Mycotoxins – Their Biosynthesis in Fungi: Ergot Alkaloids

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ABSTRACT

Biosynthesis of ergot alkaloids is discussed from the standpoint of biosynthetic precursors and intermediates as well as known biosynthetic mechanisms. Emphasis is given to work concerning regulation of alkaloid production, including the role of tryptophan as an inducer of alkaloid synthesis. A postulation is proposed to explain the significance of induction in terms of evolution and survival of the organism which also takes into account the finding that the biosynthesis of tryptophan is under regulatory control in the fungal strain which was investigated. Recent studies using protoplasts of ergot have also shown that endproduct regulation of alkaloid synthesis may be a significant phenomenon.

Mycotoxoses caused by ingestion of ergotized rye have been known for centuries. The toxic effects in man have usually resulted from eating bread made from rye flour which was contaminated with sclerotia of Claviceps purpurea (Fries) Tulasne. From the accounts of ergotism reviewed by Barger (2) and Bove (6), it is apparent that during the Middle Ages one form of this mycotoxicosis, referred to as St. Anthony’s fire or ignis sacer, produced vasoconstriction of the peripheral blood vessels, resulting in intense burning and pain in the extremities with eventual dry gangrene followed by the falling away of the affected portions of the body. Another form of the toxicity resulted in more prominent CNS effects which included hallucinations and convulsions. In connection with this, an interesting recent speculation is that the young women accused of being witches in the Salem witchcraft trials of 1692 were actually suffering from the convulsive type of ergotism (9).

MYCOLOGY

Ergot infects a wide range of wild and cultivated grasses; however, rye, Secale cereale, is a common host plant. On the plant the fungus forms a sclerotium which may have the same general configuration as the seed but is larger, dark colored, and hard. The sclerotia are the resting or overwintering stage of the fungus and in the spring they germinate, sending up flesh-colored fruiting bodies which rarely exceed 25 mm in length. These fruiting bodies produce the sexual spores (ascospores) which are carried by the wind to florets of grass inflorescences where the germinating ascospore enters the developing ovary of the flower via the stigma. After an incubation period of 2 to 7 days a sweet, sticky exudate called honey-dew is produced from each of the infected flowers. The honey-dew contains conidia and since it attracts insects, the fungus is dispersed to other flowers with resulting fresh infections. At the end of the growing season the production of conidia and the secretion of honey-dew ceases and the sclerotia are formed (27).

THERAPEUTIC USE

The mycotoxins produced by the ergot fungus are alkaloids, some of which have valuable therapeutic applications. The effects which have given ergot alkaloids importance in medicine are related to their ability to stimulate both vascular and non-vascular smooth muscle. Stimulation of the vascular smooth muscle causes constriction of the blood vessels in the vascular bed, and the resulting constriction of intracranial arteries is useful in the treatment of migraine and other types of cluster headache. Ergotamine (Fig. 1) is an agent of choice in the treatment of acute attacks of migraine.

The sensitivity of the smooth muscle of the uterus to stimulation by ergot increases along with the stage of gestation, so the resulting contractions at the end of the third trimester of pregnancy may induce labor. Midwives and physicians have used ergot for this purpose for centuries. At the present time, however, ergot compounds such as ergonovine (Fig. 1) are used only postpartum to produce firm uterine contractions and decrease uterine bleeding. They are also used to control hemorrhage associated with abortion.

An extremely potent hallucinogenic activity is exhibited by lysergic acid diethylamide (LSD). This compound was synthesized by Hofmann and Stoll (40) and Hofmann accidentally discovered its hallucinogenic activity by inhaling the crystal dust while crystallizing LSD in the laboratory (26). LSD can be considered a semi-synthetic compound, since lysergic acid (Fig. 1) is prepared from the naturally occurring alkaloids and then used as starting material for the chemical synthesis. Another drug material which is also semi-synthetic and which has important therapeutic potential is Lergotrile® (Fig. 1). This compound is synthesized using elymoclavine (Fig. 2) as a starting material. Lergotrile® is representative of a group of ergot alkaloids and related compounds that inhibit prolactin release from the anterior pituitary (17). The clinical significance of these
ERGOTAMINE

\[ R = \begin{array}{c}
\text{H} \\
\text{N} \\
\text{C} \\
\text{O} \\
\text{H} \\
\text{N} \\
\text{C} \\
\text{H}_2 \\
\end{array} \]

\[ \text{CH}_3 \]

ERGONOVINE

\[ R = \begin{array}{c}
\text{H} \\
\text{N} \\
\text{C} \\
\text{O} \\
\text{H} \\
\text{N} \\
\text{C} \\
\text{H}_2 \\
\end{array} \]

\[ \text{CH}_2 \text{OH} \]

LYSERGIC ACID

\[ R = \begin{array}{c}
\text{H} \\
\text{N} \\
\text{C} \\
\text{O} \\
\text{H} \\
\text{N} \\
\text{C} \\
\text{H}_2 \\
\end{array} \]

\[ \text{Cl} \]

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LORROTRILE

Figure 1. Examples of ergot compounds of therapeutic importance.

