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Interaction of Aflatoxin B_1 with Electron-Donating Organic Molecules, Including Aromatic Amino Acids[1]

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The interaction of the carcinogenic mycotoxin, aflatoxin B_1, with some electron-donating organic compounds including aromatic hydrocarbons, dimethylaniline, and aromatic amino acids, was studied. Spectrophotometric analysis of aflatoxin B_1 revealed that hypochromicity in the absorption around 360 nm and hyperchromicity around 385 nm were induced by dimethylaniline, hexamethylbenzene, tryptophan, and imidazole. A similar shifting of aflatoxin B_1 absorption was observed in benzene, toluene, and xylene in the presence of $ZnCl_2$. The interaction of aflatoxin B_1 with polystyrene was observed in a biphasic system. The association constants of aflatoxin B_1:DMA[4] (1:1) and of aflatoxin B_1:tryptophan (1:1) were found to be 0.64 and 22.6 liters per mole, respectively. The results suggest that charge–transfer interaction occurs between aflatoxin B_1 and these π-electron donors. Since the spectral changes on aflatoxin B_1 absorption induced by these π-electron donors are similar to those induced by nucleic acids and proteins, it is postulated that charge–transfer interaction also occurs between aflatoxin B_1 and these macromolecules. The role of such interaction in the biological activity of aflatoxin B_1 is discussed.

The biochemical and biological properties of aflatoxins have been studied extensively in recent years (*1*). Since the effect of aflatoxin is primarily on the protein synthesis system, there have been some investigations on the *in vitro* studies on the interaction of aflatoxins with macromolecules including the interaction of aflatoxin B_1 and G_1 with DNA, various nucleotides (*2–11*), histone (*8*), deoxyribonuclease (*12*) and serum proteins (*13–16*). The binding of aflatoxins B_1 and G_1 with macromolecules has also been demonstrated *in vivo* (*17*, *18*).

Upon reviewing the results obtained by previous investigators (*2–16*) we found that the changes of aflatoxin spectra after the interaction with different macromolecules were similar. The maximal hypochromicity (350–360 nm) and the maximal hyperchromicity (380–400 nm) in the aflatoxin absorption after interaction with nucleic acids or proteins were qualitatively identical. This observation led us to believe that

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[4] Abbreviations used: Afl-B_1: aflatoxin B_1; DMA: dimethylaniline; HMB: hexamethylbenzene.

there may be a common reaction mechanism for the interaction of aflatoxin with these different macromolecules. In view of the importance of charge–transfer complexes in biological systems (*19–21*) of the π-electron donating property of the nucleic acid bases adenine and guanine, which were shown to interact with aflatoxin B_1 (*6*, *22*), and of the possible electron accepting properties of aflatoxin B_1 molecules, in which the polar ketonic carbonyl groups in the cyclopentenone coumarin moiety are conjugated to the aromatic center, we have selected a group of electron-donating compounds including some amino acids and tested their ability to interact with this potent carcinogen. This paper presents evidence for the electron-accepting properties of aflatoxin B_1 based on its ability to interact with electron donors. The role of charge–transfer interaction of aflatoxin B_1 with macromolecules is discussed.

EXPERIMENTAL

Materials

Aflatoxin B_1 was prepared according to the method of Chu (*23*) and was recrystallized once from chloroform/*n*-hexane and twice from chloroform/methanol. The preparation exhibited a single spot when analyzed by tlc. A molar absorptivity of 22 400 in chloroform at 362 nm was used for aflatoxin B_1 quantitation. Hexamethylbenzene (Aldrich Chemical Co.) was recrystallized three times from chloroform/ethanol and vacuum dried. *N*,*N*-dimethylaniline (J. T. Baker Co.) was distilled under reduced pressure before using. Polystyrene was polymerized anionically with butyl lithium as an initiator, precipitated with tetrahydrofuran/methanol three times, and vacuum dried for 1 week at room temperature. The molecular weight of this polymer as determined by the viscometrical method was 200 000. L-Tryptophan (Nutritional Biochemical Corp.), imidazole (Eastman Organic Chemicals), phenylalanine (General Biochemicals), L-histidine (Eastman Organic Chemicals), chloroform (Fisher, 99.5% mole pure), methanol (Fisher, 99.5% mole pure), *n*-hexane (Fisher spectra grade), benzene, toluene, and xylene were used without any further purification.

Spectrophotometric Analyses

The visible and uv spectra of various aflatoxin B_1–ligand complexes were determined by analysis of appropriate solutions in a Cary Model 14 recording spectrophotometer or in a Beckman DU spectrophotometer with a light path of 1 cm. The temperature of the measurement was generally between 20 and 22°C.

