in vivo* activity is caused by compartmentation. Cyclopenase, for instance, is separated by membranes from its substrates cyclopenin and cyclopenol (cf. section 5).

Furthermore, the activity of secondary metabolic enzymes may be influenced by accumulating products (product inhibition by allosteric interaction or competition with the substrates at the binding sites) (cf. Floss et al. 1974, Luckner 1979b). With respect to the alkaloid metabolism of *P. cyclopium, inhibition* by products was shown *in vitro*, for instance, with the enzymes cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase, and cyclopenin *m*-hydroxylase (cf. section 3).

However, even relatively high amounts of cyclopenin-cyclopenol administered to living cells of *P. cyclopium* do not decrease the rates of alkaloid biosynthesis (B. Sept, unpublished results). Obviously, due to limited permeability of the plasma membrane (cf. section 5), inhibition by products present in the extraplasmic space plays no role in the alkaloid metabolism of *P. cyclopium*. Only trace amounts of the alkaloids, taken up by the cells, are able to reach the enzymes of alkaloid metabolism, and are transformed by these enzymes, as shown with radioactive labelled compounds (cf. section 2).

In many cases, the means by which the *in vivo* activity of secondary metabolic enzymes is regulated is not known. In hyphae of *P. cyclopium*, for instance, under conditions which abruptly reduce the growth of the hyphae but provide the cultures with a steady level of nutrients (exchange of the original nutrient solution by a diluted solution, with limited phosphate content, cf. legend to fig. 2), the rates of alkaloid formation further increase even though the *in vitro* measurable enzyme activities have reached maximum values (fig. 3). This "additional" increase is immediately stopped by inhibitors of gene expression (5-fluorouracil or cycloheximide) indicating the formation of a protein limiting the rate of alkaloid formation *in vivo*. This protein is synthesized much later than the enzymes investigated.

Probably the protein limiting the *in vivo* rate does not take part in the biosynthesis of the alkaloid precursors phenylalanine, anthranilic acid, and methionine, because feeding of these compounds to cultures at the beginning of alkaloid production shows no increase of alkaloid biosynthesis. One might speculate that intracellular channelling of precursors or co-substrates to the site of alkaloid biosynthesis requires a specific protein which limits the production rate. In accordance with this assumption we have shown that different pools of phenylalanine feeding protein and alkaloid biosynthesis (cf. section 5) exist in *P. cyclopium*.

5. Compartmentation and channelling in the alkaloid metabolism of P. cyclopium

The compartmentation of enzymes and products.—Many secondary metabolic enzymes are bound to or are associated with the plasma membrane or with the membranes of ER, dictyosomes, vacuoles and microbodies, whereas hydrophilic secondary products frequently accumulate in the non-plasmic compartments separated by these membranes from cytoplasm (e. g. vacuoles, Golgi vesicles and the extra plasmic space) (cf. Luckner 1979b, Luckner et al., in press). Corresponding to these general findings, the benzodiazepine alkaloids formed in *P. cyclopium* are accumulated in the extra plasmic space, i. e., the culture broth or the wall of those cells, e. g. the conidiospores, which have no direct contact with the nutrient medium (Nover and Luckner 1974).

The accumulated alkaloids are separated by the plasma membrane from cytoplasm, as may be demonstrated by the resistance of cyclopenin and cyclopenol present in the extra plasmic space to cyclopenase, which is located on the inner side of the plasma membrane of the conidiospores (Wilson and Luckner 1975). Artificial increase of membrane permeability, e. g., by lowering the cellular ATP level (Roos and Luckner 1977) greatly increases the rate of viridicatin/viridicatol biosynthesis (fig. 6). Cyclopenase becomes accessible to its substrates and the alkaloid metabolism of cultures of *P. cyclopium* under these experimental conditions resembles that of certain strains of *P. viridicatum* which, after a short period of cyclopenin/cyclopenol excretion, convert these benzodiazepine alkaloids into viridication and viridicatol (Roos et al. 1976).

