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De Novo Biosynthesis of Secondary Metabolism Enzymes in Homogeneous Cultures of *Penicillium urticae*

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The initiation of patulin biosynthesis in submerged batch cultures of *Penicillium urticae* NRRL 2159A was investigated at the enzyme level. In contrast to earlier studies, this study achieved a clear temporal separation of growing cells devoid of secondary metabolism-specific enzymes from nongrowing cells, which rapidly produce these enzymes. A spore inoculum, silicone-treated flasks, and two new media which supported a rapid, pellet-free, filamentous type of growth were used. In yeast extract-glucose-buffer medium, a marked drop in the specific growth rate (~0.26 h^{-1}) coincided with the appearance of the first pathway-specific enzyme, 6-methylsalicylic acid synthetase, at about 19 h after inoculation. About 3 h later, when replicatory growth had ceased entirely, the sparsely branched mycelia (length, ~550 μm) began the rapid synthesis of a later pathway enzyme, *m*-hydroxybenzyl alcohol dehydrogenase. A similar sequence of events occurred in a defined nitrate-glucose-buffer medium; 12 other strains or isolates of *P. urticae*, as well as some patulin-producing aspergilli, behaved in a similar manner. The age at which a culture produced *m*-hydroxybenzyl alcohol dehydrogenase was increased by increasing the nutrient nitrogen content of the medium or by decreasing the size of the spore inoculum. In each instance the appearance of enzyme was determined by the nutritional status of the culture and not by its age. A similar appearance of patulin pathway enzymes occurred when a growing culture was resuspended in a nitrogen-free 4% glucose solution with or without 0.1 M phosphate (pH 6.5). The appearance of both the synthetase and the dehydrogenase was arrested by the addition of cycloheximide (0.4 to 5 μg/ml) or actinomycin D (20 to 80 μg/ml). This requirement for de novo protein and ribonucleic acid syntheses was confirmed by the incorporation of labeled leucine into the dehydrogenase, and the possibility that latent or preformed proteins were being activated was eliminated.

Published reports of microbial enzymes which catalyze discrete steps in secondary metabolite biosynthetic pathways are surprisingly few, and most have been obtained from *Bacillus* and *Streptomyces* spp. (22, 24-26). In filamentous fungi, enzymes involved in the biosynthesis of the ergot (28) and benzodiazepine (57) alkaloids, enniatin B (62), β-lactams (24), aspulvinone (54), mycophenolic acid (10, 39), and patulin (15, 23, 32, 37, 38, 50) have been reported. Cell-free extracts which possess multiple activities involved in the biosynthesis of penicillic acid (3) and aflatoxin B_1 (52) have also been reported. More than three separable enzyme activities have been described for only three secondary metabolite biosyntheses, namely streptomycin (24), the benzodiazepine alkaloids (57), and patulin (50).

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This dearth of enzymology is largely due to the number and diversity of both secondary metabolites and their producers and to the generally asynchronous appearance and non-growth-associated nature of secondary metabolite biosynthesis. The dilution of effort and lack of focus on a particular secondary metabolism pathway has led to an inadequate knowledge of the individual steps of most pathways, and, even when known, these steps have often been difficult to demonstrate in cell-free preparations. This is because batch cultures are often composed of a metabolically heterogeneous population of cells and because secondary metabolism occurs at a time of metabolic upheaval, which is characterized by the accelerated turnover of many cellular constituents, particularly proteins. Given the above, it is not surprising that little molecular detail concerning the regulation of secondary metabolism is available (12, 13, 17). The interpretation of fermentation phenomena

in biochemical terms has often been based on an extrapolation from primary metabolism, usually in procaryotes.

This study is a continuation of our use of patulin biosynthesis in *Penicillium urticae* as a model of secondary metabolism. Patulin is both an antibiotic and a mycotoxin, as well as a representative of the largest and most ubiquitous class of fungal metabolites, the polyketides (56). Our knowledge of the lengthy series of reactions and intermediates which convert the first committed precursor, 6-methylsalicylic acid (6-MSA), to the end product, patulin, is nearing completion (20, 48–51). Mutants blocked at specific steps in the pathway are available (47, 48), and the list of pathway-specific enzymes (15, 23, 32, 37, 38, 50) has continued to grow. In this investigation of the appearance of patulin pathway enzymes in homogeneous, submerged cultures of *P. urticae*, we chose to use as markers the first and fourth enzymes of the pathway following:

Acetyl coenzyme A + 3 malonyl coenzyme A
$\xrightarrow{1}$ 6-MSA $\xrightarrow{2}$ *m*-cresol $\xrightarrow{3}$ *m*-hydroxybenzyl alcohol $\xrightarrow{4}$ *m*-hydroxybenzaldehyde $\xrightarrow{5}$ gentisaldehyde $\longrightarrow\longrightarrow$ patulin.

Enzymes 1 and 4 are 6-MSA synthetase and *m*-hydroxybenzyl alcohol dehydrogenase (EC 1.1.1.97), respectively. Two cultivation systems which yield a distinct separation of the replicatory growth phase from the patulin production phase are described, and the de novo synthesis of patulin pathway enzymes is shown to be governed by nutrient depletion.

MATERIALS AND METHODS

Chemicals. All biochemicals used in the preparation of cell-free extracts and in enzyme assays were purchased from the Sigma Chemical Co. [2-^{14}C]malonyl-coenzyme A, [2-^{14}C]uracil, and L-[*U*-^{14}C]leucine were obtained from the New England Nuclear Corp.

Stock cultures. The principal organism used in this and all of our previous studies of patulin biosynthesis was a spontaneous white variant (NRRL 2159A) of *P. urticae*. To ensure culture homogeneity and stability, a single-spore colony was selected from an inoculated agar plate and propagated, and the resultant spores were stored at 4°C in sterile silica gel (0.05 to 0.2 mm). A number of other cultures were used in a comparative study of the phasing of secondary metabolism. Some of these cultures were present in our laboratory as lyophilized spores on paper strips, which were stored under a vacuum at −70°C, whereas the others were fresh agar slants obtained from various culture collections. The specific cultures used are indicated in the legend to Fig. 4.

Cultivation conditions. Spores used for the inoculation of submerged cultures were obtained from surface cultures grown at 28°C on agar slants of ~15 cm^2. The slants were prepared by adding 10 ml of agar (49 g of Czapek-Dox solution agar [Difco] and 5 g of agar [Difco] per liter of deionized water) to a 8-dram (29.6-ml) vial and were later inoculated with a loopful of the silica gel stock culture. After 10 days of incubation, the slants were stored at 4°C for not more than 1 month. The spores from one slant were routinely suspended by vigorous hand shaking into 5 ml of a detergent solution (450 μl of Aerosol OT per liter; Fisher Scientific Co.).

