

# Mycotoxins - Their Biosynthesis in *Alternaria*

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## ABSTRACT

*Alternaria* produce a wide assortment of toxic and nontoxic secondary metabolites. A brief summary of the numerous secondary metabolites of *Alternaria* and their toxicity is followed by a presentation of the current view of the polyketide biosynthetic mechanism and its application to the biosynthesis of these compounds. Possible mechanisms for the biosynthesis of alternariol, alternariol methyl ether, and other dibenzo- $\alpha$ -pyrones are presented, as well as mechanisms for the biosynthesis of tenuazonic acid and altertoxin I. Bioregulation of the production of these materials by light, heat, nutrients and NADPH production, and the role of mannitol in NADPH formation are also discussed.

*Alternaria* are a large group of molds generally occurring on dead or dying plant tissue, but are often parasitic on plants (2,74). Molds of this genus require a relatively high moisture content (28 to 34% moisture) for growth, and the sustained high moisture content of leaf and stem tissue is conducive to their growth (9). *Alternaria* can cause disease in crops such as hay and silage, wheat, citrus fruits (*Alternaria citrii*), rice (rice blast disease), potatoes and tomatoes (early blight, by *Alternaria solani*), and tobacco (brown spot, caused by *Alternaria tenuis* and *Alternaria longipes*) (67).

The *Alternaria*, in addition to being plant pathogens that attack leaves and stems, also cause spoilage of mature fruits and vegetables both in the field and after harvest. *Alternaria* are weakly invasive and usually do not invade sound unblemished fruits and vegetables. Attack is usually through a skin imperfection, such as a scratch, or through the thin layer of skin under a stem cap. Fruit and vegetable tissues that have been in cold storage for extended periods of time or have not been harvested after reaching maturity are particularly prone to infection because of the weakened state of tissue (20). *Alternaria* spores can be harvested from almost all sound, high-quality, surface disinfected grain kernels. *Alternaria*, however, rarely present a problem in grain storage as the moisture content of grain is either below the organism's minimum growth level at harvest, or is dried to low moisture levels within a few hours or days after harvest (9). *Alternaria* contamination can be a problem in grain

when periods of wet, rainy weather occur during growth and harvest, or in specialty crops such as sweet corn.

*Alternaria* are of concern because they produce a wide assortment of metabolites that include many mycotoxins and phytotoxins. Both mycotoxins (24) and phytotoxins (29,37,67) have been reviewed recently. A recent handbook lists the properties of many of the toxic metabolites (11). This paper is concerned primarily with the biosynthesis of the *Alternaria* mycotoxins.

## STRUCTURES AND TOXICITY

The structures of the *Alternaria* metabolites given in Figures 1 through 4 are of several types. The dibenzo- $\alpha$ -pyrone group (Fig. 1) includes alternariol (AOH), alternariol methyl ether (AME), altenuisol (ASL), altertenuol, altenuene (ALT), and the probable dibenzopyrone derivatives altenuisin and altenuic acid II. Further work is needed to confirm the structures of altertenuol and altenuisol which were suggested after comparison with properties of similar compounds. The chemical characterization of these compounds has been reviewed (24). The nitrogen-containing group (Fig. 2) includes tenuazonic acid (TeA; a tetramic acid) cyclic polypeptides (tentoxin and AM-toxins), amides (AK-toxins) and zwitter ions (AL-toxins) (Fig. 4). TeA and tentoxin are nonspecific phytotoxins, whereas the AM-, AK- and AL-toxins are host-specific phytotoxins (37). TeA has mammalian toxicity and is considered to be one of the principal *Alternaria* toxins because of its prevalence. The anthraquinones (Fig. 3) are primarily phytotoxins, but include macrosporin, a compound formerly used as an antibiotic. Zinniol is included in this group due to its possible metabolic relation to the anthraquinones (see below). The chemistry of these compounds has been reviewed (62). *Alternaria* also produce a number of metabolites of varied structure (Fig. 4), including altertoxin-I (ATX-I), an unusual partially saturated perylene (61), and ergosta-4,6,8(14)-22-tetraen-3-one (ETO) (48).

Both the general toxicity of *Alternaria* isolated from various sources as well as the toxicity of specific compounds produced by *Alternaria* have been investigated and reported. Strains of *Alternaria* producing materials toxic to test animals when the molds were cultured on

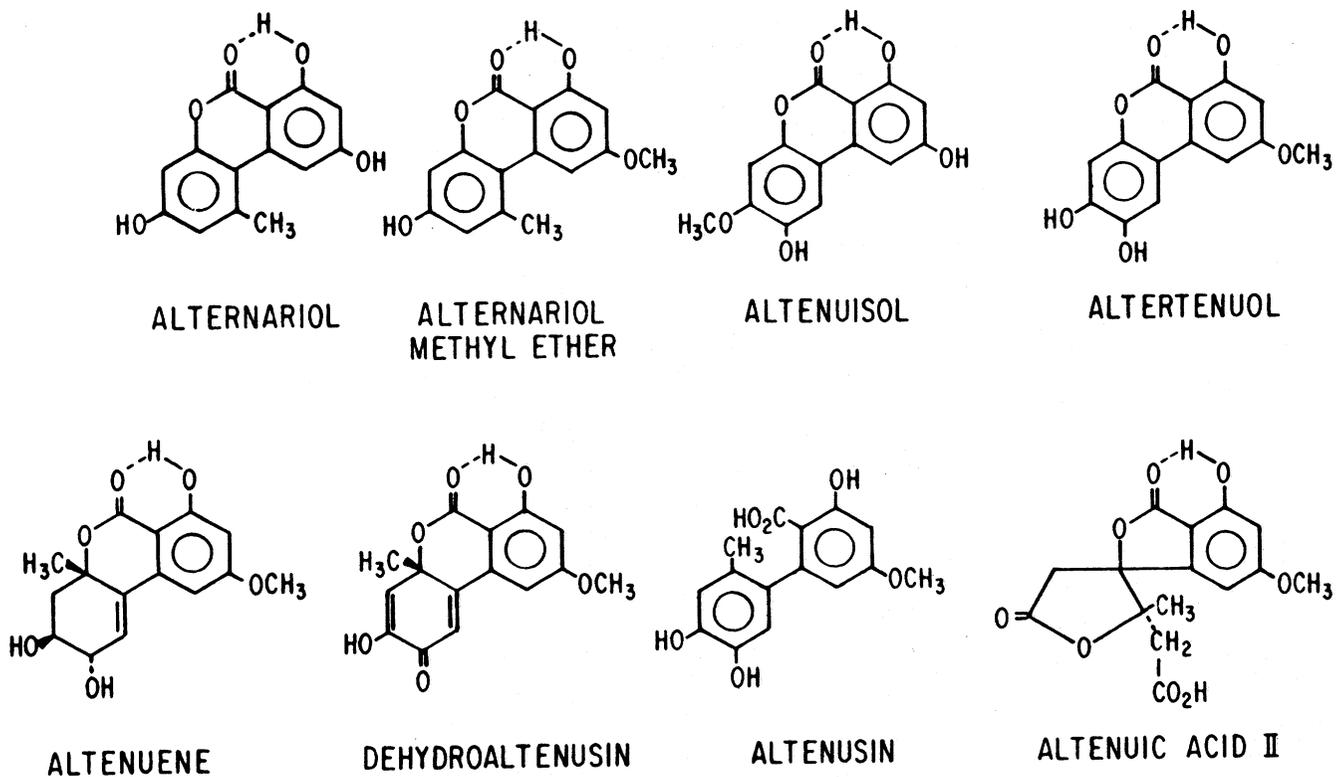


Figure 1. *Alternaria dibenzo-α-pyrone* metabolites.