Figure 2. Ergot alkaloid biosynthetic pathway.

Biosynthetic pathway. Essentially the biosynthetic precursors are tryptophan which was first found to be incorporated into the alkaloids when Mothes et al. (32) injected DL-tryptophan-\( \beta \)-\( ^{14} \)C into the internodes of rye plants which had been inoculated with ergot. Subsequent isolation and hydrolysis of the radioactive alkaloids formed in the mature sclerotia revealed that radioactivity resided in the lysergic acid. Three different groups (12, 24, 41) almost simultaneously demonstrated that mevalonic acid is efficiently incorporated into the ergoline ring system, showing the biosynthetic involvement of an isoprene unit in the form of dimethylallylpyrophosphate. Finally Baxter et al. (4) established that methionine supplies the methyl on the N-methyl group.

The first pathway-specific step in the biosynthesis is the isoprenylation of the 4-position of tryptophan to form dimethylallyltryptophan. Plieninger et al. (35) showed that radioactively labeled dimethylallyltryptophan prepared synthetically was incorporated into the alkaloids. Other workers were able to isolate dimethylallyltryptophan from ergot cultures in which alkaloid synthesis had been inhibited by exclusion of oxygen (36) or by addition of the methionine antagonist ethionine (1). Finally, the enzyme, dimethylallyltryptophan (DMAT) synthetase, which catalyzes the isoprenylation reaction, has been isolated from \textit{Claviceps} (25).

Very recent evidence indicates that the next intermediate in the pathway arises from the N-methylation of dimethylallyltryptophan to give \( N_\alpha \)-methyl-4-dimethylallyltryptophan. Barrow and Quigley (3) have isolated this compound from \textit{Claviceps}. Also, Otsuka et al. (34)
have fed N$_{\alpha}$-methyl-4-dimethylallyl tryptophan (amino-$^{15}$N, N-C$_{3}D_{3}$) to a culture of *Claviceps* and the mass spectral analysis of the isolated elymoclavine showed the presence of 29% of the molecular ion species enriched with $^{15}$N and three deuterium atoms. This result shows unequivocally that N$_{\alpha}$-methyl-4-dimethylallyltryptophan can be converted intact into tetracyclic ergolines. These findings also support earlier work by Fehr (14) in which enzymatic conversions involved in these events is still unknown; however, the biosynthetic role of the chanoclavines, tricyclic compounds found in the fungus, is known. The stereoisomers of chanoclavine are chanoclavine-I, isochanoclavine-I and chanoclavine-II (Fig. 2). Chanoclavine-I was shown by Gröger et al. (23) to be efficiently incorporated into agroclavine, elymoclavine and lysergic acid methylcarbinolamide. In addition these workers demonstrated that chanoclavine-I was not incorporated into agroclavine or elymoclavine, suggesting that N-methylation may occur before C-ring closure on the way to form chanoclavine.

The next anticipated step in the biosynthesis would be the decarboxylation of N$_{\alpha}$-methyl-4-dimethylallyltryptophan with a subsequent formation of ring C of the chanoclavines. The number of intermediates and enzymatic conversions involved in these events is still unknown; however, the biosynthetic role of the chanoclavines, tricyclic compounds found in the fungus, is known. The stereoisomers of chanoclavine are chanoclavine-I, isochanoclavine-I and chanoclavine-II (Fig. 2). Chanoclavine-I was shown by Gröger et al. (23) to be efficiently incorporated into agroclavine, elymoclavine and lysergic acid methylcarbinolamide. In addition these workers demonstrated that chanoclavine-I was not incorporated into elymoclavine with higher dilution than into agroclavine, supporting a pathway of chanoclavine-I $\rightarrow$ agroclavine $\rightarrow$ elymoclavine. As indicated in Fig. 2 neither chanoclavine-II nor isochanoclavine-I are incorporated into agroclavine or elymoclavine (15,18). A possible intermediate between chanoclavine-I and agroclavine is chanoclavine-I-aldehyde. Floss et al. (21) have demonstrated a 40% incorporation of the aldehyde into elymoclavine when the aldehyde group was labeled with tritium; however, it has not been possible to demonstrate the occurrence of chanoclavine-I-aldehyde in the fungus.