Submerged shake flask cultures were grown in 500-ml Erlenmeyer flasks containing 50 ml of medium. To increase culture homogeneity and the reproducibility of growth kinetics and yields from flask to flask, wall growth was minimized by coating all flasks with a water-repelling silicone film (Dri-film SC-87; Pierce Chemical Co.). Cultures were grown in two types of media. The first was a complex yeast extract-glucose-buffer medium containing (in grams per liter): yeast extract (Difco), 5; glucose, 40; KH_2PO_4, 13.6; citric acid, 9.8; and Na_2SO_4, 1. This medium also contained 10 ml of a trace metals solution prepared by the method of Yamamoto and Segel (61), and the pH was adjusted to 6.5 with NaOH. Yeast extract, glucose, and the remaining components were each made up separately at five times the final medium concentration and combined after autoclaving. The second medium was a chemically defined nitrate-glucose-buffer medium which was of the same composition as the medium described above, except that $NaNO_3$ (2.58 g/liter) replaced the yeast extract. Each 50-ml portion of medium was inoculated with 0.5 ml of a spore suspension prepared as described above. The final spore density was 1×10^6 to 2×10^6 spores per ml. Inoculated cultures were incubated at 28°C on a rotary shaker (model G-10 Gyrotory shaker; New Brunswick Scientific Co.; 280 rpm; 2.5-cm stroke).

Culture sampling. Cultures were sampled by withdrawing an entire flask from a batch. The mycelia were collected by suction filtration through Whatman no. 4 filter paper. The culture filtrate was removed and stored at −20°C until needed. After it was washed twice with approximately 10 ml of deionized water, the mycelial mat was peeled off the filter paper and pressed between paper towels to remove excess water; 400 mg of wet mycelium was used for enzyme analyses, and the remainder was used for a dry weight determination. Alternatively, the entire sample of wet mycelium was freeze-dried and stored in vials at −20°C for future use.

Culture growth. Two methods for following the growth of cultures were employed; these were dry weight of mycelium (biomass) per milliliter of culture and amount of mycelial protein per milliliter of culture. The latter parameter is generally considered to parallel replicatory growth better than biomass does. For a dry weight determination, the wet mycelial mat was dried overnight, either by freeze-drying or by oven-drying at 70°C. For a total mycelial protein determination, 10 to 15 mg of freeze-dried mycelium was suspended in 1 ml of 1 N NaOH and heated for 15 min in a boiling water bath. The extract was clarified by

centrifugation (10 min, 8,000 × g) and analyzed for protein by using the method of Lowry et al. (33). Bovine serum albumin (Pentex) served as a standard.

Enzyme assays. Crude mycelial extracts were prepared by glass bead homogenization in an MSK shaker (B. Braun Instruments, San Francisco). This proved to be a very effective, reproducible, and simple technique for routine use. In contrast, sonication yielded incomplete cell breakage and took longer because of the cooling intervals required. Freeze-pressing with a Hughes press was unsuitable because the enzyme *m*-hydroxybenzyl alcohol dehydrogenase is inactivated by the freezing and thawing involved in this technique. For homogenization, 400 mg of blotted fresh mycelium or 100 mg of lyophilized mycelium was added to a 50-ml homogenizing flask (Braun) together with 8 ml of cold 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.6) (23) or 100 mM Tris buffer (pH 8.0) (16, 45, 46). After a further addition of 20 g of glass beads (0.45 to 0.5 mm; Braun), the mycelium was broken by shaking the flask for 2 min in a Braun model MSK cell homogenizer at 4,000 rpm. The temperature was kept below 5°C with liquid carbon dioxide during shaking.

The determination of the mycelial *m*-hydroxybenzyl alcohol dehydrogenase content was carried out by using the TES buffer extract directly after filtration of the glass bead-containing homogenate. This assay has been described previously (23). The Tris buffer extract was used for the determination of the 6-MSA synthetase content according to the combined methods of Dimroth et al. (16) and Scott et al. (46). The glass bead-containing homogenate was filtered and centrifuged (45 min, 100,000 × g), and the supernatant was used in this assay. The assay is based on the incorporation of [2-^{14}C]malonyl-coenzyme A into 6-MSA. Quantitation of radioactivity can take place only after the separation of 6-MSA from fatty acids by thin-layer chromatography on silica gel plates (5 by 20 cm; Woelm GF_{254}) developed in benzene–dioxane–glacial acetic acid (90:25:4, vol/vol). This chromatographic step is necessary because labeled fatty acids are produced by the fatty acid synthetase present in the crude extracts. After detection under UV light, the 6-MSA spot was scraped into a vial containing 10 ml of a scintillation solution {2,5-diphenyloxazole, 7 g/liter; 1,4-bis[2-(5-phenyl oxazolyl)]benzene, 0.3 g/liter; naphthalene, 100 g/liter of scintillation-grade dioxane} and counted.

Electrophoresis. Solid sucrose (10%, wt/vol) was added to extracts (TES buffer; 45 min, 100,000 × g supernatant) prepared as described above from mycelium which had been radiolabeled with L-[*U*-^{14}C]-leucine. These extracts were then analyzed by two-dimensional polyacrylamide disc gel electrophoresis for the incorporation of [^{14}C]leucine into *m*-hydroxybenzyl alcohol dehydrogenase. The electrophoresis method used was essentially that of Ornstein (41) and Davis (11). For the first-dimension run, a 1.25% stacking gel was used over an 8-cm-long 7.5% separating gel (pH 8.9) in 5-mm (inside diameter) tubes. Samples of 200 μl were applied and electrophoresed for 2 h at 2 to 5°C by using a current of 3 mA/gel. The gels were stained with a specific dehydrogenase stain by the method of Forrester and Gaucher (21) or with amido black in 7% acetic acid. For the second-dimension run, the dehydrogenase was located by placing the first gel on a stain soaked filter paper for about 1 min. A 1- to 2-mm section containing the dehydrogenase was then cut from this gel and placed on top of a second gel. Electrophoresis conditions identical to those described above were used, except that the separating gel contained 10% acrylamide and was at least 24 h old. Radioelectrophoretograms were obtained by sectioning gels, which had been specifically stained for the dehydrogenase, into 1-mm slices with a wire gel cutter. The protein in the slices was solubilized by adding 1 ml of solubilizer (NCS [Amersham/Searle]–water [9.5:0.5, vol/vol]) and heating the mixture for 2 h at 55°C in a scintillation vial. After cooling, 10 ml of the scintillation solution described above was added. To minimize chemiluminescence, the mixture was dark adapted and cooled to 15°C for 48 h before counting.

Radiolabeling of macromolecules. Continuous labeling of mycelial protein and nucleic acids was carried out by adding L-[*U*-^{14}C]leucine (2.0 μCi; 800 ng) or [2-^{14}C]uracil (2.0 μCi; 32 μg) to 50-ml shake cultures. Samples of 2 ml were withdrawn at regular intervals and precipitated in 2 ml of 10% trichloroacetic acid. The trichloroacetic acid contained either 50 mM L-leucine or uracil, depending upon the radioactive precursor used. The trichloroacetic acid samples from [^{14}C]leucine labeling were heated for 10 min in a boiling water bath, cooled, filtered on membrane filters (type HAWP; pore size, 0.45 μm; 25 mm; Millipore Corp.), washed twice with 10 ml of 5% trichloroacetic acid containing 25 mM leucine, and finally washed with 5 ml of 95% ethanol. The trichloroacetic acid-quenched samples from [^{14}C]uracil labeling were stored at 4°C for at least 1 h before filtering and washing as described above. The 5% trichloroacetic acid in this case contained 25 mM uracil. The filters bearing the trichloroacetic acid precipitates were added directly to a vial containing 10 ml of the dioxane-based scintillation solution described above and counted.