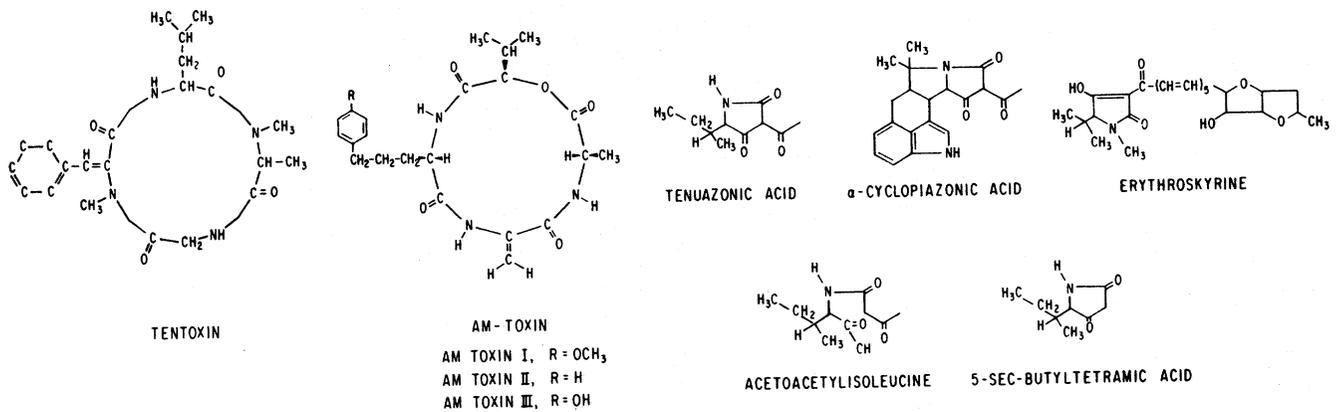


Figure 2. *Alternaria nitrogen-containing* metabolites.

neutral substrates have been isolated from grain allowed to remain in fields over the winter (30), fescue grass (75), cottonseed (14), and 53 of 60 *Alternaria* isolates from foods and feed (9). When 96 isolates of *A. longipes* obtained from tobacco were cultured on cracked corn and fed to chicks, 43 were lethal or detrimental to chicks, and crude *Alternaria* extracts from strains isolated from tobacco were lethal to mice (i.p. injection) and rats (oral) (15). In a similar experiment, the smoke from cigarettes made of alfalfa infested with *Alternaria* isolated from tobacco caused emphysema in mice, whereas tobacco or alfalfa smoke did not. Pyrolysis products of tenuazonic acid, the only *Alternaria* mycotoxin examined, did not produce this symptom (17). Specific *Alternaria* mycotoxins have been isolated from discolored pecans (44), weathered grain (43,49) and tomato paste (46), and have

been found when *Alternaria* were grown on tomatoes, apples, oranges, lemons and blueberries (58,60).

The Ames test for mutagenicity indicated AME to be weakly mutagenic to *Salmonella typhimurium* strain TA98 (-S9M), whereas AOH and TeA were inactive (47). No synergistic activity was observed with these compounds. This mutagenicity was not sufficient to account for the observed activity of the mycelium extract. Column and thin-layer chromatographic separations produced many mutagenic fractions tested against strain TA98 (-S9M). The more potent and identifiable mutagens included a material on TLC above AME that fluoresced green under 360-nm light, and gave a molecular ion in the mass spectrum at m/e 348. This material has since been identified in our laboratory as ergosta-4,6,8(14),22-tetraen-3-one (ETO). ETO widely occurs in fungi (73),

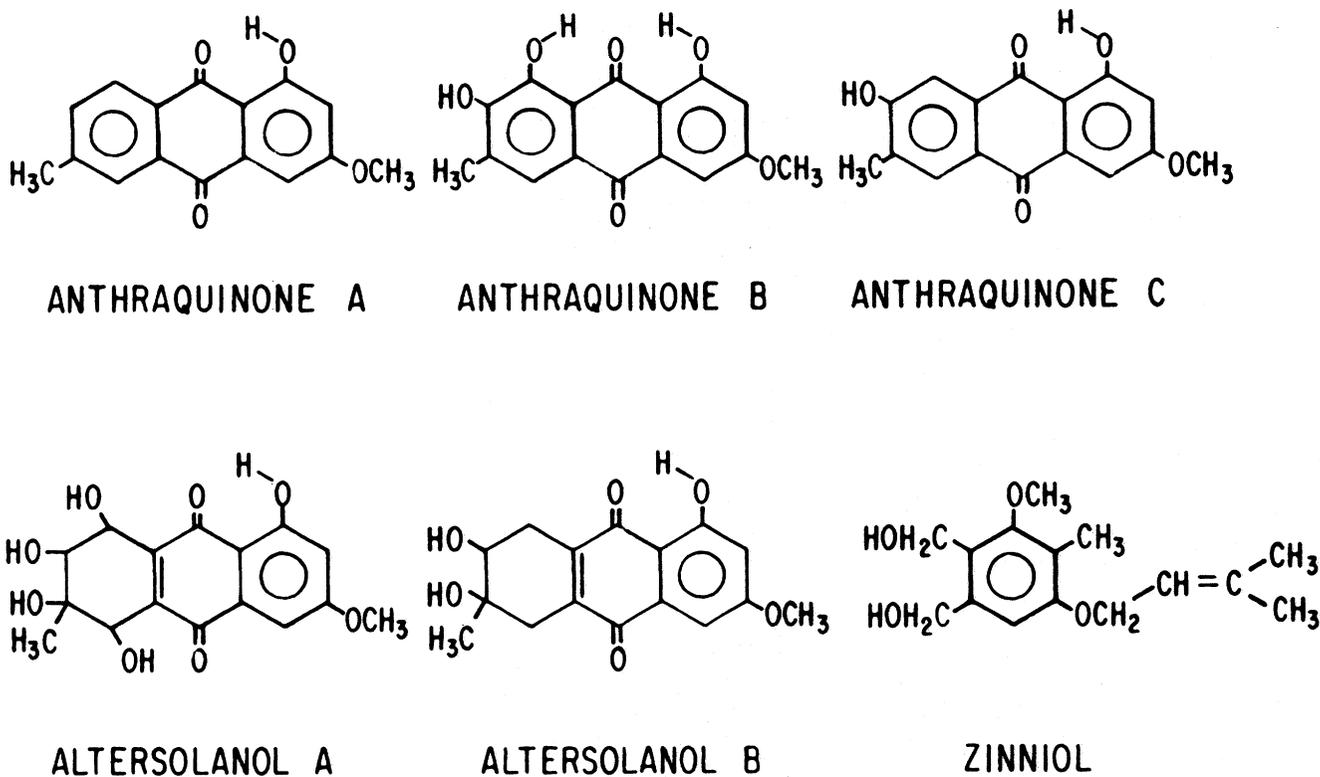


Figure 3. *Alternaria anthraquinone metabolites*.

