Aflatoxin biosynthesis in *Aspergillus parasiticus*: effect of methionine analogs

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The physiological effects of DL-ethionine and related methionine analogs upon cellular protein synthesis and methyl group transfer to aflatoxin B₁ in *Aspergillus parasiticus* were examined in growing and resting cell systems. The addition of DL-ethionine (0.02 M) to growing mycelia inhibits cellular growth some 37% after 120 h of development. Addition of ethionine before 50 h prevents any aflatoxin synthesis; however, addition of ethionine after aflatoxin production begins has little effect upon continued synthesis. The enzymes responsible for aflatoxin production are made during the transitional phase of growth (50–70 h). During this period, ethionine administration blocks protein synthesis (measured by 14C-methionine incorporation) 85% and prevents aflatoxin synthesis. Since exogenous ethionine (3.2 mM) inhibits aflatoxin B₁ synthesis about 50%, a competitive-type inhibition is indicated. Such inhibition is accompanied by the formation of a new aflatoxin B₁ derivative. The incorporation of 14C-ethyl-ethionine into this derivative indicates a transethylation of the toxin ring system in the formation of ethoxy-aflatoxin B₁. The addition of DL-ethionine and S-ethylcysteine to proliferating cells yields some of the aflatoxin B₁ derivative.

Introduction

Since the early observations of Dyer (1), considerable information has been compiled on the biochemical effects of ethionine. Ethionine interferes with methionine incorporation in protein synthesis, formation of other sulfur-containing amino acids, and transmethylation reactions (2). The biological activity of methionine, except for its incorporation into proteins, centers around mobilization and transport of the methyl group through an active form, S-adenosylmethionine (3). S-Adenosylmethionine has been isolated from yeast cells grown in the presence of ethionine (4) and participates in a transethylation reaction with L-homocysteine to form ethionine in an in vitro enzyme system derived from *Torulopsis utilis* (5).

The methyl groups of several antibiotics are derived from transmethylation reactions. Ethionine serves as an inhibitor of transmethylation leading to oxytetracycline formation in *Streptomyces viridifaciens* at concentrations producing partial inhibition of growth (6). As ethionine substitutes for methionine, transethylation takes place instead of the normal transmethylation; this reaction forms an oxytetracycline analog, the N-methyl ethyl derivative of oxytetracycline (7). A 2'-ethoxy analog of griseofulvin has been isolated from an ethionine-supplemented fermentation (8).

Since a metabolite-antimetabolite relationship between methionine and ethionine is well established for various biological molecules, it seemed likely that ethionine might interfere with aflatoxin B₁ (6-methoxydiferocoumarone) synthesis because a methylation is involved in the formation of this carcinogenic, secondary metab-
olite by *Aspergillus parasiticus*. The methoxy methyl group at the C-6 position of aflatoxin is derived from methionine (9). It was anticipated that either demethoxy or ethoxy analogs of aflatoxin B1 could be obtained with ethionine supplementation and an inhibition of toxin synthesis through ethionine antagonism. A study was made, therefore, of the effects of methionine analogs, primarily ethionine, upon protein synthesis and aflatoxin biosynthesis by *A. parasiticus* cultures.

**Materials and Methods**

**Culturing Methods**

A strain of *A. parasiticus* NRRL 2999 was maintained at room temperature on potato dextrose agar slants (20 g dextrose, 200 g potatoes, and 1 liter of distilled water) with transfers every 10 days. Spores from individual slants were used to inoculate 100 ml of a synthetic medium containing the following ingredients per liter: glucose, 50 g; (NH₄)₂SO₄, 3 g; KH₂PO₄, 10 g; MgSO₄·7H₂O, 2 g; (NH₄)₆Mo₇O₂₄·4H₂O, 0.5 mg; Fe₂(SO₄)₃·6H₂O, 0.3 mg; CuSO₄·5H₂O, 0.3 mg; ZnSO₄·7H₂O, 15 mg; and boric acid, 1 mg. The final pH of the medium was 4.6. The fungus was also cultivated in a 1% yeast extract–soybean (YES) medium (200 g sucrose, 10 g yeast extract per liter of water).