Only small amounts of cyclopenin and cyclopenol are present in native conidiospores and hyphal cells. Probably they are located in membrane-surrounded organelles, e. g., vacuoles or transport vesicles. Hence cyclopenin and cyclopenol present in the conidiospores are not attacked by cyclopenase under normal physiological conditions, but are transformed to viridicatin and viridicatol after an increase of membrane permeability (Roos et al. 1976; fig. 6). Transport by vesicles from the site of synthesis to the plasma membrane has been demonstrated with many secondary products (cf. Robinson 1977, Vassiliev 1977).

The uptake of the benzodiazepine alkaloids from the medium by suspended conidiospores is tightly coupled with their transformation by cyclopenase. Cyclopenin/cyclopenol-derivatives, which are not attacked by cyclopenase do not accumulate in the spores. Also hyphal cells, which are free of cyclopenase, neither native nor permeabilized, have any storage capacity for alkaloids present in the medium. Moreover, when the artificial permeabilization is reversed by supply of ATP, which may well cause the formation of new, intact membranes, in a medium containing cyclopenin and cyclopenol, these alkaloids are not included in the cells (W. Roos, unpublished results). Hence, after direct contact with external alkaloids, the cytoplasm is not able to constitute any storage capacity for cyclopenin and cyclopenol.

The cellular location of the enzymes of cyclopenin/cyclopenol biosynthesis is uncertain. Cyclopeptine dehydrogenase and cyclopenin *m*-hydroxylase were found to sediment with as yet unidentified membrane fractions (dictyosomes?) (W. Lerbs, unpublished results). The location of anthranilate adenylyltransferase and dehydrocyclopeptine epoxidase as yet has not been investigated.

THE CHANNELLING OF PRECURSORS AND INTERMEDIATES.—Experiments with living cells revealed that in the hyphal cells of *P. cyclopium* two pools exist for exogenous phenylalanine: a low capacity, "peripheral" pool (probably the cytoplasm), and a "central", expandable pool (obviously the vacuoles) (fig. 7). Phenylalanine present in the surrounding medium is transported by two carrier systems through the plasma membrane: a high affinity system (CS I; K_m $7 \cdot 10^{-6}$ M) and a low affinity system (CS II; K_m $4 \cdot 10^{-4}$ M) (W. Roos, unpublished results). The peripheral pool, by means of these enzymes, rapidly equilibrates with exogenous phenylalanine.

Phenylalanine present in the peripheral pool serves protein and alkaloid biosynthesis. Excess phenylalanine is accumulated in the expandable pool. This phenylalanine can be reused later in alkaloid formation but not in protein formation. Hence, with respect to alkaloid formation, two channels for exogenous phenylalanine occur: a direct low capacity pathway via the peripheral pool (primary labelling of alkaloids), and an indirect high capacity pathway from the