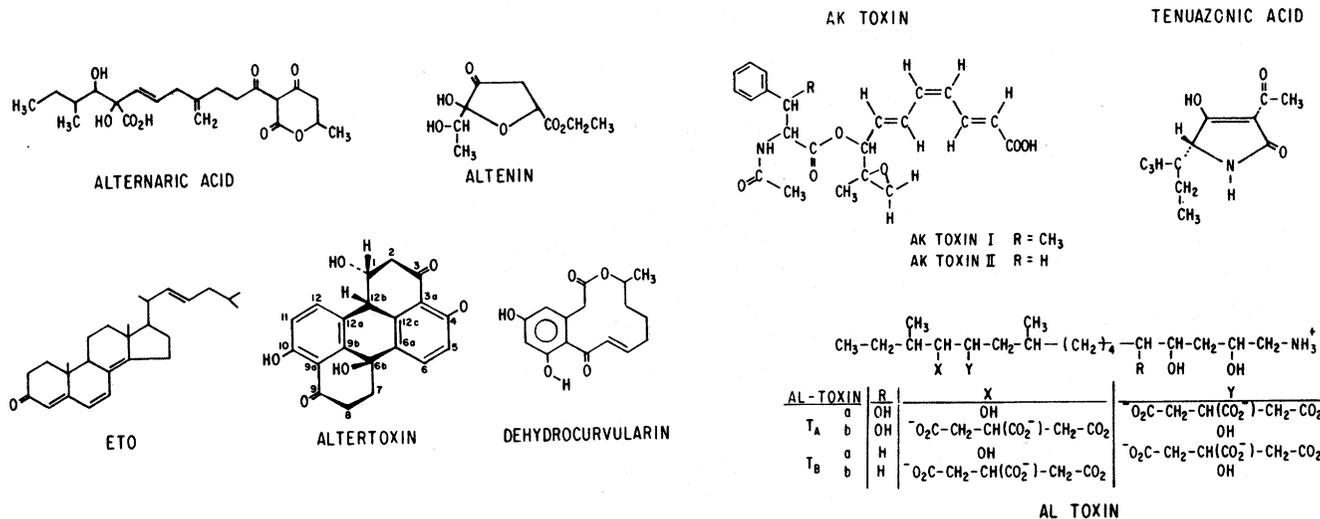


Figure 4. *Alternaria varied structure metabolites*.

and its detection in extracts of grain was suggested as a test for fungal contamination (48). ETO is frequently encountered in fruit infected with *Alternaria*, and is one of the earliest metabolites produced by the growing mold. This compound may be identical to a metabolite with similar TLC properties that was described as the primary toxicant produced by *Alternaria* isolated from apples, which exerted its toxicity particularly toward female test animals (52).

Two yellow pigments mutagenic toward strain TA98 (-S9M) were isolated by TLC (toluene-ethyl acetate-formic acid solvent), one between AME and AOH and the

other between AOH and ATX-I. Both had molecular ions at *m/e* 350. Scott and Stoltz (47) suggested that the compound between AME and AOH might be ATX-II. An orange pigment below ATX-I was found to be mutagenic. This compound was purified but was not successfully isolated.

The toxicity of a number of metabolites of *Alternaria* has been investigated (41). AOH, AME, ALT, ASL, ATX-I, altertoxin II and TeA were toxic to *Bacillus mycoides* and HeLa cells, and also displayed acute toxicity to mice. AME and AOH (the only compounds tested) were also fetotoxic and teratogenic to mice.

Onyalai, a human haematologic disease reported in Africa, may be associated with calcium/magnesium tenuazonate, known to be present in corn as a result of fungal contamination under laboratory conditions (54).

## BIOSYNTHESIS

### Polyketide synthetase

AOH and AME, and the other dibenzopyrones produced by the *Alternaria*, belong to the large group of secondary metabolites classified as polyketides. These compounds are usually of fungal origin, have diverse structures, and are classed together by apparent similarities in their mechanism of formation. There are many points of similarity between polyketide and fatty acid synthesis, both of which have been recently reviewed [polyketide (39,71); fatty acid (5,64,73)]. Fungal fatty acid synthetase (FAS), which occurs as a multi-enzyme complex,  $\alpha_6\beta_6$ , dissociates into two fragments thought to correspond to  $\alpha_4\beta_4$  and  $\alpha_2\beta_2$  (40). Pachter (39) discussed the possibility that polyketide synthetase may originate from the dissociation of FAS, with polyketide synthetase having a composition corresponding to  $\alpha_4\beta_4$ .

The current view (45) of the structure of yeast fatty acid synthetase and the processes involved in yeast fatty acid and polyketide formation is shown in Figures 5 and 6, respectively. Yeast FAS is considered to be representative of the structure of fungal FAS (73). The structure indicated in the diagram represents one  $\alpha$ - and one  $\beta$ -unit. The  $\alpha$  unit contains CON, condensing enzyme; ACP, acyl carrier protein; 1 RED,  $\beta$ -ketoacyl reductase. The  $\beta$  unit contains 2 RED, enoyl reductase; ACE, acetyl transferase; MAL, malonyl transferase; PAL, palmityl transferase; and DEH, dehydratase. Both polyketide and fatty acid synthesis involve condensation of acetyl-CoA (Ac-CoA) and malonyl-CoA (Mal-CoA) units with simultaneous release of the terminal carbon of the malonyl unit. For polyketide synthesis, the condensations continue without hydrogenation until a poly- $\beta$ -ketomethylene chain of the required length is assembled. At this point the activated methylene units of the polyketides have been assumed to react spontaneously with carbonyl groups by an aldol or Claisen condensation to yield the required aromatic compounds which are then released from the enzyme surface. However, the current view is that the cyclization

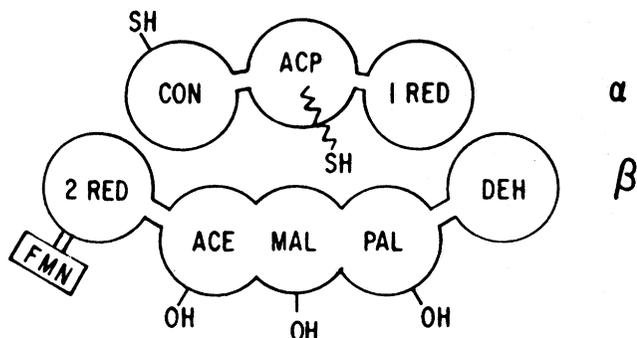


Figure 5. Proposed structure of yeast fatty acid synthetase.

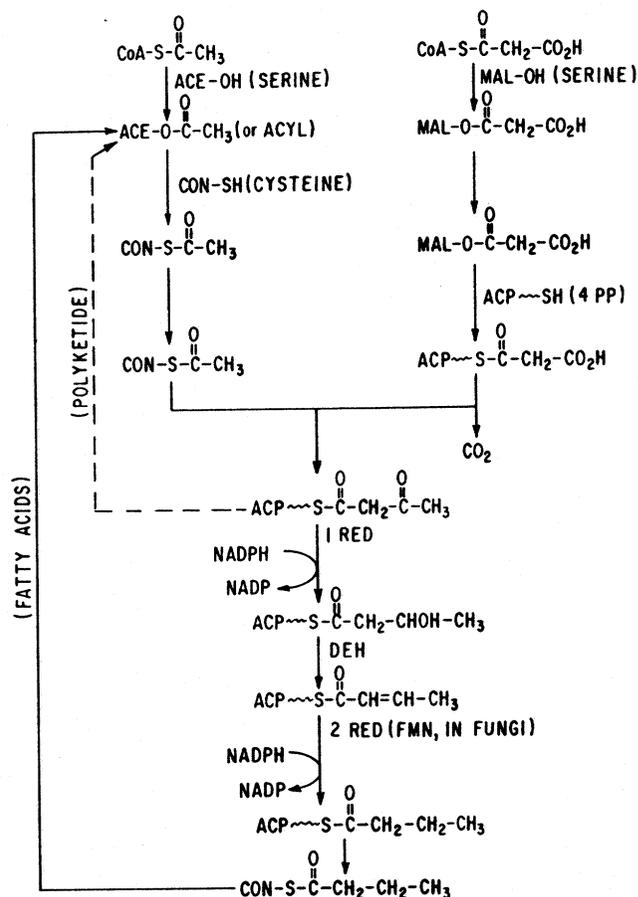


Figure 6. Processes of fatty acid and polyketide formation.

must be controlled so that only the correct reactive sites are brought into proximity to form the desired aromatic product. It has been proposed that the compound shifts to form a *cis*-double bond at the chain-folding site so that the three bonds are in a *syn* position (16). This premise was tested by examining the pattern of incorporation of  $CD_3CO_2H$  into several fungal metabolites, including AOH. The crucial double bond could be formed either from dehydration of a reduced carbonyl group (occurring during removal of an oxygen atom) or from keto-enol equilibrium when the oxygen is retained. The latter reaction would lead to dilution of the labeled deuterio atom. This was observed in AOH formation, which was consistent with the idea expressed above (1).