Concerning the biosynthetic mechanism of alkaloid formation, there is evidence that two isomerizations in the isoprenoid moiety takes place during formation of the tetracyclic ergolines (19). Mevalonic-2-14C acid labels the trans-carbon atom of dimethylallylpurinephosphate and, as can be seen in Fig. 2, the trans-carbon atom of the isoprenoid moiety of agroclavine is also labeled. Rather than this labeling pattern being a result of a biosynthetic mechanism without isomerization it is, instead, a result of a cis-trans isomerization going from chanoclavine-I to agroclavine, and experiments with mevalonic acids stereospecifically tritiated at C-4 indicate that another cis-trans isomerization occurs earlier in the pathway.

Using both sclerotia and cultures of the ergot fungus, elymoclavine has been shown to be the precursor of lysergic acid derivatives (33). Several investigators (29,31) have demonstrated that lysergic acid serves as a precursor to the peptide alkaloids and the various peptide side chains of these compounds arise from the respective amino acids.

**INDUCTION**

In 1964 it was suggested that tryptophan not only serves as a precursor to the ergot alkaloids but may also act as an inducer of the alkaloid-synthesizing enzymes (20). This proposal resulted from observations that tryptophan stimulated alkaloid production if added early during the fermentation period before the onset of alkaloid synthesis, but not when it was added after the start of alkaloid synthesis. It was also demonstrated that methyl analogs of tryptophan which were not alkaloid precursors had a stimulatory effect on alkaloid production. In addition it was found that mycelium grown in the presence of tryptophan or tryptophan analogs retained an ability to produce more alkaloid than the controls even after it had been transferred into fresh culture medium not containing tryptophan or its effectors.

Other evidence supporting the possibility of induction was provided by Bu'Lock and Barr (8) when they found that protein synthesis was necessary to maintain alkaloid production. In addition, using tryptophan-supplemented cultures, they found that the second differential of the alkaloid production curve, which would indicate the rate of the appearance and disappearance of an enzyme(s) limiting the rate of alkaloid synthesis, closely paralleled the experimental curve for internal tryptophan concentration, suggesting a direct relationship between the rate of synthesis of this enzyme and the amount of tryptophan within the mycelium. In this regard, we observed an increase in the endogenous free-tryptophan pool before the onset of alkaloid production (19).

Other laboratories and our own have also provided evidence supporting an induction of ergot alkaloid synthesis by tryptophan. The stimulation of alkaloid synthesis by addition of tryptophan at the beginning of the growth phase has been observed by Vining (42). He found that addition of radioactively labeled L-tryptophan during the period of rapid alkaloid synthesis resulted in an efficient incorporation of radioactivity into alkaloids; however, there was only a slight increase in yield of alkaloid, leading Vining to suggest that tryptophan stimulates alkaloid production through the activity of the alkaloid-synthesizing enzyme system.

Employing a culture medium which did not contain yeast extract and carrying out parallel fermentations with tryptophan and 5-methyltryptophan added at the beginning of the culture period, we (37) were able to obtain a greater stimulation of alkaloid production over the control than had previously been reported (20). At day 11 in the culture period the tryptophan-containing cultures showed a six-fold increase in alkaloid and the 5-methyl-tryptophan-containing cultures a four-fold increase over the control cultures.

Whereas the methyl-substituted tryptophans used in these earlier works were never as active as tryptophan itself in increasing alkaloid production, we obtained an analog, thiotryptophan [β-(1-benzothien-3-yl)-alanine] which, as seen in Fig. 3, consistently equaled or exceeded
tryptophan in its ability to increase alkaloid production. Thiophryptophan contains a sulfur atom which substitutes for the indolic nitrogen atom of tryptophan, and experimental evidence indicates that it does not serve as a substrate for the alkaloid-synthesizing enzymes (28). Cultures which were induced with tryptophan, 5-methyltryptophan, and thiophryptophan were sampled daily over the course of the fermentation. The alkaloid titer of the cultures was determined and cell-free extracts were prepared from mycelium and assayed for activity levels of the first pathway-specific enzyme for alkaloid synthesis, DMAP synthetase. The results from these assays are illustrated in Fig. 3 and 4 and show a clear parallel between DMAP synthetase levels and alkaloid production leading us to believe that the induction effect involves de novo synthesis of this enzyme (28).