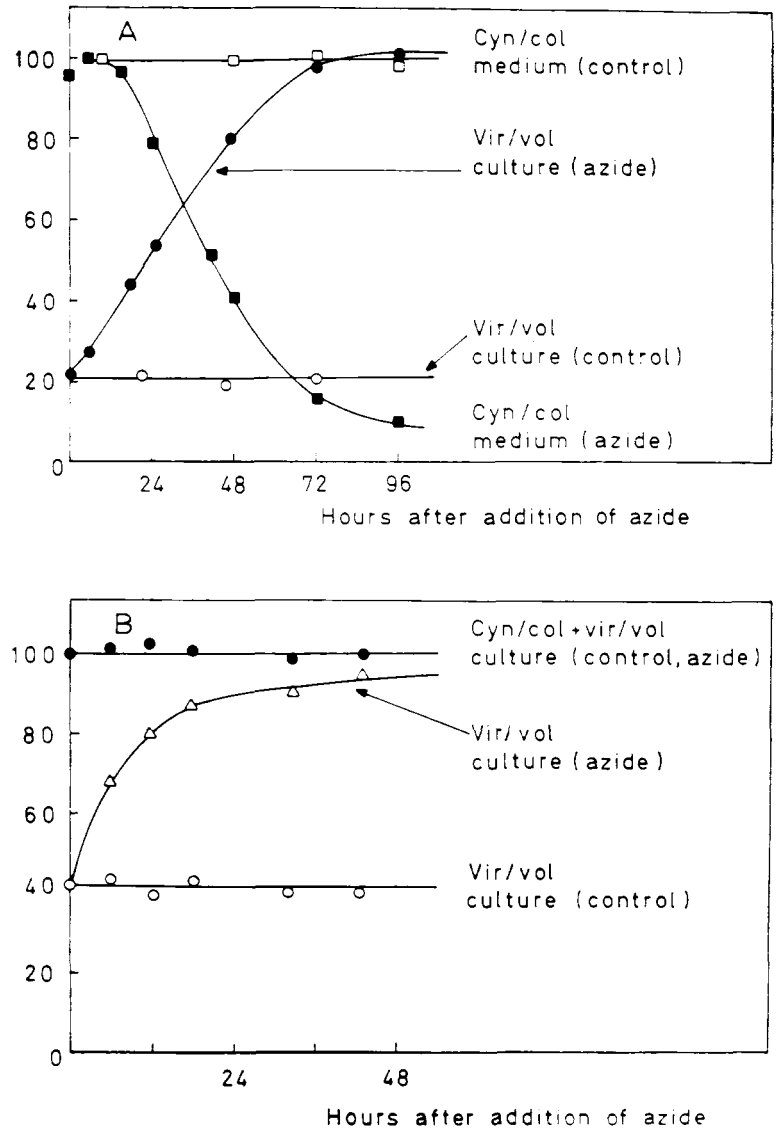

FIG. 6. Transformation of cyclopenin/cyclopenol by cyclopenase after increase of membrane permeability (From Roos et al. 1976).

P. cyclopium was grown emerged as batch culture on NL I (cf. fig. 2). 7 days after inoculation 1 mM azide was added to the culture medium causing reduction of the cellular ATP-level and by this way increase of membrane permeability.

A: Disappearance of cyclopenin/cyclopenol in the medium accompanied by the increase of the viridicatin/viridicatol content of the cultures (100 = 1.4 μmol alkaloids/cm² culture area).

B: Transformation of cyclopenin/cyclopenol present in the cells in viridicatin/viridicatol after treatment with azide (100 = 0.24 μmol alkaloids/cm² culture area).

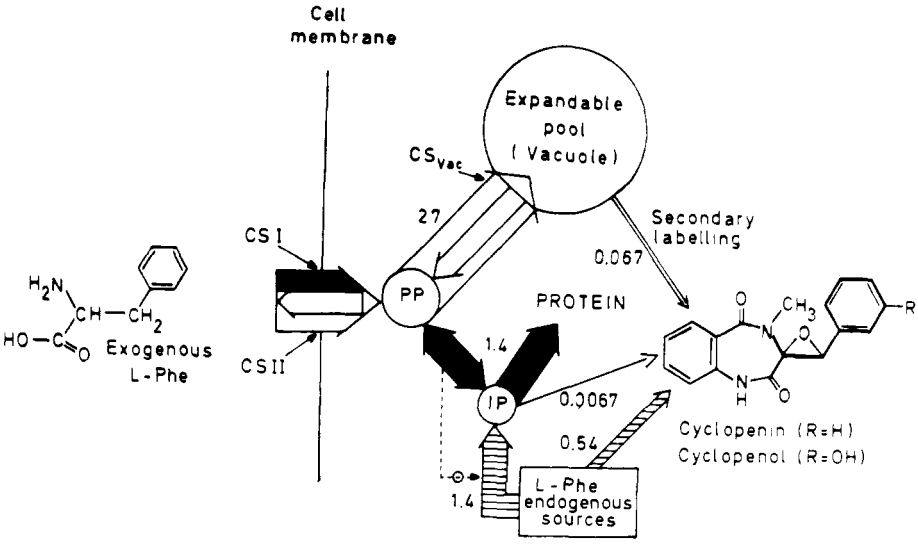

FIG. 7. Channelling of exogenous phenylalanine in *P. cyclopium* (From Nover 1979, Nover et al. 1979).