The nature of the actual reaction of ring closure is completely unknown, although flavin mononucleotide (FMN) may be involved in ring closure in the formation of aromatic compounds, as well as the reduction of the  $\beta$ -keto groups in fatty acid synthesis.

Fatty acid synthesis is more complex, as the keto groups must be reduced to methylene units before the next addition can occur. This reduction, which occurs in two later stages in the cycle, utilizes NADPH as a proton source. In polyketide biosynthesis, the keto groups are generally retained and appear in the aromatic ring structures as phenolic groups. When oxygen is removed, the process is thought to utilize the same cycle as FAS. Ad-

ditional oxygens may be introduced into polyketides through mixed-function oxygenases.

Use of labeled precursors revealed that Ac-CoA furnishes the first two carbon atoms at the methyl end of the polyketide molecule, whereas the remainder of the molecule is formed from Mal-CoA. The entire process is thought to occur on the surface of the synthetase molecule as no polyketide intermediates have been found. The incorporation of labeled carbon from acetate is in alternate carbons as would be expected from a head-to-tail condensation of acetate units.

#### Studies on AOH and AME biosynthesis

The currently accepted view of AOH formation is shown in Figure 7. As there is no loss of oxygen other than the atoms lost in the condensation to form aromatic rings, NADPH is not involved. The sequence of ring formation has not been established, but utilization of 1-<sup>14</sup>C acetate by the intact organism (68) resulted in incorpora-

tion of <sup>14</sup>C into alternate carbons of AOH. This pattern is consistent with the single-chain, head-to-tail structure postulated as an intermediate. The resemblance to orsellinic acid is apparent. Orsellinic acid and its ethyl ester were not incorporated, but this does not eliminate the possible involvement of compounds such as  $\gamma$ -orsellinyl-triacetic acid as intermediates (68,69). Mechanical disruption of mycelia of *A. tenuis* (18) produced a crude cell-free solution that could synthesize AOH by incubation with acetyl CoA and malonyl CoA. Incubation of the crude extract with labeled acetyl CoA and nonlabeled malonyl CoA produced AOH with 83% of the total radioactivity in the CH<sub>3</sub>-C positions and the remainder evenly distributed, thus indicating that the alternariol molecule was formed from a single sequence of malonate condensations. These authors also considered the synthesis to proceed through an orsellinic acid-type arrangement. This crude cell-free preparation could also synthesize lipid material (probably fatty acids) when supplied

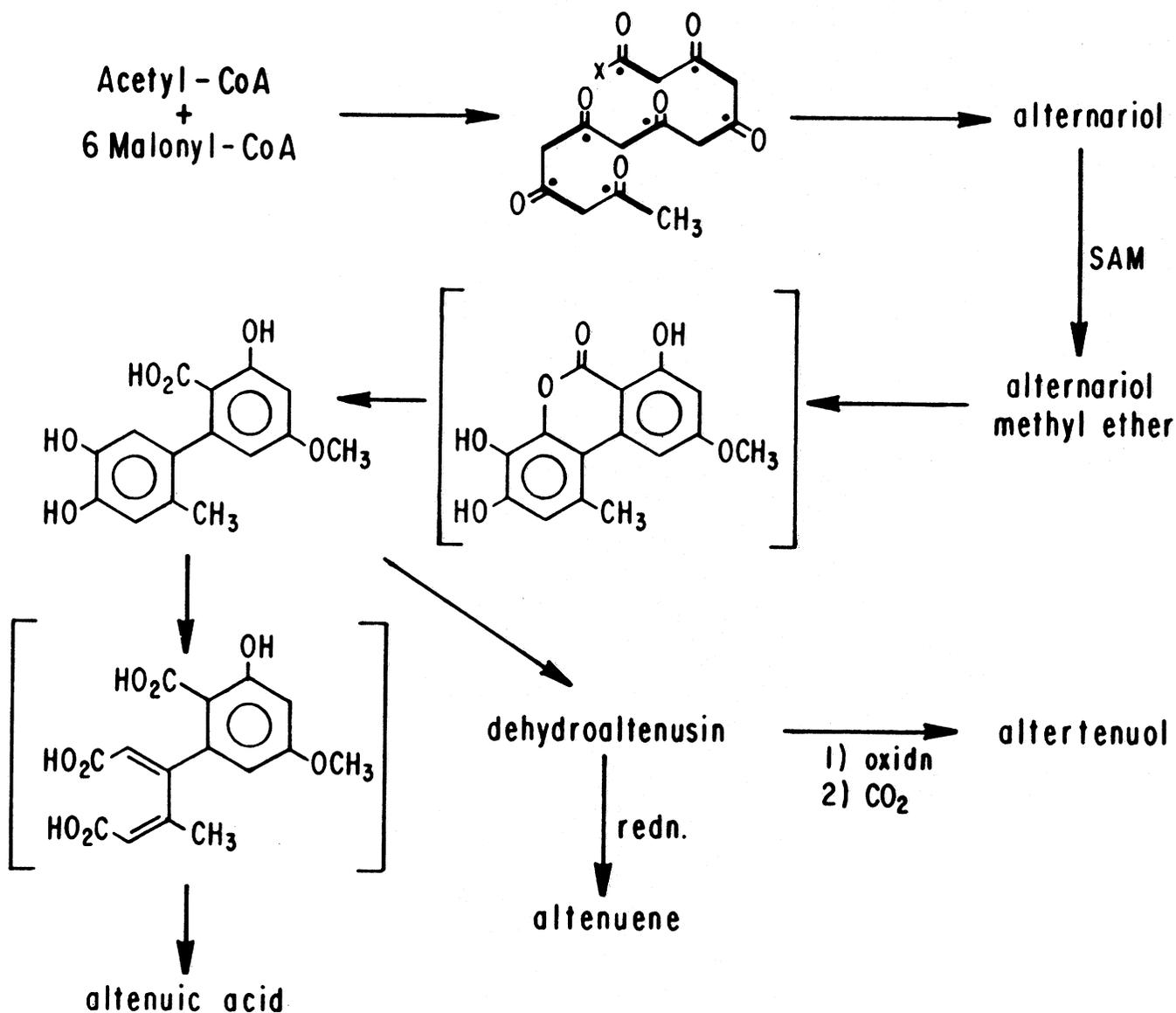


Figure 7. Current view of dibenzo- $\alpha$ -pyrone formation.

with NADPH. The crude enzyme solution was further purified by ethanol precipitation and gel filtration to yield a 30-fold purified enzyme solution. No production of radioactive substances other than AOH could be observed, supporting the hypothesis of the formation of enzyme-bound intermediates in the formation of aromatic compounds.