Detection of secondary metabolites. Thin-layer chromatography was used to indicate qualitatively the presence of patulin pathway metabolites in the medium at various stages of cultivation. The method has been described previously (47, 48).

Patulin bioassay. Patulin production profiles were determined by using an agar plate diffusion assay (27). Sterile, liquified agar medium (streptomycin assay agar; 25.5 g/liter; Difco) was held at 55°C and inoculated with a spore suspension of the test organism *Bacillus subtilis*. The inoculated assay agar (95 ml) was poured to a thickness of about 2 mm into a square aluminum frame (22 by 22 by 1.5 cm), which was mounted on a glass plate with petroleum jelly. After the agar had solidified, a uniformly spaced grid of 16 wells (inside diameter, 11 mm) was punched out with a corkborer. Test samples of 0.1 ml were added to the wells. Each plate held duplicate samples of four patulin concentrations (0.5, 1.0, 2.0, and 4.0 μmol/ml) and four culture filtrates. The plates were incubated for 10 to 12 h at 28°C. Plots of the logarithms of the four patulin concentrations versus inhibition zone diameters gave straight lines.

Glucose determination. Culture filtrates were an-

alyzed for residual glucose by using a Glucostat reagent kit (Worthington Biochemicals Corp.).

RESULTS

Development of cultivation conditions which support distinct metabolic phasing. The absence of secondary metabolism (e.g., antibiotic or mycotoxin biosynthesis) from the replicatory growth phase of a fungal culture and its presence during the stationary growth phase are common observations and are the basis of the trophophase (replicatory growth stage)-idiophase (production stage) terminology of Bu'Lock et al. (7). Unfortunately, this metabolic phasing is not always distinct, and actively growing cultures often exhibit secondary metabolism. This is most probably due to the existence of a metabolically heterogeneous population of mycelial cells, some of which are engaged in replicatory growth and others of which, having been subjected to the conditions which initiate secondary metabolism, are engaged in this nongrowth-associated pursuit. Although increases in the biomass of a culture often continue after replicatory growth ceases, a comparison of the kinetics of secondary metabolite production and dry weight accumulation is still informative since replicatory growth is usually responsible for the initial two-thirds of the increase in dry weight and its cessation is usually accompanied by a detectable decrease in the rate of biomass accumulation. In all previous cultivations of *P. urticae* in simple nitrate-glucose medium (7, 8, 29) or in more complex yeast extract-nitrate-glucose medium (16, 20, 21) there appeared to be a significant overlap of the growth and production phases. In the belief that the initiation of the synthesis of secondary metabolism enzymes would be most clearly observed in homogeneous cultures which exhibited a distinct separation of growth and production phases, we began this study with a search for improved conditions of cultivation. A significant improvement in the trophophase-idiophase separation was achieved by using a spore inoculum, silicone-treated shake flasks, and new media which supported a rapid pellet-free (i.e., filamentous) growth. A mycelial inoculum invariably resulted in a significant overlap of the growth and production phases, whereas a spore-inoculated nitrate-glucose-buffer medium exhibited good phasing in shake flasks but not in a 5-liter fermentor (GrootWassink, unpublished data).

Two media were used for the routine testing of various fermentation variables and metabolic effectors. Fermentation patterns of shake flask cultures in yeast extract-glucose-buffer medium and nitrate-glucose-buffer medium are shown in Fig. 1A and B, respectively. Both media supported ideal filamentous growth. Reproducibility between cultures grown in the complex medium was good; however, a considerable number of flasks with erratic growth yields were generally rejected from batches of cultures grown in the chemically defined medium. Germination times were 8 to 9 and around 18 h in these two media, respectively. Replicate experiments showed that both types of culture exhibited an exponential growth phase, with specific growth rates of 0.26 h^{-1} (mass doubling time, 2.7 h) and 0.15 h^{-1} (mass doubling time, 4.6 h), respectively. Exponential growth ceased at about 19 and 33 h in these two media, respectively. Total mycelial protein (i.e., milligrams of protein per milliliter of culture) indicated that postexponential replicatory growth only occurred in the yeast extract-containing medium. This was most likely due to the successive exhaustion of two classes of nitrogen compounds in the yeast extract. The dry weight increase, after the accumulation of protein ceased, can be ascribed to lipid accumulation and cell wall thickening.

Both types of culture showed marked differences in their pH and residual glucose profiles. In the yeast extract medium excess glucose was present throughout the fermentation, whereas in the nitrate medium glucose exhaustion occurred during the early stationary growth phase. In both media the pH dropped after replicatory growth had ceased and glucose utilization continued. Other experiments showed that patulin

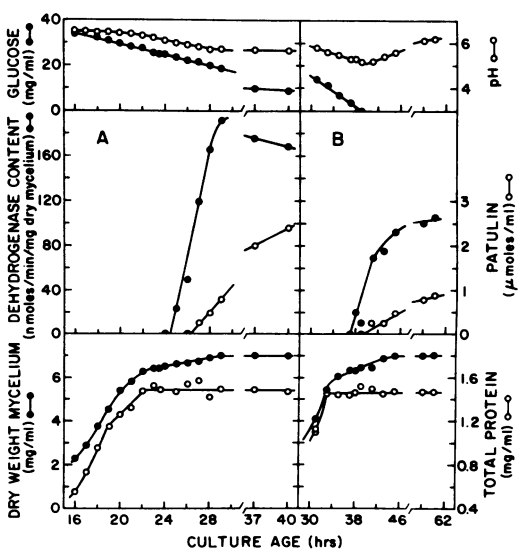

FIG. 1. *Time course of shake flask cultures grown in yeast extract-glucose-buffer medium (A) and nitrate-glucose-buffer medium (B) from a spore inoculum.*

biosynthesis was dependent upon the presence of an external C source but not an N source. As sole C source, the common medium component citrate would not support the growth of either spores or mycelium or the production of secondary metabolism enzymes and hence patulin. Thin-layer chromatography of culture filtrates demonstrated that relatively large quantities of glucose had been converted into gluconic acid, as has been reported earlier (18, 31). This secondary C source may have been utilized after glucose itself was exhausted (9).

The appearance of secondary metabolism was followed by monitoring the cellular content of *m*-hydroxybenzyl alcohol dehydrogenase, the fourth enzyme of the patulin pathway. In either cultivation system the enzyme appeared abruptly, soon after cessation of the replicatory growth phase. In the yeast extract medium (Fig. 1A) maximum levels of dehydrogenase were reached within 5 to 6 h. This rapid appearance of enzyme suggested the presence of a metabolically homogeneous population of cells engaged in a relatively synchronous initiation of secondary metabolism. Microscopic examination showed that this is indeed probable since at 22 h, when replicatory growth had ceased and secondary metabolism had begun, the culture possessed a simple hyphal morphology (Fig. 2). These mycelial filaments were on the average only about 550 μm long and possessed only a few short, primary branches on one side of the parent spore. Trinci (55) found that for *Penicillium chrysogenum*, hyphal structures of this length consisted of communicating compartments which freely exchanged cellular material. Thus, apart from the gradient of organelles commonly found in growing hyphal tips, a 22-h-old culture should possess a significant degree of intrahyphal as well as interhyphal homogeneity at the metabolic level.