The amount of phenylalanine incorporated in the alkaloids and in proteins depends on the developmental stage of *P. cyclopium*. It is maximum with respect to the proteins in the growth phase (cf. fig. 2) (2.7 pmol/cm² culture area . sec, 48 hours after inoculation), but zero with respect to the alkaloids. In the phase of maximum alkaloid formation (i.e. in cultures with discontinuous replacement of the nutrient solution 7–10 days after inoculation, cf. fig. 2) it is only 0.4 pmol/cm² culture area . sec relative to the proteins, but about 10 pmol/cm² culture area . sec relative to the alkaloids. The fig. represents the phenylalanine fluxes 72 hours after inoculation, i.e. at an intermediate metabolic state.

Full arrows: pathway of exogenous phenylalanine predominating at low concentrations: open arrows: pathway of excess exogenous phenylalanine not incorporated in proteins; hatched arrows: pathway of endogenously produced phenylalanine (biosynthesis and/or protein degradation). The numbers besides the arrows give an estimate of the capacity of the individual channels in pmol phenylalanine/cm² culture area . sec.

CS I and CS II: high affinity and low affinity carrier systems in the plasma membrane; CS_{vac}: supposed carrier system in the vacuole membrane; PP: speculative peripheral pool in the cytoplasm which is in rapid equilibrium with the internal pool (IP).

expandable pool (secondary labelling of the alkaloids). The relative contributions of these two channels vary with the concentration of exogenous phenylalanine, the time of incubation, etc. Under all experimental conditions, however, in contrast to protein biosynthesis, about 90% of the phenylalanine incorporated into the alkaloids is recruited from endogenous sources, i. e., *de novo* synthesis and protein degradation (Nover 1979, Nover *et al.* 1979).

The intermediates of alkaloid biosynthesis also undergo channelling. This is indicated for example, by the lack of hydroxylation of cyclopeptine and dehydrocyclopeptine fed to cultures of *P. cyclopium in vivo*, although these compounds serve as substrates of cyclopenin *m*-hydroxylase *in vitro* (cf. section 3). Under the experimental conditions used the compounds are incorporated into cyclopenin and cyclopenol, i. e., enter the metabolic pathway of alkaloid biosynthesis. Probably enzyme complexes which show preference in the transformation of endogenously produced intermediates are involved in the biosynthesis of the alkaloids.

Recently an increasing number of such complexes has been detected in secondary metabolism (for summaries cf. Luckner 1979b, Luckner et al., in press).

6. Alkaloid metabolism and the development of *Penicillium cyclopium*

The phase dependance of alkaloid formation.—The development of *P. cyclopium*, like that of other organisms (cf. Luckner et al. 1977, Nover et al. 1978), proceeds as a program including a large number of subsequent steps of differential gene expression. To focus attention on some of its outstanding characteristics (germination, growth, cell specialization including expression of alkaloid formation), it may be divided into germination phase, trophophase, and idiophase. These periods are not to be understood as absolutes (cf. Bu'Lock 1975). They include many steps of differentiation as demonstrated, for instance, with the varying starting times in submerged cultures of the most important features of cell specialization: conidiation and alkaloid formation (Schröder 1978) and with the fact that alkaloid biosynthesis and spore formation themselves comprise a chain of differentiation steps. The three above-mentioned phases reveal the following features:

(a) The germination phase (lasting about 24 h). During this phase, spore dormancy is overcome. Proteins characteristic of the conidiospores, e. g., cyclopenase, which in dormant spores are stable for long periods, are degraded. The spores swell and form a germ tube. Germination needs oxygen and certain easily usable nutrients, e. g., glucose, whereas, during the later developmental phases other carbon sources like citric acid, mannitol/sorbitol, ethanol, and glycerol may also be consumed. This is obviously due to the fact that certain catabolic enzymes are only inducible in hyphal cells of a certain developmental state onwards as was shown for β-galactosidase (Ininger and Nover 1975) and invertase (W. Roos, unpublished results).