Both malonylpantetheine and S-malonyl-N-caprylsteamine could be substituted for malonyl CoA in AOH synthesis and lipid formation. For acetate to function as a precursor, CoA was required as the thiol moiety. The purified enzyme solution also possessed O-methyltransferase activity. The methyl group of S-adenosyl methionine was transferred to both alternariol added to the incubation mixture as well as to the alternariol synthesized de novo from malonyl CoA and acetyl CoA. In each case, the product formed was alternariol monomethyl ether (AME).

The properties of this purified enzyme were studied (51). The optimum activity was obtained at pH 7.8 to 7.9 at 28°C. Studies on the reaction velocity with different concentrations of malonylpantetheine and acetyl CoA revealed that the enzyme's optimum activity occurs with a 6:1 ratio of malonylpantetheine:acetyl CoA. The  $K_m$  for malonylpantetheine and acetyl CoA was  $2.4 \times 10^4$  and  $1.8 \times 10^5$  M, respectively. Acetyl CoA had considerably greater affinity for the enzyme than acetylpantetheine. Free CoA acted as a competitive inhibitor. The presence of the usual metal ion cofactors or EDTA (a chelating agent) had no effect on activity. The enzymatic activity was inhibited by the sulfhydryl-inhibiting ions  $Zn^{+2}$  and  $Cu^{+2}$ , as well as by p-chloromercuribenzoate, phenylmercuriacetate and iodoacetamide which have a similar effect, showing the importance of -SH groups in the enzyme. The -SH compounds, glutathione and cysteine, activated the enzyme.

The negative effects of increasing amounts of malonyl CoA could indicate an affinity of malonyl CoA for the acetate-binding site on the enzyme. Acetylpantetheine does not have any evident affinity for the malonate-binding enzyme. The addition of citric acid intermediates had no effect on the reaction velocity, whereas poly- $\beta$ -keto compounds inhibited AOH formation.

In the scheme presented in Figure 7 for formation of the dibenzo- $\alpha$ -pyrone metabolic products of *Alternaria* (24), AOH was considered to be the precursor of most of the dibenzo- $\alpha$ -pyrones produced by this genus. The formation of AME from AOH in the presence of SAM (S-adenosyl-methionine) has been established (51) but the remainder of the scheme is hypothetical. Alternusin, formed from AME, could give rise to the remaining dibenzo- $\alpha$ -pyrones by the indicated routes.

In contrast to the above results, Stinson and Moreau (59) have found that cell-free extracts of mycelia of *Alternaria alternata* under similar conditions are capable of utilizing  $1\text{-}^{14}\text{C}$  Ac-CoA for biosynthesis of AOH, whereas  $2\text{-}^{14}\text{C}$  Mal-CoA is not incorporated. These results indicate that AOH biosynthesis may involve addi-

tional factors than those usually considered to be involved.

Several other pathways for AOH biosynthesis are at least theoretically possible. The benzopyrone nucleus and oxygenation patterns of AOH and related compounds are suggestive of the structure of 7-oxygenated coumarins (6). The structure of AOH has similarity to p-coumaric acid and umbelliferone, whereas altertenuol has a marked resemblance to caffeic acid and esculetin. Where required, further oxygenation of simple coumarins can occur either before closure of the lactone ring (42) or subsequent to formation of the lactone (6). However, the benzopyrone structure of coumarins has been shown to be derived from shikimic acid which would lead to randomization of labeling from  $1\text{-}^{14}\text{C}$  acetate, whereas distinct alternate labeling is observed in AOH (18,69). In vivo feeding studies with  $^{14}\text{C}$ -labeled cinnamic, coumaric and benzoic acids indicated that *Alternaria* metabolize these compounds by  $\beta$ -oxidation of the side chain to yield acetate and the corresponding benzoic acids. The phenylalanine- and tyrosine ammonia-lyases, which would convert these amino acids to the corresponding cinnamic acids, could not be detected in crude extracts of the mycelium (36).

Another possibility is a biosynthetic pathway similar to the aflatoxin biosynthesis by *Aspergillus* (55). The mechanism, based on  $^{13}\text{C}$ -NMR data, involves bond cleavage and rearrangement of the anthraquinone structure present in versicolorin A to form the xanthone (dibenzo- $\gamma$ -pyrone) structure present in sterigmatocystin. This compound in turn undergoes ring cleavage and rearrangement to form the dibenzo- $\alpha$ -pyrone structure present in aflatoxin B<sub>1</sub>. The cleavage of the aromatic ring of versicolorin also resembles the gentisaldehyde ring cleavage in patulin biosynthesis (4). The corresponding mechanism for dibenzo- $\alpha$ -pyrone formation in *Alternaria* is presented in Figure 8. The pattern of introduction of labeled acetate precursors into the altersolanol A molecule was established by  $^{13}\text{C}$ -NMR (63). The tetrahydroanthraquinone *Alternaria* metabolite shown in Figure 8, altersolanol A, has an oxygenation pattern that could readily give rise to AME by this mechanism, and is known to be the source of macrosporin. The xanthone proposed as an intermediate has not been isolated, but zinniol, another *Alternaria* metabolite isolated from the *Alternaria* strain that produced altersolanol A, has a structure and  $^{13}\text{C}$ -distribution similar to the proposed intermediate (63). Both this mechanism and the single-chain polyketide mechanism (18,69) would produce, with  $1\text{-}^{14}\text{C}$ -labeled acetate, the same labeling pattern in AOH. The  $^{13}\text{C}$ -NMR investigation of the incorporation of 2-C-labeled and di-labeled acetate into AOH would serve to distinguish the two mechanisms and is currently being investigated in our laboratory.

The scheme shown in Figure 8 starting with altersolanol A (a prearomatic  $\beta$ -methylanthraquinone) necessitates the loss of the  $\beta$ -methyl group, which could give rise to altertenuol, and subsequent introduction of the 6-methyl of AME and AOH. Although  $\beta$ -methylan-

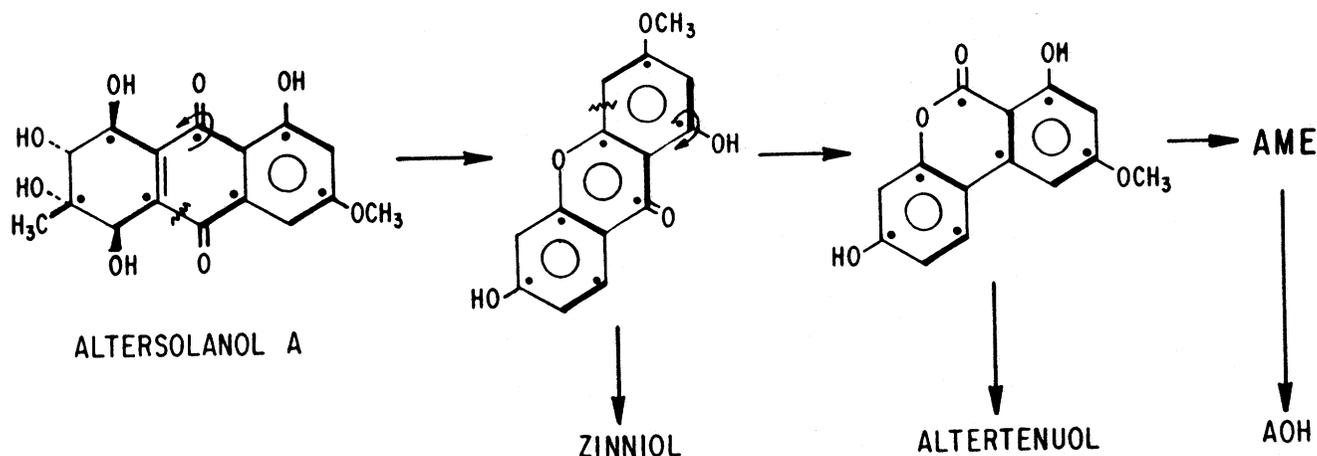


Figure 8. Alternative pathway for formation of dibenzo- $\alpha$ -pyrones from an anthraquinone.

thraquinones are more abundant in nature,  $\alpha$ -methylanthraquinones also are found, with  $\alpha$ - and  $\beta$ -compounds occurring even in the same plant (33). An  $\alpha$ -methylanthraquinone analog of altersolanol A (if produced by *Alternaria*) could be the origin of the quaternary carbon of altenuene, dehydroaltenusin and altenusin. The structures of several unidentified anthraquinones produced by an altenuene-producing strain of *A. alternata* is being investigated in our laboratory.