Examination of culture filtrates by thin-layer chromatography revealed that 6-MSA appeared in the yeast extract medium at about 21 to 22 h, which is just before the appearance of the dehydrogenase. Patulin and *m*-hydroxybenzyl alcohol, as well as the related metabolites toluquinol and gentisyl alcohol, all appeared simultaneously a few hours after the enzyme was first detected. The rates and yields of patulin production in these shake flask cultures were as low as 25% of those obtained in fermentor cultures. The low oxygen transfer rate in shake flasks is the most likely explanation for this, since patulin biosynthesis takes place via an oxygen-dependent pathway.

Sequential appearance of patulin pathway enzymes. Based on metabolite analyses, Bu'Lock et al. (8) postulated that the enzymes of the patulin pathway appear in a sequential order, each of them being induced by its respective substrate. Although an overlapping sequence of metabolite profiles in the medium has been observed by using gas-liquid chromatography (38; J. Neway and G. M. Gaucher, unpublished data), this sequence of metabolite appearance outside the cells cannot be confidently extrapolated to the sequence of enzyme appearance inside the cells. Therefore, in separate experiments we compared the actual production profiles of 6-MSA synthetase and *m*-hydroxybenzyl alcohol dehydrogenase for both complex and chemically defined media (Fig. 3A and B, respectively). In both media the times of appearance of the two enzymes were separated by an interval of 3 to 4 h. It may be noted that the initial appearance of 6-MSA synthetase generally coincided with a marked drop in the specific growth rate. Thus, in the yeast extract medium (Fig. 3A) the enzyme appeared at the beginning of the postexponential linear growth phase, whereas in the nitrate medium (Fig. 3B) it appeared at the beginning of the stationary phase. The question of whether the observed sequential appearance of these two enzymes is the result of induction by pathway metabolites will be considered in a later communication.

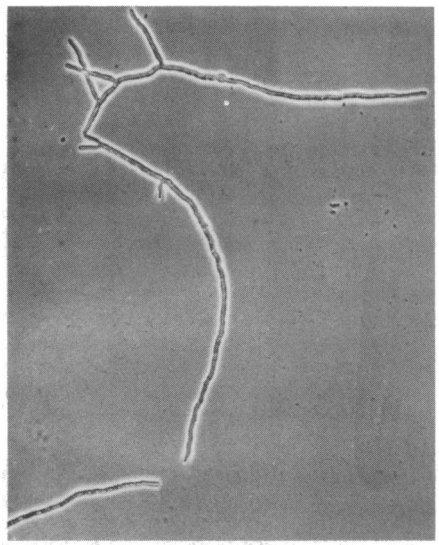

FIG. 2. *Phase-contrast light micrograph of a 22-h-old mycelium from a P. urticae shake culture grown in yeast extract-glucose-buffer medium.* ×190.

General occurrence of metabolic phasing. A comparative study was conducted to establish the general occurrence of metabolic phasing in patulin-producing fungi. Such information would also ensure that our strain (NRRL

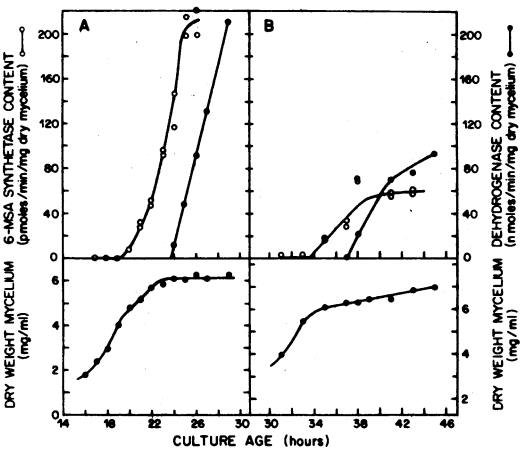

FIG. 3. *Sequential appearance of 6-MSA synthetase and m-hydroxybenzyl alcohol dehydrogenase in shake flask cultures grown in yeast extract-glucose-buffer medium (A) and nitrate-glucose-buffer medium (B).*

2159A) is representative of other *P. urticae* strains and that the lack of phasing in previously reported cultivation systems (7, 8, 16, 20, 21, 29) was not due to the variety of strains used. Growth and *m*-hydroxybenzyl alcohol dehydrogenase profiles for several patulin-producing *Penicillium* and *Aspergillus* strains were compared with those of *P. urticae* NRRL 2159A. The production of patulin by these strains was substantiated by thin-layer chromatography. The fungi were grown from spores in shake flasks containing yeast extract-glucose-buffer medium. The *Penicillium* cultures were analyzed at 2-h intervals between 20 and 30 h after inoculation. The *Aspergillus* cultures were tested up to 50 h because of slower growth rates and higher growth yields. The organisms examined and, where possible, references to use by other investigators are given in the legend to Fig. 4. To exclude variations due to differences in inoculum size, the cellular dehydrogenase content for each strain was plotted as a function of growth rather than culture age. As indicated in Fig. 1 and 3, *P. urticae* NRRL 2159A did not produce dehydrogenase until replicatory growth had ceased at a dry weight of about 6 mg/ml. In Fig. 4 this is expressed as a sudden vertical rise in dehydrogenase content when the dry weight reached this value. In all of the 12 other strains or isolates of *P. urticae* growth cessation and dehydrogenase appearance occurred at the same time, and except for Norstadt strain no. 4, all cultures of *P. urticae* achieved a very similar growth yield. It is of interest to note that strains which had resided for some time in culture collections behaved in a manner identical to those which were much more recent soil isolates (40). Of the *Aspergillus* strains tested, one (NRRL 4763) was very similar to *P. urticae*, one (NRRL 10) exhibited more non-replicatory growth and the others had higher growth yields but much lower dehydrogenase contents. In all cases, however, the dehydrogenase was produced only after a marked change in the growth rate. Thus, the data in Fig. 4 suggest that a phased appearance of patulin biosynthetic enzymes is of general occurrence and that *P. urticae* NRRL 2159A is a truly representative strain.