To obtain the full induction effect, time-course studies show that tryptophan must be added to cultures during the first 24 h of the fermentation, and that a 12-h exposure period is sufficient for the maximum effect (38). The effects, higher enzyme levels and greater alkaloid synthesis, are observed after the end of the active growth phase, which is 4-6 days later. Apparently the gene expression for induction is programmed by tryptophan early in the growth phase but suppressed in some way until later in the culture period. The suppression may be linked to phosphate inhibition, since it is known that in the presence of increased levels of phosphate alkaloid synthesis is almost completely repressed (12). Addition of ten times the normal level of inorganic phosphate to normal or thiophryptophan-induced cultures at any time up to day 11 of the fermentation will block any further alkaloid synthesis (38); however, high levels of tryptophan can partially overcome the phosphate inhibition of alkaloid synthesis (28).

**REGULATION OF TRYPTOPHAN BIOSYNTHESIS**

The central role of tryptophan in ergot alkaloid biosynthesis has led us to investigate the degree to which tryptophan biosynthesis is regulated in the fungus. Since effectors of tryptophan such as 5-methyltryptophan usually inhibit tryptophan biosynthesis either through feedback inhibition or repression, the fact that in ergot these compounds stimulate the formation of alkaloids indicates a lack of regulation in tryptophan synthesis. As indicated in Table 1, we (28) found when we assayed for activity of certain key enzymes of tryptophan biosynthesis in induced cultures that the biosynthesis of tryptophan is under regulatory control in *Claviceps* species, strain SD 58. It appears that thiophryptophan is rather ineffective as a substitute for tryptophan in end-product regulation. As might be expected, tryptophan is the most effective in inhibiting the enzymes, whereas 5-methyltryptophan approaches the ability of the parent compound in controlling tryptophan biosynthesis. These results could also explain why 5-methyltryptophan is consistently much less effective in
stimulating ergot alkaloid synthesis than tryptophan, whereas thirottryptophan is equally as effective or, in some experiments, more effective than the parent compound (Fig. 3). Apparently, 5-methyltryptophan, while triggering formation of alkaloid synthesizing enzymes, limits tryptophan synthesis by false feedback inhibition and/or repression resulting in a limitation of precursor tryptophan for alkaloid synthesis.

**EVOLUTIONARY SIGNIFICANCE OF INDUCTION**

It is reasonable to speculate that tryptophan induction of alkaloid biosynthesis in ergot has offered a selection advantage for the organisms in evolution. One of the more attractive hypotheses as to what this advantage might be is that proposed by Bu'Lock (7), who states that it is not the secondary metabolites (alkaloids) in themselves that are important but rather the actual activity of secondary biosynthesis. The argument is that during the growth phase of the organism there is an absence of secondary biosynthesis, not because of a lack of appropriate precursors, but because of the absence of the required enzymes. When growth is halted by some circumstance such as the using-up of a nutrient essential to growth, an imbalance results in primary biosynthesis and this may initiate formation of enzymes of secondary metabolism. If primary metabolites accumulate to a certain level, metabolic regulation phenomena such as feedback inhibition or repression would shut down the biosynthetic machinery of primary metabolism. Repression would result in a cessation of the synthesis of enzymes; however, if some of the primary metabolites are siphoned off into secondary metabolism, this may allow for the continued de novo synthesis of a limited amount of the enzymes of primary metabolism which would be a distinct advantage in maintaining the integrity of the cell and thus increase the survival opportunities for the organisms.

In the case of ergot, as illustrated in Fig. 5, during the growth phase tryptophan is utilized in the formation of protein. When growth ceases, tryptophan would no longer be incorporated into protein and would be expected to accumulate to levels that would shut down its biosynthesis. Indeed, we (28) have obtained evidence that exogenously fed tryptophan and 5-methyltryptophan do inhibit the tryptophan biosynthetic pathway (Table 1). However, since tryptophan also induces alkaloid synthesis, it is possible that enough tryptophan is removed from the endogenous tryptophan pool through alkaloid biosynthesis, so that the pool does not reach levels that would cause repression of tryptophan biosynthesis. This would be a stabilizing influence since a consequence of this would be that primary metabolism would continue to operate and the substrates for protein synthesis would continue to be formed.