#### Tenuazonic acid biosynthesis

Tenuazonic acid is one of the most abundant of the metabolites of *Alternaria*. It is a tetramic acid that apparently is secreted by the mold, as it is present in the filtrate of submerged cultures (54). Tenuazonic acid is produced by a number of organisms, including *A. tenuis* (54), *A. alternata* (34), *Pyricularia oryzae* Cavara (72) and *Phoma sorghina* (54). Other naturally occurring mycotoxins containing the tetramic ring structure include  $\alpha$ -cyclopiazonic acid, isolated from *Penicillium cyclopium*, *Aspergillus versicolor* and *Aspergillus flavus* Link, and erythroskyrine, which is a mycotoxin produced by *Penicillium islandicum* Sopp (Fig. 2). The biosynthesis of these compounds has been reviewed recently (26). Incorporation of labeled compounds into these tetramic acids is consistent with the view that these are formed by condensation of an amino acid (L-isoleucine, L-tryptophan or L-valine) with units derived from acetate.  $\alpha$ -Cyclopiazonic acid also contains an isoprene unit derived from mevalonic acid.

Incorporation of labeled precursors (57) indicated that tenuazonic acid was formed from one molecule of L-isoleucine and two acetate units. Details of the polyketide assembly and final condensation with the amino acid to form the tetramic acid ring system have not been established. It has not been shown whether the active form involved in condensation was isoleucine or a precursor. Presuming isoleucine is involved, the biosynthesis could proceed either via acetoacetylisoleucine or 5 sec-butyltetramic acid. If the latter is the case, acetoacetic acid would not be involved, and acetates would be involved sequentially. No tests were done to investigate the direct

utilization of isoleucine, acetoacetic acid, or either of the presumed intermediates. Acetoacetic acid, if involved, is probably formed via a nonpolyketide pathway similar to that observed in hepatic cells. Polyketide or fatty acid synthetases would not be expected to participate because fungal polyketide and fatty acid intermediates are firmly bound to the synthetases and are not released until the complete molecule is assembled. When the culture media was supplemented with L-valine and L-leucine, the organism formed the corresponding tetramic acid, i.e., 3-acetyl-5-isopropyltetramic acid and 3-acetyl-5-isobutyltetramic acid (19). These results are consistent with formation of the tetramic acid from the amino acid instead of its precursor.

#### ATX-I biosynthesis

No studies have been done on the biosynthesis of ATX-I, the structure of which was recently reported as a perylene (61). If ATX-I is a polyketide, it cannot be formed from a single chain. This would be consistent with other perylene derivatives reported as fungal metabolites.

The distribution of labeled carbon in elsinochrome A, a perylene produced by *Elsinae* and its imperfect form, *Phyllosticta caryae*, indicates that it is formed from a dinaphthyl intermediate derived from two polyketide chains, each involving one acetate and six malonyl units (8). Another fungus, *Daldinia concentrica*, apparently produces another perylene via a dinaphthyl intermediate derived from two butyrophenone units known to be biosynthesized from acetate (3).

Two *Alternaria* pigments with perylene structures have been isolated and their biosynthesis investigated (38).

## BIOREGULATION

#### Effect of light on mycotoxin production by *Alternaria*

Excellent reviews have been written on the effect of light on fungal development and metabolism (50,66). White light inhibited production of AOH and AME by *A. alternata* in the late growth phase, although mycelial weight was not significantly affected (53). Exposure to

12 h of illumination during the exponential growth phase at days 3 and 4 almost completely inhibited the AOH and AME production which occurred from days 5 to 7. An unidentified red-brown pigment was observed to accumulate under these conditions, and it was suggested that under the influence of the light, the fungus uses acetyl-CoA and malonyl-CoA for the production of this red pigment.

Subsequently, alternariol production in *A. alternata* found to be generally inhibited by light as seven isolates were found to respond by lower AOH and AME production (22). Continuous blue light between 400 to 500 nm decreased AOH and AME production 69% and 77%, respectively, whereas continuous red light (550 to 750 nm) had no effect on toxin levels. Other metabolic pathways were affected by the light treatments, as total lipids were increased 25% when mycelium was treated by blue light as compared to darkness or red light, and exposure to white or blue light, but not red light or darkness, caused production of red-brown pigments. Incubation in continuous white light caused production of  $\beta$ -carotene by *A. alternata*. This compound was absent in culture grown in darkness. The mechanisms responsible for these phenomena remain obscure, although several possibilities are apparent. These include:

(a) Application of blue light halts sporulation, and developing conidiophores redifferentiate to form sterile mycelium (32). This may be of possible significance to mycotoxin production in *A. alternata*, as mycotoxin production was associated with strains having highly branched mycelia (23).

(b) Biosynthesis itself is a nonmorphogenic biochemical process that could be affected. One factor of possible significance is the long-range action between time of exposure to light (3 to 4 d, during the rapid growth phase) and observed effect on chemical action (5 to 7 d) (50). This could be indicative of either regulation of enzyme synthesis by formation of new messenger RNA or the prevention of formation of enzymic material during this period that would not be utilized until later.

(c) Blue light has been shown in *Penicillium isariforme* to change respiration from the Embden-Meyerhof pathway (EM) to the pentose phosphate shunt (PPS). A similar effect in *Alternaria* would have the effect of creating a pool of triose, pentose, hexose and heptose phosphates that would undoubtedly affect secondary metabolism and polyketide biosynthesis (21). This shift from EM to PPS would also increase the level of NADPH, which would favor fatty acid synthesis instead of polyketide formation.

(d) Absorption in the effective region between 400 and 500 nm resembles the absorption spectra of both carotenes and flavins, both of which have been suggested as receptors for the characteristic blue absorption of fungi. Flavins are the current favorites, but the question is far from settled (13). Although exposure to continuous white light caused production of  $\beta$ -carotenes by *Alternaria* (23), this could be a protective response. Carotenes are often considered to be photoprotectants (31).

Involvement of light-exposure with FMN function has

been indicated with other organisms. Spectrophotometric measurements of mycelium of *Neurospora crossa* irradiated with moderate amounts of blue light showed change in absorbance at 560 nm, which indicated the reversible photoreduction of a b-type cytochrome (35). Dose-response relations as a function of actinic wavelength showed that the receptor pigment was not cytochrome b (Cyt b), but rather a pigment absorbing maximally at about 460 nm, which absorbed the light and mediated the photoreduction of Cyt b. Long-term irradiation with high-intensity blue light caused irreversible bleaching in the 460-nm region together with loss of the reversible light-induced absorbance change. The absorption spectrum of the bleached pigments resembled that of a flavin.