Effect of concentration of N source on the appearance of *m*-hydroxybenzyl alcohol dehydrogenase. The experiments described here were carried out to achieve the following objectives. The first objective was to establish a direct relationship between depletion of the N source of the medium and the onset of secondary metabolism. Bu'Lock et al. (7) were unable to identify a nutritional limitation as the physiological factor causing the development of secondary metabolism in *P. urticae*. A second objective was to obtain information on whether intrinsic factors, such as ageing, can initiate secondary metabolism. Brown and Vass (5) and Martin

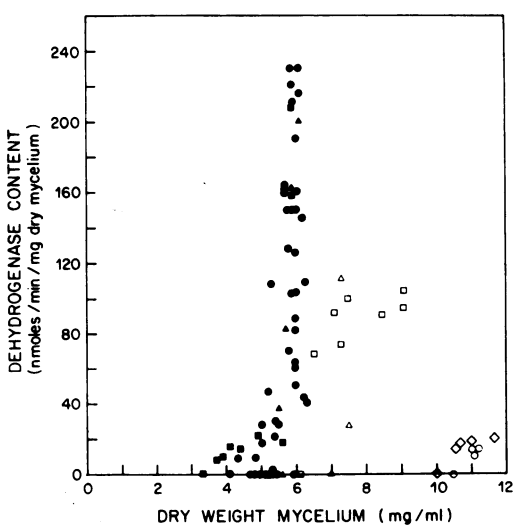

FIG. 4. *Relationship between stationary growth phase and production of m-hydroxybenzyl alcohol dehydrogenase in shake cultures of patulin-producing fungi grown in yeast extract-glucose-buffer medium. See text for cultivation and analytical details. The fungi tested were P. urticae NRRL 2159A (▲) (4, 21, 29, 46), 11 other P. urticae strains: (●) (NRRL 2159, NRRL 2329, NRRL 992, NRRL 989 [=CBS 384.48] [16, 38], IMI 59511, and Norstadt no. 1 through 3 and 5 through 7 [40], P. urticae Norstadt no. 4 (△) (40), Aspergillus niger Norstadt (○), Aspergillus giganteus NRRL 10 (□) (19) and NRRL 4763 (■), and Aspergillus clavatus NRRL 1980 (◇) (58).*

and McDaniel (35) have suggested that in microbial cultures which consist of "older" and "younger" hyphal cells, the elapsed time between formation of a new cell and the onset of secondary metabolism in that cell is fixed under given culture conditions. This was called the maturation time of a cell.

Cultures were grown in media containing increasing concentrations of yeast extract as the sole N source. Production profiles of biomass and dehydrogenase were determined for each concentration (Fig. 5A). The linear relationship (Fig. 5C) between the growth yield and the yeast extract concentration (above 2 mg/ml) indicates that the yeast extract contained the growth-limiting nutrient(s).

If the onset of secondary metabolism is determined by environmental factors, such as exhaustion of the N source, a predictable relationship should exist between the concentration of the N source and the time of dehydrogenase appearance. The nature of this relationship depends upon the growth kinetics (i.e., exponential and/or linear growth) which precedes the appearance of the dehydrogenase. Assuming exponential growth, then each doubling of the yeast extract concentration should extend the growth phase and delay the dehydrogenase appearance by one doubling time, which is 2.7 h in this medium. Thus, a linear relationship between the log of the yeast extract concentration and the time of dehydrogenase appearance should then hold true. It is clear from Fig. 5A that the enzyme appearance was increasingly delayed as the yeast extract concentration was increased. However, a logarithmic relationship (Fig. 5B) appeared to be restricted to yeast extract concentrations below 4 to 5 mg/ml. In cultures with higher concentrations, the enzyme appeared somewhat earlier than expected, suggesting that inhibitory factors had interfered with normal exponential growth. These results indicate that for about 2 to 5 mg of yeast extract per ml, a depletion of the N source initiated secondary metabolism. Intrinsic ageing or cell maturation did not appear to cause this initiation.

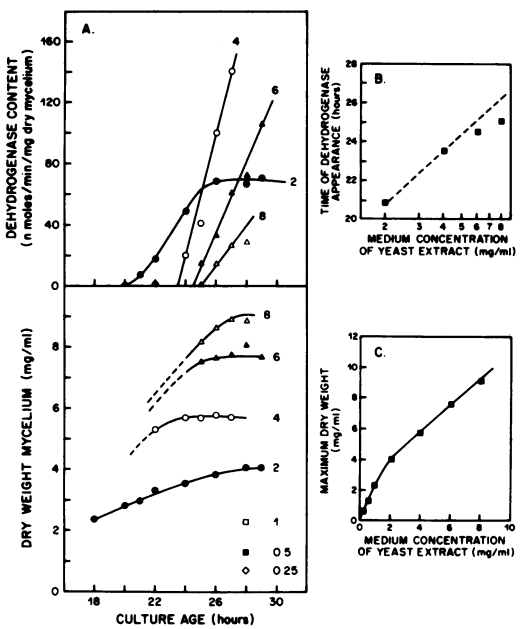

FIG. 5. *Effect of the yeast extract concentration of the medium on the appearance of m-hydroxybenzyl alcohol dehydrogenase. Shake flask cultures were grown in yeast extract-glucose-buffer medium containing 0.25, 0.5, 1, 2, 4, 6, and 8 mg of yeast extract per ml. For yeast extract concentrations of 2 to 8 mg/ml the time of dehydrogenase appearance was obtained by extrapolating the linear production profiles (A) to zero dehydrogenase content. These times of appearance were plotted against the log of the yeast extract concentration (B) and compared with a plot (dashed line) calculated from the specific growth rate (0.26 h^{-1}) of cultures in this medium. The slope of the calculated line is equal to the doubling time (2.7 h) of these cultures. Dry weight profiles (A) provided growth yields for each yeast extract concentration, and the linear relationship between these two variables is shown (C).*

The above results, which were obtained by using the complex yeast extract-glucose-buffer medium, were confirmed by a similar experiment with the defined nitrate-glucose-buffer medium (Fig. 6). Within the range of concentrations used, nitrate was the growth-limiting nutrient (Fig. 6C). Again, the higher the concentration of this single N source, the later the appearance of the dehydrogenase. The experimental points obtained by plotting the time of appearance against the log of the nitrate concentration were found (Fig. 6B) to be grouped about a calculated line, which had a slope equal to the doubling time of the culture (4.6 h).

Effect of inoculum size on the appearance of *m*-hydroxybenzyl alcohol dehydrogenase. Varying the culture age at which critical N depletion occurs may also be accomplished by varying the size of the inoculum. If a culture exhibits ideal exponential growth, then a doubling of the inoculum size shortens the culture age at which nutrient limitation becomes effective by one doubling time.