**END-PRODUCT INHIBITION**

It has been shown by Heinstein et al. (25) that DMAT synthetase isolated from *Claviceps* species SD 58 is inhibited by physiological concentrations of agroclavine and elymoclavine, the end products of the alkaloid biosynthetic pathway in this strain of the organism. A later enzyme in the pathway, chanoclavine-I-cyclase, was also inhibited slightly by elymoclavine (13). Anthranilate synthetase was also sensitive to inhibition by elymoclavine (30). Since millimolar concentrations of elymoclavine accumulate in the culture medium towards the end of the fermentation, it is possible that end-product inhibition could stop the production of alkaloids. We (11) conducted experiments to determine whether end-product regulation was a significant phenomenon in vivo. Our results using intact cells of the fungus were variable; however, utilizing protoplasts of the fungus we found a significant difference of incorporation of L-(methylene-14C)-tryptophan into alkaloids in the presence of physiological levels of elymoclavine as compared to controls without elymoclavine (Fig. 6). In addition DMAT synthetase activity was measured in cell-free extracts of protoplasts which had been exposed to elymoclavine and which had been carefully dialyzed to remove residual alkaloid. As shown in Fig. 7, there is little difference in the enzyme activity level between the controls and the treated protoplasts; consequently, it appears that elymoclavine acts through an inhibition of DMAT synthetase rather than a repression of enzyme synthesis.

**TABLE 1. Effect of tryptophan and its analogs on enzyme activities in Claviceps species. SD 58.**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Enzymes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAH synthetase</td>
<td>Anthranilate synthetase</td>
</tr>
<tr>
<td>4mM DL-Tryptophan</td>
<td>49(^a)</td>
<td>24</td>
</tr>
<tr>
<td>4mM 5-Methyl-DL-tryptophan</td>
<td>60</td>
<td>34</td>
</tr>
<tr>
<td>4mM DL-Thiotryptophan</td>
<td>92</td>
<td>94</td>
</tr>
</tbody>
</table>

\(^a\)Obtained from five-day-old mycelium.

\(^b\)Expressed as percent of enzyme activity as compared to enzymes from control cultures which contained no additives.
Figure 6. Incorporation of L-tryptophan into ergot alkaloids by protoplasts of Claviceps prepared from 4-day-old mycelium The incubation consisted of flasks with 0.7 M KCl ( ) and with 4 mM potassium succinate ( ) respectively as controls; and with 4 mM elymoclavine succinate ( ). Each flask was sampled at 4, 12, and 24 h after the addition of 2 μ Ci L-(methylene-14C)-tryptophan.

ACKNOWLEDGMENT

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REFERENCES


Figure 7. The effect of removal of residual alkaloid from enzyme preparations obtained from protoplasts. (A) Incorporation of L-tryptophan into ergot alkaloids by protoplasts of Claviceps using the same conditions as described in Fig. 6. Incubation consisted of control with 4 mM potassium succinate ( ) and with 4 mM elymoclavine succinate ( ). (B) DMAT synthetase activity in enzyme extracts dialyzed for 20 h to remove residual alkaloid. Incubations were the same as in (A).

Drug-Resistant Organisms, cont. from p. 820

Workers in a penicillin factory had 100 percent of nasal staphylococci carriers with resistant organisms while in the general public 88 percent of carriers had penicillin susceptible organisms.

In another study, the sensitivity pattern of Staph. aureus isolated from animals was checked. Of 621 samples about two-thirds were resistant to at least one of the 11 drugs tested.

Resistance was more frequent to penicillin (60%) followed by streptomycin (34.7%) and tetracycline (32.8%) in treated animals.

Two especially significant discoveries were 1) the high prevalence of resistance to penicillin, streptomycin, and tetracycline regardless of the host of origin, and 2) the low to negligible prevalence of resistance to the other antimicrobial agents among isolates from dogs and horses.

Other organisms of equal concern are E. coli, which are normal inhabitants of most animal intestines. It, under certain conditions, may attack its host with serious or fatal results. Resistance in this organism complicates treatment.

Its widespread presence and the ability to transfer resistance to other organisms are additional complications.

Grumbles stresses that he is not advocating discontinuing use of antibiotics where needed and effective. He is cautioning that in view of present knowledge added care should be exercised in their use.

One possibility is the development of parallel antibiotic usage—those used in animal feeds would not be used in treatment of disease.

Two such antibiotics are presently coming into use for swine and poultry feed.

Editor’s Note — Any questions regarding this column should be addressed to Science Writer, Dept. of Agricultural Communications, Texas A&M University, College Station, Texas 77843.