For resting-cell suspension experiments, mycelial pellets (13 g wet weight per 100 ml) were suspended in resting-cell media composed of, per liter: glucose, 15 g; KH₂PO₄, 5 g; MgSO₄·7H₂O, 1 g; KCl, 1 g; and trace elements.

For experiments measuring protein and aflatoxin biosynthesis, filtered mycelia were washed with sterile 1% NaCl solution. Protein determinations were also made by the method of Lowry (12). Aflatoxin B1 synthesis was measured according to methods previously described.

The mycelial pellets from each assay tube were washed by filtration, oven-dried for 30 min, and weighed. The spent assay medium and pellet washings from filtration were extracted by a standard CHCl₃ extraction method for B1 (10). These extracts were evaporated to dryness on a flash evaporator; then the residue was redissolved in a given volume of CHCl₃. Aflatoxins B1 and B2a, and the ethoxy derivative were separated on preparative thin-layer Silica Gel G-HR₂ (Brinkmann Instruments, Inc., Westbury, Mass.) chromatoplates with 20% acetone in CHCl₃ as used as developer. The aflatoxin bands were scraped off, the toxins eluted with CHCl₃, and radioactivity determined in Bray’s scintillation fluid with a Packard Tricarb dual channel liquid scintillation counter.

**Incorporation Assay**

The incorporation data for B1 and the derivative were given in disintegrations per minute by correcting for quenching in the system with the channels ratio method. Aflatoxin B1 concentration was also measured spectro-photometrically (10, 11) by its absorbancy at 360 μm. Mycelial pellets (1.0–1.5 mg aliquots, dry weight) from a 50-h culture of strain NRRL 2999 were transferred to test tubes containing 1.25 μcurie ¹⁴C-(CH₃)-methionine (5 μcurie/m mole) in 5-ml volume of YES medium. After this addition, DL-ethionine solution was added, while an appropriate volume of H₂O was added to the control tubes. Incubations were carried out at 30°C and terminated at various times. The mycelial pellets were washed by filtration, oven-dried for 30 min, and weighed. The dry mycelium samples were ground with 60-mesh glass beads to a fine powder. The material was diluted with 30 ml of H₂O, plus 5 ml of 5% trichloroacetic acid. Tubes were heated at 85°C for 10 min and allowed to cool. Insoluble material from each tube was centrifuged out at 9000 g for 10 min and washed twice with 10 ml of 95% ethanol. The pellet material was transferred to scintillation vials and assayed for radioactivity in a scintillation counter after the addition of 15 ml of Bray’s scintillator solution. Protein determinations were also made by the method of Lowry (12). Aflatoxin B1 synthesis was measured according to methods previously described.

The radioactivity in the O-methyl group of aflatoxin B1 can be determined by conversion into methyl iodide by the Zeisel reaction (13). This degradative method was applied to the determination of an ethoxy group on the new aflatoxin derivative. Unlabeled aflatoxin B1 (10 mg), plus ethionine-labeled B1 derivative, was refluxed for 20 min with a solution of HI (5 ml of 57% v/v) and red phosphorus (100 mg). The apparatus was swept with N₂ to liberate any C-1 (methyl) or C-2 (ethyl) iodides which were bubbled through H₂O and trapped in acetone (10 ml) at −20°C. Anhydrous trimethylamine (1.0 ml) was added and the precipitate resulting was obtained by centrifugation. It was washed with acetone and ether and crystallized from ethanol. The crystals were dissolved in H₂O and the sample was counted for radioactivity.

A comparison of the excitation–emission spectra of aflatoxin B1 and the ethoxy derivative was carried out on an Aminco-Bowman spectrophotofluorometer.

L-Methionine-methyl-¹⁴C and L-ethionine-ethyl-¹⁴C were purchased from New England Nuclear Corp., Boston, Mass. Methionine analogs were purchased from Calbiochem, Los Angeles, Calif. (DL-ethionine, DL-α-methylmethionine, S-ethyl-L-cysteine, L-methionine sulfoxide, DL-methionine sulfone, and S-methyl-L-cysteine).