Cell-free extracts of the mycelium contained Cyt b and Cyt c in the oxidized state but did not facilitate the light-induced changes in absorbance. However, when FAD or FMN was added to the solution and incubated for a few minutes, both cytochromes became photoreducible. In view of the possible relation between FAS and polyketide synthetase, it is interesting that both yeast and *Penicillium patulum* FAS contain flavin (25,45).

Similarly, cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. form a naphthoquinone, shikonin, when grown in the dark, but exposure to light prevents accumulation of this compound unless FMN is added to the culture. It has been suggested that FMN was a cofactor of an enzyme involved in formation of the aromatic ring, and that light destroyed the FMN (65). FMN may play a similar role in *Alternaria* during the formation of the chemically similar anthraquinones, which are possible precursors of AOH.

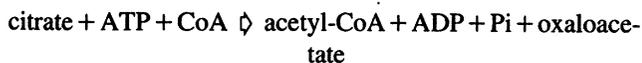
(e) Another possible primary effect might be on the concentration of cAMP (3',5' cyclic monophosphate). In *Phycomyces*, irradiation caused a decrease in cAMP within 1 min (10). Interestingly, addition of cAMP greatly stimulates mycotoxin production by *Aspergillus* (70).

#### *Relation of NADPH to AOH biosynthesis*

The polyketide metabolites AOH and AME may be produced in large amounts by *Alternaria*, with AOH comprising up to 10% of the dry weight of mycelia (69). *Alternaria* have also been reported to produce large amounts of lipids by FAS activity (27). As both polyketides and lipids are derived from Mal-CoA and Ac-CoA, the supply of both materials would soon be exhausted. The inference is that *Alternaria* almost certainly use vigorous metabolic pathways such as the tricarboxylic acid cycle (TCA) to replenish these materials.

Hult and Gatenbeck (27) investigated the roles of various carbon sources in the biosynthesis of AOH and lipids in the presence of (-)-hydroxycitrate, which is an inhibitor of ATP citrate lyase. The results are summarized in Figure 9. Both the synthesis of the polyketide AOH and fatty acid require Ac-CoA and Mal-CoA. Mal-CoA is formed in the cytosol by addition of CO<sub>2</sub> to Ac-CoA, whereas Ac-CoA is formed in the mitochondria from pyruvate resulting from glycolysis. The Ac-CoA formed

in the mitochondria is combined with oxaloacetate to form citrate, which is transported to the cytoplasm by the citrate-malate shunt. Once citrate is in the cytoplasm, Ac-CoA is regenerated by ATP-citrate lyase, which catalyzes the reaction:



(-)-Hydroxycitrate is a known inhibitor of ATP-citrate lyase. Hult and Gatenbeck (27) found that, with glucose as the carbon source, hydroxycitrate inhibited both fatty acid and AOH biosynthesis, presumably by causing a shortage of cytoplasmic Ac-CoA.

When the acetate was used as the precursor, (-)-hydroxycitrate did not change AOH formation and only fatty acid formation was diminished. This indicated the presence of an acetyl-CoA synthetase in the cytoplasm that utilized acetate. Although free acetate is a rare substrate in nature, Stumpf (64) provides many examples of plant acetyl-CoA synthetases occurring in the cytosol that utilize acetate.

Since the main difference between AOH and fatty acid biosynthesis is the requirement for cytoplasmic NADPH, (-)-hydroxycitrate must influence the production of this material. Hult and Gatenbeck (27) investigated two reactions of the TCA cycle that can serve as a source of cytoplasmic NADPH for fatty acid synthesis in (-)-hydroxycitrate-inhibited cells. In the first reaction, cytoplasmic oxaloacetate, formed together with acetyl-CoA from the action of ATP-citrate lyase upon citrate, is reduced to malate by cytoplasmic dehydrogenase. Malate, in turn, is partially utilized in forming pyruvate and NADPH. This reaction, however, is self-limiting as a source of NADPH because it leads to depletion of the malate which is necessary for the shuttle system to transport citrate from the mitochondrion to the cytoplasm. In confirmation of this explanation, Hult and Gatenbeck (27) found that addition of malate to (-)-hydroxycitrate-inhibited cells increased production of fatty acid.

The second NADPH-producing reaction of the TCA cycle that was investigated involved isocitrate brought to the cytosol by the isocitrate/malate shunt system. As malate is not consumed, it does not have the self-limiting

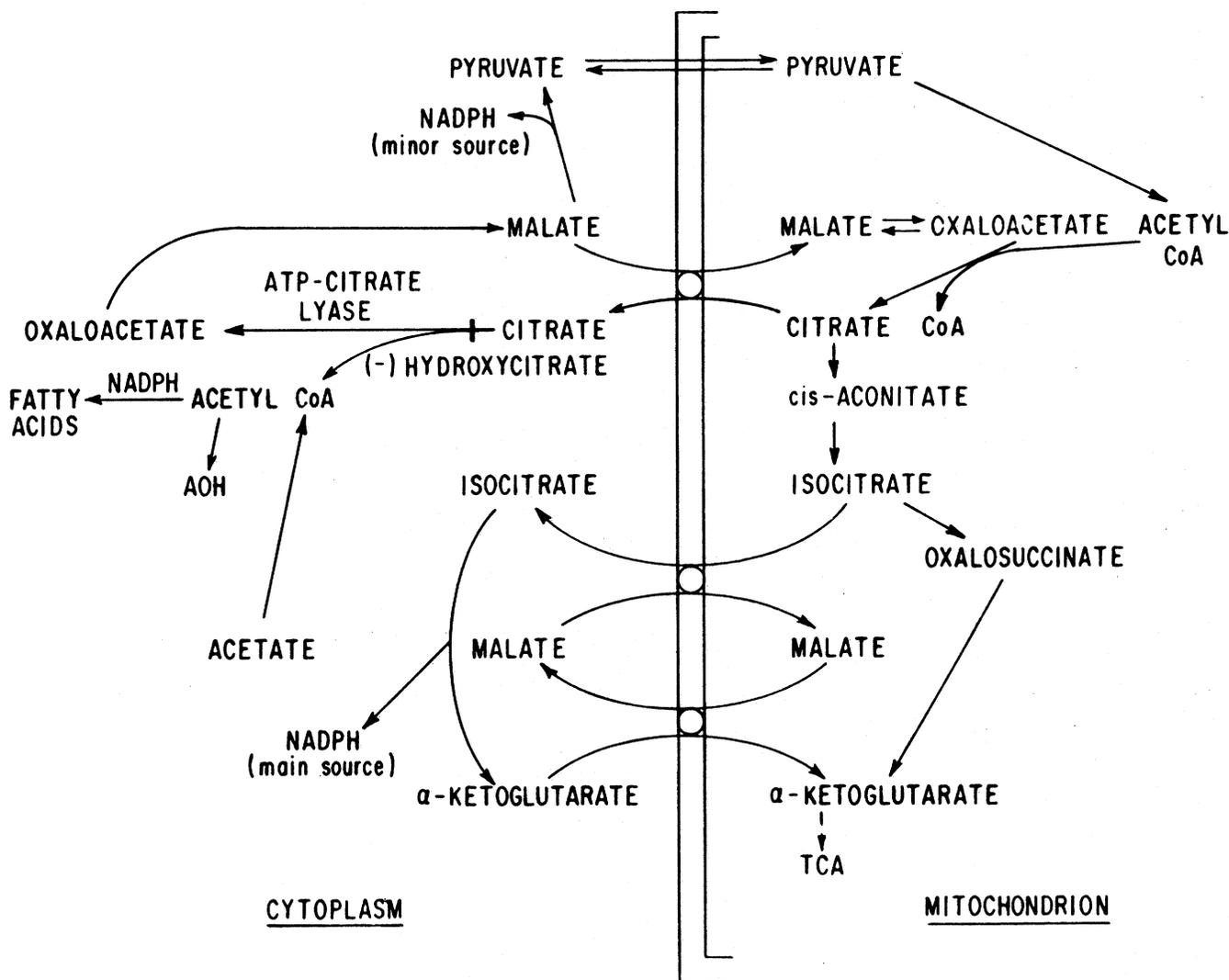


Figure 9. Effect of (-)-hydroxycitrate on alternariol and lipid formation.