(b) The trophophase. This phase is characterized by rapid growth and branching of the hyphae. Due to the high frequency of hyphal branching at the beginning of the trophophase, dry weight and protein increase approximately exponentially. The synthesis and content of nucleic acids and proteins show maximum values (Nover and Luckner 1974).

(c) The idiophase. Characteristics of idiophase development are the formation of the specialized cells of the penicilli and the conidiospores and the biosynthesis of the alkaloids. In emerged cultures, conidiation is concentrated at the surface of the mycelium. In submerged cultures, probably any hyphal tip is able to form a penicillus.
In batch cultures, the idiophase is terminated by the exhaustion of nutrients. In cultures with replacement of nutrients, all metabolic reactions gradually cease due to the general processes of senescence.

By means of macrocolonies it has been demonstrated that each hyphal cell passes from the state of growth to that of the idiophase independent of the differentiation of its neighbour cells (Luckner 1977). This relative independence exists though all cells may be derived from a single conidiospore and, thus, are connected with each other at least indirectly. In this respect, *P. cyclopium* resembles the more highly developed multicellular organisms which are able to exist only because the cells are at the same time integrated by regulation into the whole organism, but still have a definite possibility of independent differentiation (Luckner 1977).

During the transition from the trophophase to the idiophase in hyphae, at least four processes have to be gone through before alkaloid biosynthesis really takes place (Voigt et al. 1978, N. Schwelle, B. Sept, unpublished results):

(a) Formation of a precursor of the enzyme anthranilate adenylyltransferase (terminated about 48 h p. i.) (cf. section 4)

(b) Activation of anthranilate adenylyltransferase (48–144 h p. i.) (cf. section 4)

(c) Synthesis of cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase and cyclopenin *m*-hydroxylase (48–144 h p. i.) (cf. section 4) and

(d) Formation of the protein limiting the *in vivo* rate of alkaloid biosynthesis (72–216 h p. i.) (cf. section 4).

These processes cannot be blocked by inhibitors of DNA biosynthesis, indicating that in the hyphae already existing cells become specialized with formation of the means necessary for secondary product biosynthesis. This feature is frequently found in microorganisms, but also occurs in higher plants and animals (cf. Luckner et al. 1977).

Due to the synchronous appearance of the activities of anthranilate adenylyltransferase (thought to be representative of cyclopeptine synthetase, cf. section 3.) as well as of cyclopeptine dehydrogenase, dehydrocyclopeptine epoxidase, and cyclopenin *m*-hydroxylase at the beginning of the idiophase, the alkaloids cyclopeptine, dehydrocyclopeptine, cyclopenin, and cyclopenol appear and accumulate simultaneously in the culture medium (Framm et al. 1973).

In contrast to hyphal cells, conidiospores contain the enzymes of benzodiazepine alkaloid biosynthesis as constitutive proteins (Voigt et al. 1978; Luckner, 1979a; cf. fig. 5). These enzymes are synthesized during cell division in the conidia-producing cells, the phialides.

Some very important features of alkaloid metabolism are expressed, however, only during the subsequent maturation of the spores. They are embedded in the program "spore ripening", which in emerged cultures includes the following stages (Nover and Luckner 1974, Voigt et al. 1978):

(a) Formation of the melanins which incrust the cell wall and give the mature spores their green color and their resistance against uv radiation (Ha-huy-Ke and Luckner 1979, Bartsch et al. 1979).

(b) Cyclopenin-cyclopenol biosynthesis which increases gradually in the first period of conidiospore maturation and then ceases, obviously due to lack of nutrients (Voigt et al. 1978; fig. 5). This form of behavior resembles that of the hyphal cells in batch cultures (cf. section 6.).

(c) The appearance of cyclopenase activity which is a late event in spore maturation (fig. 5). As mentioned in section 4, this process is not accompanied by protein *de novo* synthesis. Cyclopenase under normal physiological conditions remains metabolically inactive (cf. section 4.).