**Results and Discussions**

**Biphasic Growth Pattern of the Organism**

A typical growth pattern of *A. parasiticus* in synthetic media is depicted in Fig. 1, showing the onset of toxin synthesis after 60–70 h of culturing. The synthesis of aflatoxin is accelerated during the transitional period (70–90 h) marked by a decrease in rate of growth (cell mass), protein synthesis, and RNA synthesis. Aflatoxin B₂ₐ formation occurs at 80 h when the pH drops below 3.5. Aflatoxin B₂ₐ is a hydroxy-deriva-
ative of aflatoxin B₂ and is formed rather readily by acid catalysis. After 110 h, aflatoxin B₁ synthesis is curtailed because the pH of the medium has reached 2.2; however, aflatoxin B₃a (1,2-dihydrorifurocoumarone) synthesis increases slightly under these acidic conditions.

Effect of Ethionine upon Mycelia and Aflatoxin Yield

DL-Ethionine was added to A. parasiticus cultures after 49 h of mycelial development. Table I shows that, with 2.0 mM DL-ethionine, inhibition of aflatoxin B₁ formation was pronounced. Increasing the DL-ethionine concentration to 4.0 mM did not significantly alter this effect. Although the inhibition of toxin synthesis is great at the 2.0 and 4.0 mM levels, there is little effect, if any, upon the final dry weight of the mycelium (97%). Therefore, the effect upon toxin synthesis does not result simply from decreased cell synthesis.

When DL-ethionine was used at high levels, inhibition was pronounced not only upon aflatoxin formation, but also upon cellular synthesis. With 20.0 mM inhibitor, the dry weight mycelial yield was 63% of that of the control; the aflatoxin B₁ level (µg) was 10% of controls. Hence, there is a considerable effect upon aflatoxin B₁ formation beyond that due to inhibition of cell synthesis. Using an inhibitor level of 50 mM yielded total inhibition of growth and toxin synthesis.

Effect of DL-Ethionine upon Protein Synthesis

Figure 2 shows that the rate of protein synthesis in a developing culture of A. parasiticus decreases rapidly between 70 and 80 h. Corresponding to this change in protein synthesis is the onset of aflatoxin B₁ synthesis. In this experiment 50 mM DL-ethionine and ¹⁴C-methionine was added to the culture at 50 h and the effect on protein synthesis determined up to 90 h of culture development. Protein synthesis was inhibited 85% relative to the control as measured by total ¹⁴C-methionine incorporation into trichloroacetic acid insoluble material over the 40-h experimental period. Furthermore, the addition of DL-ethionine at 50 h prevents any aflatoxin synthesis. This block in aflatoxin synthesis indicates that further protein synthesis is required in the transitional phase of growth shift from primary to secondary metabolism to make the necessary or additional enzymes responsible for aflatoxin formation.

![Graph](image1)

**TABLE 1**

<table>
<thead>
<tr>
<th>Ethionine, mM</th>
<th>Dry weight of mycelium, mg</th>
<th>Aflatoxin B₁, µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>500</td>
<td>802</td>
</tr>
<tr>
<td>0.2</td>
<td>498</td>
<td>810</td>
</tr>
<tr>
<td>0.6</td>
<td>490</td>
<td>800</td>
</tr>
<tr>
<td>2.0</td>
<td>470</td>
<td>187</td>
</tr>
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<td>4.0</td>
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<td>10.0</td>
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<td>14.0</td>
<td>380</td>
<td>140</td>
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<tr>
<td>18.0</td>
<td>340</td>
<td>112</td>
</tr>
<tr>
<td>20.0</td>
<td>315</td>
<td>80</td>
</tr>
</tbody>
</table>

*Ethionine was added to the growth flasks at 49 h. Dry weight and aflatoxin B₁ formation were measured at 120 h.*

![Graph](image2)
TABLE 2
Ethionine inhibition of aflatoxin B₁ synthesis in a resting-cell system

<table>
<thead>
<tr>
<th>DL-Ethionine, mM</th>
<th>Aflatoxin B₁, µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1,200</td>
</tr>
<tr>
<td>0.8</td>
<td>1,270</td>
</tr>
<tr>
<td>1.6</td>
<td>790</td>
</tr>
<tr>
<td>3.2</td>
<td>400</td>
</tr>
<tr>
<td>4.8</td>
<td>160</td>
</tr>
</tbody>
</table>

*Mycelial pellets were harvested from a 1% yeast extract–sucrose (YES) growth medium at 96 h, washed, filtered, and resuspended in a resting-cell medium (15 g wet weight/100 ml), and incubated for 24 h in the presence of DL-ethionine.