Calculation of Association Constant (K) and Molar Absorptivity (ϵ)

The concentration dependence of the absorption spectrum of aflatoxin B_1–ligand complex was analyzed by the method of Benesi–Hildebrand (*24*) and of Ketelaar et al. (*25*). In this analysis, a 1 : 1 complex is assumed. The equilibrium may be written as:

$$(\text{Af}) + (\text{Donor}) \rightleftharpoons (\text{Af–D})\ \text{complex} \tag{1}$$

The association constant for complex formation, K, is:

$$K = [\text{Cx}]/\{[\text{Af}]_0 - [\text{Cx}]\}\{[\text{D}]_0 - [\text{Cx}]\}, \tag{2}$$

where $[Af]_0$ and $[D]_0$ represent the initial aflatoxin and donor concentration, respectively, and [Cx] represents the concentration of complex. Equation (2) may be rearranged:

$$[Af]_0/[Cx] = 1/K \cdot 1/[D]_0 + [Af]_0/[D]_0 + 1 - [Cx]/[D]_0. \quad (3)$$

If ϵ_λ^{Af} is the molar absorptivity of aflatoxin at the wavelength of measurement, then for a 1-cm pathlength of solution the absorbance a is:

$$a = \epsilon_\lambda^{Cx}[Cx] + \epsilon_\lambda^{Af}[Af] = \epsilon_\lambda^{Cx}[Cx] + \epsilon_\lambda^{Af}\{[Af]_0 - [Cx]\}. \quad (4)$$

An apparent molar absorptivity of aflatoxin at a wavelength, may be defined as

$$\epsilon_\lambda^{app} = a/[Af]_0. \quad (5)$$

If Eqs. (3), (4), and (5) are combined, and the approximation is made that in Eq. (3) the term $[Af]_0/[D]_0$ and $[Cx]/[D]_0$ approach to zero for solution where $[D]_0 \gg [Af]_0$, then

$$1/\epsilon_\lambda^{app} - \epsilon_\lambda^{Af} = 1/K(\epsilon_\lambda^{Cx} - \epsilon_\lambda^{Af}) \cdot 1/[D]_0 + 1/\epsilon_\lambda^{Cx} - \epsilon_\lambda^{Af}. \quad (6)$$

If the complex is 1:1 as assumed in the derivation, a plot of $1/(\epsilon_\lambda^{app} - \epsilon_\lambda^{Af})$ vs $1/[D]_0$ should be linear and yield $1/(\epsilon_\lambda^{Cx} - \epsilon_\lambda^{Af})$ as the intercept, and the product $K(\epsilon_\lambda^{Cx} - \epsilon_\lambda^{Af})$ as the reciprocal of the slope. The apparent association constant, K, and the molar absorptivity, thus, can be determined.

RESULTS

Interaction of Dimethylaniline and Hexamethylbenzene with Aflatoxin B_1

When *N,N*-dimethylaniline was added to aflatoxin B_1 solution in chloroform, the uv spectrum of aflatoxin B_1 was altered by an increase in absorption at 385 nm and a decrease at 360 nm. A slight yellow color was observed in the concentrated solution (10^{-3} *M* of Afl[4]-B_1 and 0.8 *M* DMA). The difference spectra of Afl-B_1–DMA in chloroform above 370 nm are shown in Fig. 1. Difference spectra below 370 nm were not recorded because the aflatoxin blank at this high concentration gave an absorbance which greatly affected the measurement at 370 nm and below. A similar change was also observed in the Afl-B_1–HMB system, and the difference spectrum is shown in Fig. 2. The dependence of concentration on the complex formation for Afl-B_1–DMA is illustrated in Fig. 3. Since the plot in Fig. 3 yielded a straight line, the complexes other than the 1:1 molar ratio were absent. The apparent association constant (K) and molar absorptivity (ϵ) for the complex as derived from this calculation were found to be 0.64 liters $mole^{-1}$, and 3560, respectively. The molar absorptivity of aflatoxin alone at 385 nm ($\epsilon_{385\,nm}^{Afl\text{-}B_1}$) of 1820 was used.

Interaction of Afl-B_1 with Polystyrene

Since the absorption of both components in an interaction system were more or less modified in solution, the complex formation was studied in a heterogeneous phase system where one component, the donor in this case, remained insoluble and the acceptor in solution. Polystyrene which has a pendant aromatic nucleus on the hydrocarbon backbone was used in this study. Polystyrene was added to aflatoxin B_1