feature of the first method for NADPH production and may be the principal source of NADPH in uninhibited cells. In this reaction, malate is involved in a shuttle to transport isocitrate (formed by the TCA cycle) from the mitochondria to the cytoplasm where isocitrate is oxidized by cytoplasmic dehydrogenase to form  $\alpha$ -ketoglutarate and produce NADPH. Addition of isocitrate also stimulated production of fatty acids in (-)hydroxycitrate-inhibited cells in the same manner as addition of malate. The effect of (-)hydroxycitrate by its role as a chelating agent has not been investigated. It has not been established if the inhibitor penetrates into the mitochondrion itself where it could effect the events of the TCA cycle.

#### Role of mannitol in NADPH formation

Mannitol is one of the main metabolic products of *Alternaria*. Mannitol comprises 15% of the cell dry weight of 4-d cultures and decreases to 9% of the cell dry weight after 8 d (28). Mannitol is simultaneously formed and utilized at a high turnover rate, which suggests that mannitol serves both as a carbon source and as a storage form of reducing power. For the latter use, the net result of one turn of the mannitol cycle (Fig. 10) is:



In confirmation of the importance of this cycle, it was shown by kinetic studies that over 50% of the fructose-6-phosphate formed proceeded through the mannitol cycle. The amount of NADPH thus formed was calculated to be sufficient to fulfill the total need of NADPH for fat synthesis.

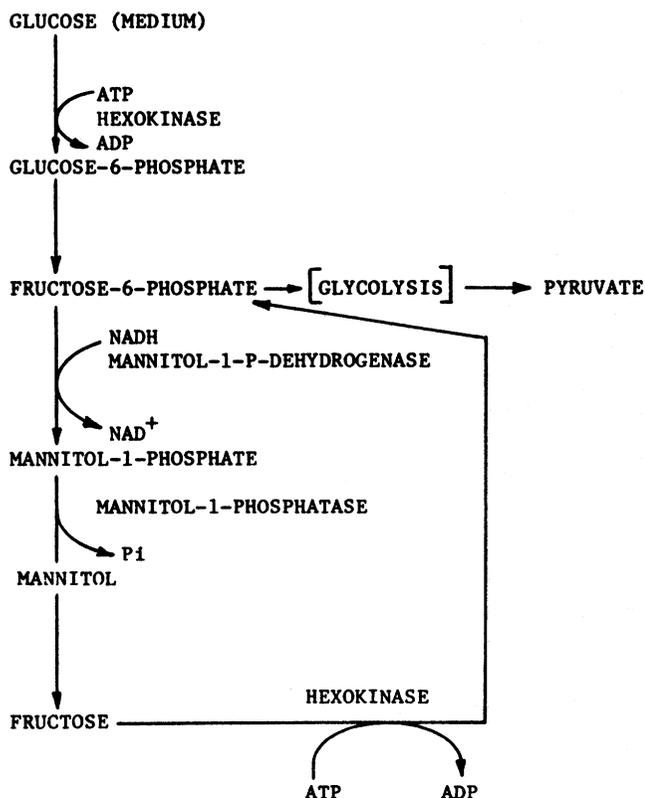


Figure 10. Mannitol cycle in *Alternaria alternata*.

Mannitol also served as a carbon source for both AOH and fatty acid biosynthesis; however, at peak AOH production at 8 d, mannitol and glucose were equivalent as carbon sources for both AOH and fatty acid biosynthesis. The operation in mammalian systems of this and other shuttle systems for conveying reducing equivalents from cytosol to mitochondria and their effect upon the regulation of oxidative metabolism has been reviewed recently (12).

#### Effect of substrate on mycotoxin production

AOH production is decreased if yeast extract or yeast extract plus Czapek-Dox broth is added to rice used as a substrate (7). The conventional theory of mycotoxin formation suggests that limiting the supply of an essential nutrient triggers the change in metabolism to a mature metabolic pattern with its attendant mycotoxin production pattern. In this case, yeast extract would eliminate the deficiency of the essential nutrient.

In a somewhat different vein, Zn and Cu were found to suppress the formation of AOH in *A. tenuis*. These elements were thought to react with sulfhydryl groups in the synthetase, thus causing inhibition of AOH synthetase (51).

#### Effect of temperature

Very little information is available on the effect of temperature on mycotoxin production by *Alternaria*, although poikilothermic organisms adapt to cold temperatures by increasing their unsaturated lipid content (73). Toxin formation was observed in grain overwintered in the field, from which toxin-producing strains of a number of fungi, including *A. tenuis*, were later isolated (30).

The optimum temperature for TeA production is 20°C, on both yeast extract-sucrose medium and cottonseed, although TeA production occurs at all temperatures between 10 and 30°C. TeA has been produced on cottonseed having as low as 14.97% moisture content, with highest TeA production at 37.5% moisture (76).

#### SUMMARY

The *Alternaria* are a large group of molds which include many species that are plant pathogens and food spoilage organisms. *Alternaria* produce a wide assortment of both mycotoxins and phytotoxins. The mycotoxins are structurally diverse, and include a large number of dibenzo- $\alpha$ -pyrones of the alternariol family. The phytotoxins are also structurally diverse and include large numbers of anthraquinones and tetrahydroanthraquinones. Many *Alternaria* mycotoxins still have not been chemically identified.

The *Alternaria* dibenzoquinones and anthraquinones are produced by the polyketide mechanism, which has many points in common with fatty acid biosynthesis. This review presents the current view of the enzymes and processes involved in polyketide and fatty acid synthesis. Fungal fatty acid synthetase occurs as a multi-enzyme complex having the formula  $\alpha_6\beta_6$  which readily dis-

sociates into fragments that may be  $\alpha_4\beta_4$  and  $\alpha_2\beta_2$ . The relation of the larger fragment with polyketide synthetase, which has a molecular weight that corresponds to  $\alpha_4\beta_4$ , has not been established.

The currently accepted view of AOH biosynthesis is that it proceeds by a classic polyketide mechanism involving a single enzyme-bound polyketide chain assembled from one acetyl-CoA and six malonyl-CoA units to yield a product with a distinct alternate labeling pattern. A biosynthetic pathway involving anthraquinone rearrangement similar to the biosynthesis of aflatoxin by aspergilli should produce the same labeling pattern in AOH with  $1-^{14}\text{C}$  acetate as that predicted from the accepted mechanism. Biosynthesis of tenuazonic acid, another important metabolite of AOH, involves one isoleucine unit and two acetate units.

Bioregulation of mycotoxin production is influenced by nutritional deficiencies, exposure to light, internal cellular metabolism affecting NADPH supply, and temperature. The interrelationship of these factors and their relative importance in the organism growing in the wild are imperfectly understood. Both polyketide and lipid biosynthesis utilize Ac-CoA and Mal-CoA, but lipid biosynthesis also requires NADPH. Control of the relative proportions of these metabolites, which are produced by this branched pathway, involves more than simple availability of NADPH. As yet, there is no evidence of competitive inhibition whereby lipids interfere with mycotoxin formation by inhibiting an early step of the mycotoxin pathway.

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