TABLE 3
Effect of exogenous DL-ethionine upon the incorporation of ¹⁴C-CH₃ of methionine into aflatoxin B₁

<table>
<thead>
<tr>
<th>DL-Ethionine, mM</th>
<th>Aflatoxin B₁, dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25 000</td>
</tr>
<tr>
<td>3.0</td>
<td>19 000</td>
</tr>
<tr>
<td>6.0</td>
<td>13 500</td>
</tr>
</tbody>
</table>

*Mycelial pellets were harvested from a YES growth medium at 96 h, washed, filtered, and resuspended in a resting-cell medium (1.3 g/100 ml), containing 5 curie ¹⁴C-CH₃-methionine, and incubated for 24 h in the presence and absence of DL-ethionine.

We have also observed that the addition of DL-ethionine after aflatoxin formation begins (75 h) has little effect upon continued synthesis at the ethionine levels tested. Therefore, once the aflatoxin-synthesizing enzymes are formed, the inhibitor is no longer as effective against the activity of these enzymes.

Ethionine can be readily incorporated into proteins (14, 15) and does inhibit the formation of some enzymes (16, 17). Spižek and Janeček (18) report that the addition of ethionine to logarithmically growing cultures of Escherichia coli suppresses β-galactosidase activity. Since ethionine addition inhibits β-galactosidase activity in both constitutive and inducible strains, the effect appears to be upon enzymatic synthesis rather than induction. The aflatoxin-synthesizing enzyme system appears to be formed during the transitional period of growth and is under metabolic control, possibly of a repressed nature. The addition of ethionine at the transitional phase of growth may well block de novo protein synthesis required for the synthesis of an enzyme or enzymes responsible for aflatoxin formation.

**DL-Ethionine Inhibition of Aflatoxin B₁ Synthesis in a Resting-Cell System**

The results of exogenously adding DL-ethionine to a resting-cell medium containing previously washed mycelial pellets are given in Table 2. The influence of increasing molar concentrations of DL-ethionine in a resting-cell system was measured against a control flask containing no exogenous DL-ethionine. The synthesis of aflatoxin B₁ in the resting-cell system was completely unaffected at 0.8 mM DL-ethionine concentrations; however, 1.6 mM DL-ethionine blocked B₁ synthesis 34%. Increasing DL-ethionine concentrations to 3.2 and 4.8 mM yielded aflatoxin B₁ inhibitions of 67 and 87%, respectively. These inhibitions of toxin synthesis compare favorably with those in growing-cell cultures of A. parasiticus. Since the methoxyl-group of aflatoxin B₁ originates from the methionine CH₃ group, an experiment was conducted to measure the inhibitory effect of DL-ethionine upon ¹⁴CH₃ group transfer from ¹⁴C-CH₃-methionine as donor (Table 3). A resting-cell medium system was used to measure the incorporation of ¹⁴C-CH₃-methionine into aflatoxin...
B₁ in the presence of DL-ethionine. In the presence of 3.0 mM DL-ethionine, there was a 25% inhibition in ¹⁴C-methionine incorporation and about 50% at 6.0 mM DL-ethionine. The discrepancy observed of only a 50% inhibition of ¹⁴C-methionine incorporation into aflatoxin B₁ compared to 87% total aflatoxin inhibition at 4.8 mM as shown in Table 2 has not been resolved. However, the data do show that DL-ethionine not only causes an inhibition in protein-synthesizing activity, but also appears to interfere with toxin synthesis through a blockage of CH₃ group transfer.