The growth and dehydrogenase profiles of cultures grown from various spore densities in yeast extract-glucose-buffer medium are shown in Fig. 7. The time of dehydrogenase appearance shifted forward as the inoculum size was in-

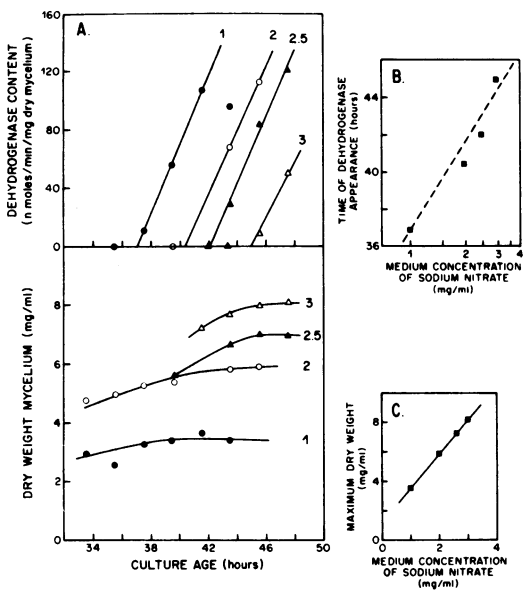

FIG. 6. *Effect of the sodium nitrate concentration of the medium on the appearance of m-hydroxybenzyl alcohol dehydrogenase. Shake flask cultures were grown in nitrate-glucose-buffer medium containing 1, 2, 2.5, and 3 mg of sodium nitrate per ml. The times of dehydrogenase appearance were obtained by extrapolation (A) and were plotted against the log of the sodium nitrate concentration (B) and compared with a plot (dashed line) calculated from the specific growth rate (0.15 h^{-1}) of cultures in this medium. The slope of the calculated line was equal to the doubling time (4.6 h) of these cultures. Dry weight profiles (A) provided growth yields for each sodium nitrate concentration, and the linear relationship between these two variables is shown (C).*

creased. A logarithmic relationship (Fig. 7, inset) was, however, limited to inoculum sizes above about 0.4 ml/culture. The enzyme appeared earlier than expected at lower inoculum sizes. Nevertheless, these results again indicate that the enzymes of secondary metabolism appear, within limits, in response to N source depletion and independent of the age of individual cells.

De novo synthesis of enzymes. The experiments described above showed that 6-MSA synthetase and *m*-hydroxybenzyl alcohol dehydrogenase activities appeared only at the end of the exponential growth phase and that net protein synthesis was not required for the production of these enzymes. Since enzyme activity rather than enzyme protein was assayed in these experiments, the de novo syntheses of these proteins could have occurred before as well as after the cessation of exponential growth. In the former case a decrease in growth rate may have simply led to an activation of preformed proteins. Indirect evidence for the de novo syntheses of these two enzymes was sought in experiments with the eucaryotic protein synthesis inhibitor cycloheximide. In an earlier study Light (30) showed that the incorporation of [^{14}C]leucine into total protein was completely inhibited within a few minutes of adding cycloheximide (8 μg/ml) to a growing culture of *P. urticae*. Cycloheximide was also shown to exert both stimulatory and inhibitory effects on the appearance of 6-MSA synthetase. Figure 8 shows the effect of cycloheximide on the biosynthesis of the dehydrogenase. A series of cycloheximide concentrations was used in order to determine the lowest effective concentration and thus avoid secondary inhibitory effects. When added 2.5 h before the normal appearance of the enzyme, cycloheximide (0.4 to 5 μg/ml) increasingly delayed its appearance. Note that the organism was able to recover from the effects of

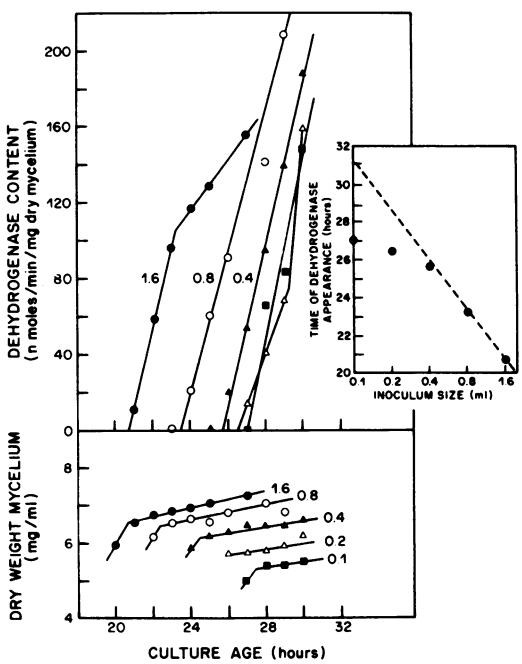

FIG. 7. *Effect of inoculum size on the appearance of m-hydroxybenzyl alcohol dehydrogenase. Shake flasks containing 50 ml of yeast extract-glucose-buffer medium were inoculated with 0.1, 0.2, 0.4, 0.8, and 1.6 ml of a standard spore suspension prepared as described in the text. The times of dehydrogenase appearance were obtained by extrapolating the linear production profiles to zero enzyme content and were plotted against the log of the inoculum size and compared with a plot (dashed line) calculated from the specific growth rate (0.26 h^{-1}) of cultures in this medium. The slope of the calculated line was equal to the doubling time (2.7 h) of these cultures.*

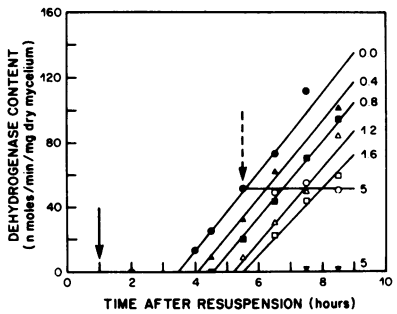

FIG. 8. *Effect of cycloheximide on the biosynthesis of m-hydroxybenzyl alcohol dehydrogenase. A 5-liter New Brunswick Microferm fermentor containing 3 liters of yeast extract-glucose-buffer medium was inoculated with the spores from three agar slants of P. urticae as described in the text. After 20 h of growth at 28°C with a dissolved oxygen tension greater than 40% of saturation, the mycelium was harvested, washed, and suspended to a density of 8 mg (wet weight) of mycelium per ml in a resting cell medium containing only glucose (40 g/liter) and KH_2PO_4 (0.1 M, pH 6.5). Cell suspensions (50 ml) were dispensed into shake flasks and incubated as previously described. Cycloheximide at 0.4 to 1.6 and 5 μg/ml was added 1 h later (solid arrow) and at 5 μg/ml 5.5 h later (dashed arrow).*

small doses (i.e., 0.4 to 1.6 μg/ml) of inhibitor and that an immediate interruption occurred when cycloheximide was added about 2 h after dehydrogenase appearance had begun. In a similar experiment cycloheximide immediately arrested the appearance of 6-MSA synthetase (Fig. 9). Clearly, the appearance of both secondary metabolism enzymes required de novo protein synthesis.

Direct evidence for de novo synthesis of the dehydrogenase was obtained by radiolabeling the enzyme with [^{14}C]leucine. The radiolabeled amino acid was added to a resting cell culture at a time when the dehydrogenase content was 160 nmol/min per mg of dry mycelium (73% of maximum level). The incubation was continued for 1 h, at which time the enzyme level had risen to 190 nmol/min per mg of dry mycelium. The purpose of adding the radiolabel at such a late stage in the enzyme production phase was to increase the preferential incorporation into the dehydrogenase. Crude extracts of radiolabeled mycelium were analyzed for labeled dehydrogenase by using two-dimensional polyacrylamide gel electrophoresis (Fig. 10). Amido-black staining of the first-dimension gel revealed a large number of protein bands, whereas staining with the specific dehydrogenase stain revealed only one band. The radioelectrophoretogram (Fig. 10A) shows a distinct peak at the location of the dehydrogenase, suggesting that the enzyme was indeed radiolabeled. To improve the resolution of the proteins in the dehydrogenase area, a second-dimension run was performed with a 10% rather than a 7.5% acrylamide separating gel. To do this, a 1 to 2-mm section of the first-dimension gel containing the dehydrogenase was placed on top of the second-dimension gel. The radioactivity remained with the dehydrogenase band (Fig. 10B).