Spore maturation also proceeds with conidia separated from the mold colonies, demonstrating that it does not depend on interaction with hyphal cells (El Kousy et al. 1975, Bartsch et al., 1979). Conidia detachment in *P. cyclopium* is, therefore, an example of the so-called quantal cell cycles, in which daughter cells are determined during their formation to express certain qualities at further maturation that are not shown by the mother cells (cf. Holtzer and Rubinstein 1977).

7. Signals influencing alkaloid metabolism

The signals which affect secondary product formation may be subdivided into signals acting directly on the control of secondary product formation and signals which influence secondary metabolism only indirectly e. g., by affecting the programmes of differentiation and development of the producer organisms in which secondary product formation is integrated (Luckner et al. 1977, Nover et al. 1978). Alkaloid biosynthesis in cultures of *P. cyclopium* is influenced by signals acting indirectly, like special nutrients, phenylalanine and its derivatives and certain endogeneously produced hormone-like compounds.

The influence of nutrients on alkaloid biosynthesis and development.—The formation of many secondary products is suppressed by a surplus of nutrients, e. g., glucose or other easily metabolizable carbon sources, by phosphate, ammonia etc. (cf. Demain 1968, 1972, Grisebach 1975). With *P. cyclopium*, this phenomenon may be observed in submerged cultures. In glucose-containing nutrient solutions, under the normal conditions of cultivation, no alkaloids are formed and synthesis of penicilli and conidiospores is suppressed, i. e., idiophase development is barred. This suppression does not occur if sorbitol/mannitol instead of glucose, are used as carbon sources (Schröder 1976). It is overcome also if citrate and Ca^{++} are added to glucose-containing nutrient solutions (Schröder 1978), an effect which obviously is caused by stabilization of the pH in the culture broth (Schröder and Schlette 1978).

In emerged cultures, the inhibitory effect of glucose on alkaloid formation is interfered with by the morphological organization of the mycelial mat, which obviously limits nutrient supply at the upper side of the mycelium. Furthermore, this kind of growth makes possible contact of the upper hyphae with air, which is one of the most important triggers of idiophase development in *Penicillium* and other molds (Morton 1961, Rurian and Bianchi 1972). As a result, in emerged cultures of *P. cyclopium*, sporulation and alkaloid biosynthesis are suppressed by surplus glucose only to a certain extent (Luckner et al. 1977). This action demonstrates that, due to the morphological structure, a regulatory principle which under the natural conditions of development may be unfavorable is overcome.

The way in which glucose affects alkaloid metabolism and sporulation is unknown. It may be expected that, like suppression by glucose of β-galactosidase induction in *P. cyclopium* (Ininger and Nover 1975), it is not mediated by cyclic-AMP as in bacteria, but is regulated at the posttranslational level.

The indirect action of hormone-like signals and phenylalanine derivatives on the expression of alkaloid formation and other idiophase characteristics.—Analysis of the low molecular weight fraction of *P. cyclopium* has shown the occurrence of hormone-like compounds in the young trophophase hyphae, which increase the rates of alkaloid biosynthesis and spore formation (Dunkel et al. 1976, S. Khalil, unpublished results). It has been demonstrated that the compounds increase the *in vitro* measurable activity of cyclopeptine dehydrogenase indicating that an enlarged enzyme amount rather than an enhanced precursor supply causes the higher rates of alkaloid production (S. El Kousy, unpublished results).

There is a short period of competence for the hormone-like compounds at the beginning of the growth phase, i. e., long before the idiophase characteristics really appear (Dunkel et al. 1976). Similar phases, during which secondary product formation can be determined (determination phases), have been found with other microorganisms, too, (cf. Luckner et al. 1977).

After addition of the mycelial factors to cultures of *P. cyclopium* the young hyphae show faster growth; their rates of protein and nucleic acid synthesis as well as the protein content show higher values. However, at the beginning of the idiophase, these parameters are similar to those of the controls (Dunkel et al. 1976). Thus the effects of the compounds on alkaloid metabolism and sporulation cannot be due to a general acceleration of primary metabolism during the idiophase, and the action of the hormone-like effectors on the expression of alkaloid biosynthesis and sporulation is, therefore, an indirect one.