Production of an Aflatoxin Analog in an Ethionine-supplemented System

Another observation made in the DL-ethionine inhibition experiments was the occurrence of a new, fluorescent aflatoxin metabolite in ethionine-supplemented fermentations. In thin-layer chromatography (t.l.c.) analysis, a minor amount of this new fluorescent component was found at Rᵢ 0.72 as compared with an Rᵢ of 0.69 for aflatoxin B₁. This new compound represented neither a product of autocatalytic degradation nor an extractable product of the mycelia per se, since the compound was not detected in the control flasks minus DL-ethionine. The mobility of the new compound in the chromatograms suggested it was not 6-demethyl-oxydifurocoumarone because of its increased polarity and its subsequent Rᵢ (0.72) in the t.l.c. solvent system used to separate the aflatoxins. Its production only in the presence of ethionine suggested that perhaps an ethyl group was being incorporated into the aflatoxin molecule. Its production only in the presence of ethionine suggested that perhaps an ethyl group was being incorporated into the aflatoxin molecule.

This hypothesis was tested by the addition of radiochemically pure ¹⁴C-1-ethyl-ethionine to the fermentation media. The newly isolated product was strongly labeled; however, there was a small amount of radioactivity in the aflatoxin B₁ fraction. This residual radioactivity in the B₁ possibly may be explained on the basis of a small amount of ethyl labeling of aflatoxin B₂, which could then possibly migrate in the B₁ zone on preparative t.l.c. plates. Since the aflatoxin analog was devoid of radioactivity when ¹⁴C—CH₃-methionine was added to the ethionine-supplemented fermentation, a methoxy group must be missing in the C-6 position.

Ultraviolet absorption and fluorescence spectra showed the new compound to be an aflatoxin similar to aflatoxin B₁. Radioactive material was isolated from fermentations supplemented with 1-¹⁴C-ethyl-ethionine and subjected to Zeisel degradative studies. The presence of ¹⁴C-label indicated that possibly an ethyl substitution had taken place at C-6. The structures of aflatoxin B₁ and the 6-ethoxy-aflatoxin analog (6-ethoxydifurocoumarone) appear in Fig. 3.

Incorporation of the ethyl from ethionine into the new compound prompted the testing of other methionine and ethionine analogs as possible donors: i.e., S-ethyl-L-cysteine, L-methionine-DL-sulfoxide, DL-methionine sulfoxone, S-methyl-L-cysteine, and DL-α-methylmethionine (Table 4). When S-ethyl-L-cysteine was added, the new product was formed; however, it did not show the inhibition of aflatoxin B₁ productivity caused by DL-ethionine. All the other analogs tested did not inhibit B₁ synthesis nor did they form any new aflatoxin derivatives. Apparently, the specific ethionine structure is required for transmethylation, perhaps by competitive inhibition of transmethylation, which would possibly explain the inhibition of total aflatoxin B₁ synthesis. The S-ethyl-L-cysteine analog may be capable of causing transmethylation but not through a competitive response to the methyl transfer reaction. Therefore both ethionine and S-ethyl-L-cysteine probably compete with methionine in transmethylation, but only ethionine inhibits aflatoxin biosynthesis.

Since the concentration of DL-ethionine used in inhibition experiments was high and if a true metabolite-antimetabolite competitive inhibition occurred, there should be a fairly large yield of the ethoxy-analog or the 6-demethoxy aflatoxin. It was estimated by densitometer readings at 362 mp that the new component represented about 3–5% of the total aflatoxin B₁ present. The transmethylation enzyme may be partially stereospecific for methyl group transfer; thus, ethionine may be capable of competing for the active sites yielding the total toxin inhibition which occurs, although little ethyl transfer can be attained by this transmethylation system. The system is also subject to possible differences in membrane permeability to methionine and the ethyl analog. It was thought that the high concentrations of DL-ethionine may effect the trans-
port of methionine into the cell; however, at DL-ethionine levels of 20.0 mM there is considerable increase in biomass and incorporation of $^{14}$C-methionine into protein. The total effect of ethionine upon methionine transport is still a matter of conjecture and needs resolution.