De novo synthesis of mRNA. The sudden appearance of secondary metabolism enzymes in cultures of *P. urticae* could be due to a delayed processing or translation of precursor or mature mRNA, respectively. In either case the de novo synthesis of RNA as well as protein would not be a necessary prerequisite to enzyme appearance. The highly specific transcription inhibitor actinomycin D was used to examine this possibility. To test the relative effectiveness of actinomycin D on both RNA and protein syntheses in *P. urticae*, additions of labeled uracil and L-leucine to exponentially growing cultures were followed 1 h later by addition of the inhibitor. Figure 11 shows that the rate of incorporation of [^{14}C]uracil decreased faster than did that of [^{14}C]leucine when actinomycin D was added. This differential effect suggests that the primary action of the antibiotic in *P. urticae* is the inhi-

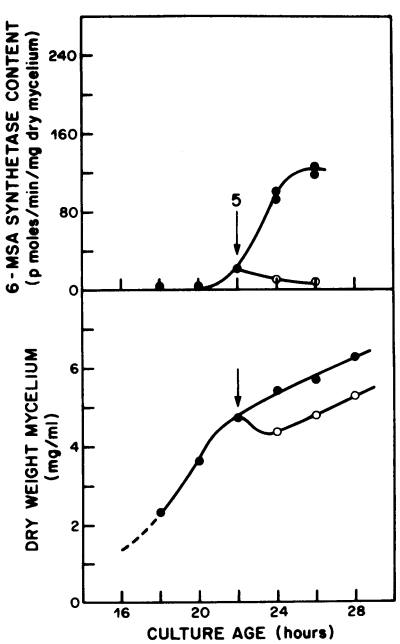

FIG. 9. *Effect of cycloheximide on the biosynthesis of 6-MSA synthetase. Standard shake cultures were grown in yeast extract-glucose-buffer medium, and cycloheximide at 5 μg/ml was added 22 h (arrow) after inoculation* (○).

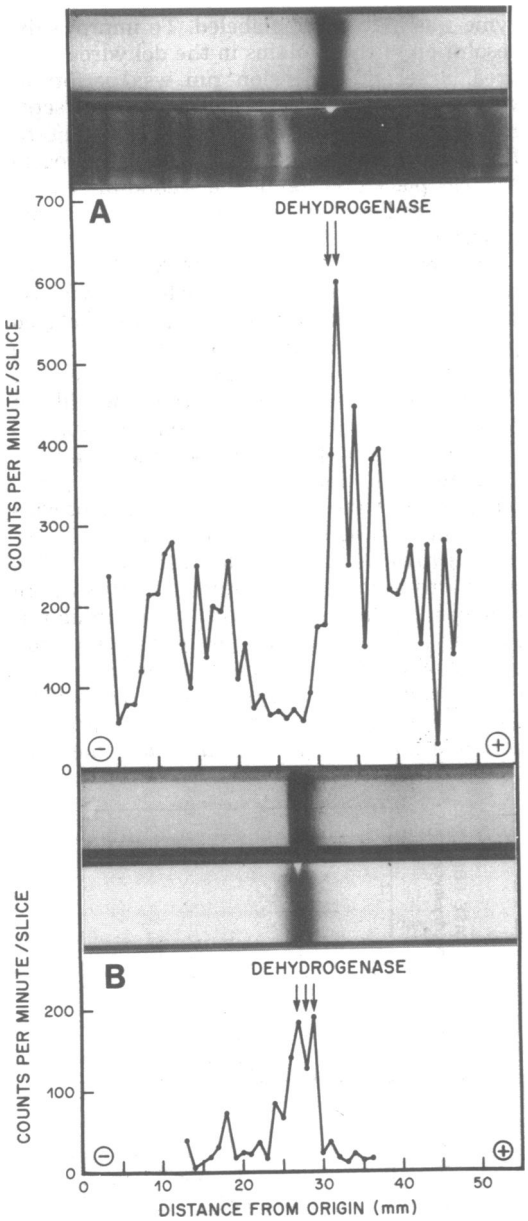

FIG. 10. *Radiolabeling of m-hydroxybenzyl alcohol dehydrogenase. A 50-ml shake culture (21.5 h in yeast extract-glucose-buffer medium) was harvested, washed, and suspended in a resting cell medium containing all components of the growth medium except yeast extract. At 4.5 h after suspension 10 μCi (4.0 μg) of L-[U-^{14}C]leucine was added, and the culture was shaken for an additional 1 h, after which the labeled mycelium was collected, washed, and lyophilized. As described in the text, an extract prepared from dry mycelium was subjected to electrophoresis. (A) First-dimension electrophoretogram. (B) Second-dimension electrophoretogram. In both pictures the top gel was stained specifically for the dehydrogenase, and the bottom gel was stained for protein in general.*

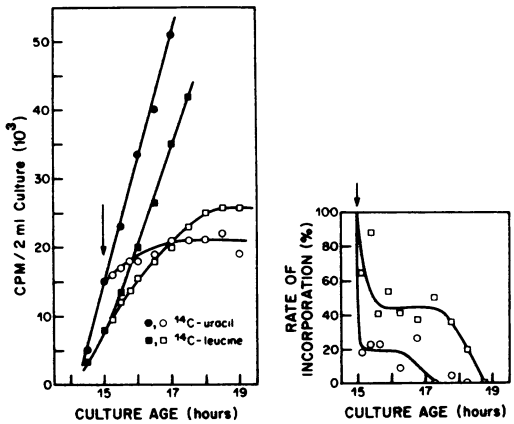

FIG. 11. *Effect of actinomycin D on general protein and RNA syntheses in P. urticae shake cultures grown in yeast extract-glucose-buffer medium. Continuous labeling of protein or RNA was accomplished by adding L-[U-^{14}C]leucine (2 μCi; 800 ng) or [2-^{14}C]uracil (2 μCi; 32 μg) to 14-h-old cultures (■ and ●). Actinomycin D (80 μg/ml) was added (arrow) 1 h later (□ and ○). The trichloroacetic acid-precipitable radioactivity in 2-ml samples was determined as described in the text.*

bition of RNA synthesis. A subsequent loss of existing mRNA via turnover could result in the observed cessation of protein synthesis.

The effect of actinomycin D on the appearance of 6-MSA synthetase and *m*-hydroxybenzyl alcohol dehydrogenase is shown in Fig. 12. Increasing concentrations of the inhibitor completely blocked the synthesis of 6-MSA synthetase and markedly decreased the rate of dehydrogenase synthesis. The patulin pathway intermediate *m*-hydroxybenzyl alcohol was added in this experiment to compress the times of appearance of the two enzymes and to ensure that, if necessary, substrate induction of the dehydrogenase would occur. The phenol advances the appearance of the dehydrogenase by approximately 2 h (unpublished data). The preferential inhibition of the synthetase over the dehydrogenase could have been due to a variety of causes, the simplest being the increased opportunity for loss of the inhibitor before dehydrogenase-specific transcription. These and the previous results clearly show that the appearance and continued syntheses of these enzymes in *P. urticae* require the de novo syntheses of both RNA and protein.