It is of interest that the administration also of the precursor amino acid phenylalanine during the determination phase may increase alkaloid formation during the idiophase. The same effect is caused by certain phenylalanine analogues which cannot be incorporated into the alkaloids (Dunkel et al. 1976). Administration of phenylalanine during the idiophase itself will not enhance the rate of alkaloid production (Dunkel et al. 1976, Nover et al. 1979). Furthermore, ethionine-resistant mutants which produce excess amounts of phenylalanine during the idiophase show rates of alkaloid biosynthesis which resemble that of the wild-type strain (Müller et al. 1978). All these results demonstrate that the influence of phenylalanine on alkaloid biosynthesis is an indirect one, similar to that of the hormone-like signals mentioned above.

8. The alkaloid metabolism of mutants with altered developmental program

There are two types of developmental mutants frequently found after treatment of conidiospores of *P. cyclopium* with mutagens (Schmidt et al. 1878):

(a) With relatively high frequency strains occur in which all idiophase characteristics, including alkaloid biosynthesis, are not expressed. This indicates that the idiophase development can be blocked at central points.

(b) In a second type of mutants, the expression of only one or some of the idiophase characteristics is reduced. Others are formed almost unchanged. For instance, the rate of alkaloid biosynthesis in the hyphae or the sporulation and, within the program of spore maturation, benzodiazepine alkaloid formation, cyclopenase activity, or pigmentation may be reduced without a resultant influence on the other parameters of the idiophase program.

These results indicate that from the main road of the developmental program by-paths branch off and that the formation of the alkaloids and other secondary products is part of these by-paths. A similar situation is encountered also with bacteria. On the one hand, mutational blocks may inhibit the whole process of sporulation at different stages, including the formation of the characteristic secondary products built during spore maturation (peptide antibiotics, dipicolinic acid, sulfolactic acid and spore pigments). On the other hand, specific blockage of secondary product synthesis and other "secondary" characteristics of sporulation may occur without inhibiting the formation of intact spores (for a summary cf. Luckner et al. 1977).

9. The integration of alkaloid metabolism in the developmental program of *P. cyclopium*

Summarizing the results of the foregoing sections, there are three arguments indicating that alkaloid biosynthesis is integrated into the developmental program of *P. cyclopium* (Luckner 1977):

(a) Its phase dependence, i.e., the fixation of its expression in relation to other chemical and morphological parameters expressed during the mold-develop-

ment (cf. section 6) and the lack of alkaloid formation if the development is stopped by physiological means (cf. section 7) or by mutation (section 8) before the stage of alkaloid synthesis is reached;

(b) The influence on alkaloid metabolism of hormone-like compounds generally affecting idiophase development (cf. section 7) and

(c) The determination of the rates of alkaloid biosynthesis at an early stage of the mold-development, the determination phase, i.e., long before alkaloid formation is really expressed (cf. section 7).

It has been shown, however, that the integration of the alkaloid metabolism into the programs of cell specialization and development is not an absolute one. By drastic changes of the physiological conditions, e. g., by submerged instead of surface cultivation (cf. section 7) as well as by mutations (Schmidt et al. 1978) the time during which alkaloid metabolism is expressed within the developmental program may be changed. This pliability seems to be due to the location of secondary metabolism on side-branches of the developmental program (cf. section 8) and renders possible the flexibility of secondary product formation, which may be of significance for the producer organism as well as for the investigation and the manipulation of secondary metabolism.

10. Has alkaloid formation any importance for the producer strains of *P. cyclopium?*

In general the importance of secondary product formation for the producing organisms may exist in two fields:

(a) The formation of secondary products may be significant, as a process permitting detoxication of waste products which otherwise would disturb general metabolism.

(b) The secondary products formed are useful as physiologically active compounds, as metabolic signals involved in the regulation of metabolism (e. g. certain hormones), or as factors participating in the manifold ecological relations by means of which the producer organisms are related to the other organisms of their biotope (cf. Harborne 1977).

For the synthesis of a large group of secondary products, however, none of these possibilities has as yet been substantiated, perhaps due to lack of appropriate experiments. Their formation has been considered as a deviation from the purely utilitarian purpose and has been explained as a play of nature or the so-called luxury of metabolism.

In *P. cyclopium,* no evidence exists that the alkaloids themselves or the process of their synthesis have any function in the regulation of cell metabolism. On the one hand, strains have been found which are free of alkaloids (Cunningham and Freeman 1953, M. Luckner, unpublished results). On the other hand, in submerged cultures in which alkaloid biosynthesis begins at a relatively late developmental stage or is not expressed at all, penicilli formation and sporulation proceed normally (cf. section 7). Furthermore, mutants of *P. cyclopium* in which alkaloid biosynthesis is strongly reduced or shifted to later developmental stages still grow and sporulate (Schmidt et al. 1978). Moreover, under laboratory conditions, the non- or low-alkaloid-producing strains formed by spontaneous segregation of the high producers (cf. Schmidt et al. 1978) easily overgrow their parent strains. This was shown also with viridicatin-producing strains of *P. crustosum* and *P. viridicatum* (Cunningham and Freeman 1953, Taniguchi and

Satomura 1970). Hence, under laboratory conditions, in which ecological relations are without importance, alkaloid production seems to be a disadvantage.

Nevertheless, the thorough control of alkaloid formation by regulation of enzyme amount and enzyme activity, by compartmentation and channelling and the integration of alkaloid formation in the developmental program of *P. cyclopium* indicate that selection pressure rests on alkaloid biosynthesis. Relative to the above discussion, this seems to be explicable if the formation of the alkaloids is, for instance, of ecological advantage for the producer organism. However, with respect to the alkaloids of the cyclopenin-viridication group, as with most other groups of secondary products, no systematic research on ecological activities has as yet been performed. The only alkaloid investigated even superficially is viridicatin, which was shown to have antibiotic activity against gram-positive bacteria, e. g., *Bacillus subtilis* and *Staphylococcus aureus* (Taniguchi and Satomura 1970) as well as against *Mycobacterium tuberculosis* (Cunningham and Freeman 1953). The alkaloid, furthermore, inhibits shoot and root formation in rice seedlings (Taniguchi and Satomura 1970). Probably these actions are caused by the metal ion-chelating properties of viridicatin (Taniguchi and Satomura 1970).

11. CONCLUSIONS

The foregoing remarks have shown that the formation of the alkaloids of the cyclopenin-viridicatin group in *P. cyclopium* exhibits some of the most characteristic features of secondary product formation, i.e.: Restriction of the synthesis of the alkaloids to a limited group of organisms; formation by specific enzymes; strict regulation of the amount and *in vivo* activity of the enzymes; compartmentation of enzymes, precursors, intermediates and products; expression during cell specialization or the *de novo* formation of specialized cells by integration in the programs of differentiation and development of the producer organisms; and lack of significance for the synthesizing cell itself, but possible importance for the producer organism as a whole.

Whereas some aspects of alkaloid metabolism are now relatively well known, in terms of chemistry, biochemistry and enzymology, other aspects are still obscure. These latter aspects include comparatively simple fields such as the regulation of the *in vivo* activity of the enzymes and the raison d'être of alkaloid biosynthesis. There is still a lack of understanding of the most complicated problems of the mode of action of the signals influencing alkaloid metabolism and of the molecular mechanisms of its integration into the program of differentiation and development. The latter may only be understood when the molecular organization of these programs is elucidated. Information in this central field of biochemistry is, however, still in its infancy (Nover et al. 1978).

ACKNOWLEDGMENTS

The author wishes to express his appreciation to his colleagues Dr. Nover (Institute of Plant Biochemistry of the Academy of Sciences of the GDR, Halle) and Drs. W. Lerbs and W. Roos (Section of Pharmacy of the Martin-Luther-University Halle) for their helpful suggestions.

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