DISCUSSION

In contrast to earlier studies (7, 8, 16, 21, 29), this study shows that a complete separation of the replicatory growth and secondary metabolite production phases can be achieved in batch cultures of *P. urticae*. A significant overlap in these

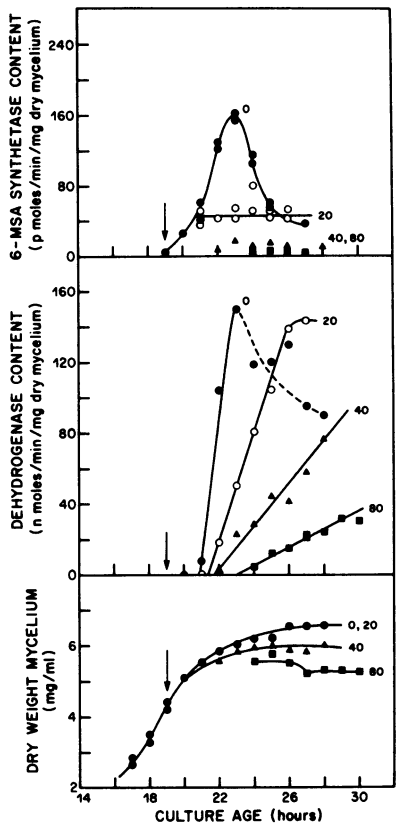

FIG. 12. *Effect of actinomycin D on the biosynthesis of 6-MSA synthetase and m-hydroxybenzyl alcohol dehydrogenase. Shake cultures (25 ml) were grown in yeast extract-glucose-buffer medium. At 19 h (arrow) the inhibitor at concentrations of 0, 20, 40, and 80 μg/ml was added together with 0.5 mM m-hydroxybenzyl alcohol.*

phases appears to be due primarily to a lack of cellular homogeneity. This is in turn due to a variety of factors. The inoculum used is critical. We have found that a mycelial inoculum which possesses secondary metabolism enzymes retains this activity during replicatory growth in fresh medium, whereas an inoculum devoid of these enzymes quickly develops this activity if transferred from a complex to a nutritionally simpler medium. As in this latter case, a nutritional step-down can also occur when an inoculum is grown in a medium containing both a complex organic nitrogen source, such as yeast extract, and a simple inorganic nitrogen source, such as nitrate (16, 21). In both cases these cultures exhibit growth-associated secondary metabolism and metabolic heterogeneity. The more obvious heterogeneity of a pellet as opposed to a filamentous type of growth is also common. Thus, a mycelial inoculum which is not homogenized before use (16, 21) generally yields a culture of pellets. Even with a spore inoculum, pellet formation in *Aspergillus* and *Penicillium* is promoted by low inoculum size (i.e., spore concentration), mild agitation, adhesion to vessel surfaces, low medium viscosity, the absence or presence of trace metals or chelating agents, and by media possessing a high pH (pH 6 to 8), a high C/N ratio, or a simple inorganic, as opposed to a complex organic, nitrogen source (2). These generally poor growth conditions promote the high frequency of branching, which is characteristic of pellets and which leads to a compact core of cells whose access to substrate is severely limited (44). For example, oxygen limitation in *Penicillium* occurs in pellets only 0.2 mm in diameter (43). The marked phasing or "metabolic differentiation" described in this study resulted from the cellular homogeneity attained in shake cultures grown from a spore inoculum in silicone-treated flasks containing carefully designed media. These growth conditions were also successful in yielding essentially identical phasing in a variety of patulin-producing isolates of *P. urticae* and other fungi.

After the metabolic homogeneity and synchrony which are essential prerequisites to any study of the events which initiate secondary metabolism were achieved, subsequent experiments clearly demonstrated that a decrease in the specific growth rate of a culture was routinely accompanied by the rapid appearance of the first pathway enzyme, 6-MSA synthetase. Several hours later the fourth enzyme, *m*-hydroxybenzyl alcohol dehydrogenase, appeared. This initiation of secondary metabolism was independent of the age of individual cells and of the specific growth rate attained during the prior exponential growth phase (i.e., 0.26 and 0.15 h^{-1} in yeast extract and nitrate media, respectively). It is clear from these and other experiments (60; unpublished data) that a variety of environmental factors (e.g., aeration) can decrease the specific growth rate of a culture and initiate secondary metabolism in *P. urticae*. Of these, the exhaustion of nutrient nitrogen is the best documented and the most reproducible. Nitrogen catabolite repression has received little attention (17) despite its marked effect on the biosynthesis of nitrogen-free, acetate-derived secondary metabolites. The appearance of the polyketides trihydroxytoluene (59) and bikaverin (6) in *Aspergillus fumigatus* and *Gibberella fujikuroi*, respectively, has been correlated with the depletion of ammonium ion or glycine from the medium, and the low carbon/nitrogen ratio found in dairy products has been correlated with low yields of patulin (53). In contrast to this, the biosynthesis of the amino acid-derived β-lactam

cephalosporin is suppressed by carbohydrate and ammonium ion (1) but is stimulated by amino nitrogen, such as urea or amino acids. None of these studies examined the effect of nitrogen nutrients on the appearance of secondary metabolism enzymes.

Cells of *P. urticae* growing exponentially in either yeast extract- or nitrate-glucose medium exhibited normal dehydrogenase production profiles when resuspended in a 4% glucose solution with or without 0.1 M KH_2PO_4 (pH 6.5). A nitrogen source is not required, and hence no net protein synthesis is observed. An extracellular carbon and energy source (i.e., glucose) and an intracellular turnover of existing protein are the essential prerequisites for the initiation and maintainence of secondary metabolism in *P. urticae*. It is also clear that neither catabolite repression by glucose (14, 22) nor inhibition by phosphate (36) affect this initiation. The marked inhibitory effect of both cycloheximide and actinomycin D on the appearance and subsequent accumulation of both the synthetase and the dehydrogenase indicate that both transcription and translation are required and that the initiation of the synthesis of patulin pathway enzymes is controlled at the level of the gene. The incorporation of radiolabeled leucine into the dehydrogenase further supported the de novo synthesis of the enzymes themselves and eliminated the possibility that the appearance of these activities was simply due to an activation of preexisting protein. Earlier suggestions (8, 42) that 6-MSA synthetase is constitutive and remains latent until different nutritional conditions result in its activation are incorrect and probably originate from the carry-over of secondary metabolism in the mycelial inoculum. The suggestion that a disaggregation of fatty acid synthetase yields 6-MSA synthetase (42) is also incompatible with our results and those of Lynen et al. (34). Significant future progress in our understanding of the regulation of secondary metabolite (e.g., antibiotic and mycotoxin) biosynthesis will require an examination of the intracellular events occurring in truly homogeneous cell populations. This in turn will require a detailed knowledge of the pathways, enzymes, and genetics of secondary metabolism.

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