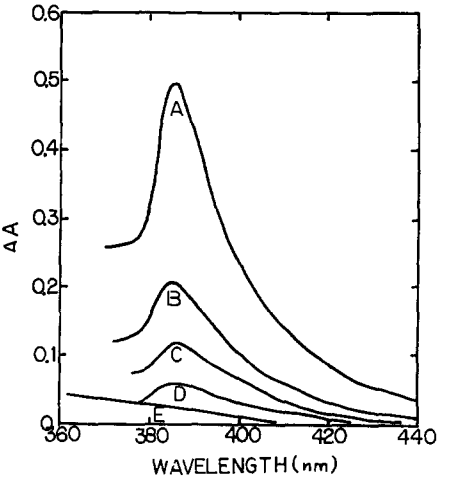

FIG. 1. Difference spectra of aflatoxin B_1–dimethylaniline in chloroform. The concentration of aflatoxin B_1 and DMA are 1.074×10^{-3} and 7.9×10^{-1} *M* for solution A. The concentrations in solutions B, C, and D were 66.7, 50, and 33.3% of A. Slope E represents the absorption of DMA (7.9×10^{-1} *M*) alone. Aflatoxin B_1 (curves A–D) alone was used as the blank in the determination, and absorptions due to free DMA were not compensated.

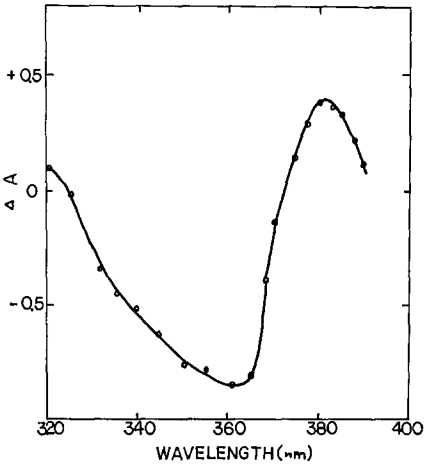

Fig. 2. Difference spectrum of aflatoxin B_1–hexamethylbenzene in chloroform. The aflatoxin B_1 and HMB concentration used in this measurement were 5.51×10^{-5} *M* and 1.0 *M*, respectively. Aflatoxin B_1 (5.51×10^{-5} *M*) was used as the blank in this measurement.

solutions (50:50 of chloroform and methanol) of appropriate concentration. The aflatoxin concentration in the supernatant fraction was determined 1 day after mixing. In a typical experiment, the aflatoxin B_1 concentration was decreased by 2.4% when 0.96 *M* styrene unit (in the polystryene form) was added to 2.95×10^{-5} *M* of aflatoxin B_1. Assuming the disappearance of aflatoxin in the supernatant fraction is due to its

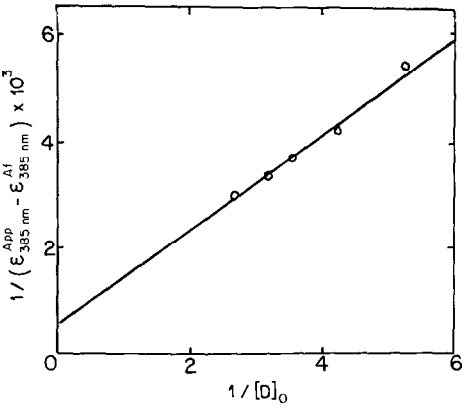

FIG. 3. Determination of apparent dissociation constant (K) and molar absorptivity ($\epsilon^{385\,nm}_{1\,cm}$) for aflatoxin B_1–dimethylaniline complex. The concentration for aflatoxin B_1 varied from 1.56×10^{-4} M to 4.16×10^{-4} M and for DMA from 1.41×10^{-1} to 3.77×10^{-1} M. All measurements were performed at 385 nm.

binding to the pendant aromatic nucleus of the swollen polystyrene through the charge–transfer interaction, the apparent association constant in this free aflatoxin–complex equilibrated solution was 2.6×10^{-2} liters mole^{-1}.

Interaction of Aflatoxin B_1 with Aromatic Hydrocarbons

The interactions of aflatoxin B_1 with aromatic hydrocarbons such as benzene, toluene, and xylene are difficult to demonstrate because the absorption below 300 nm of these hydrocarbons greatly affects spectral measurements, and also possibly because of the weak interactions. The possibility of enhancement of electron acceptability for

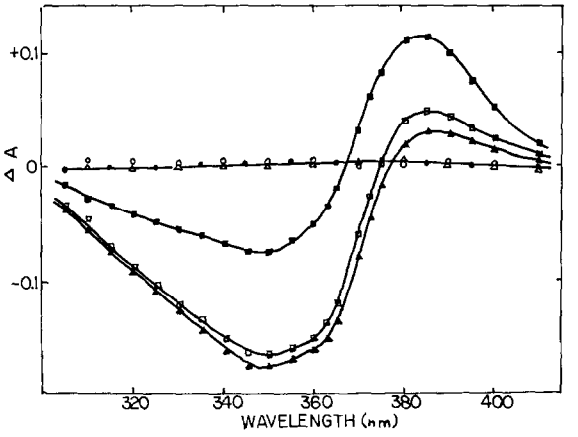

FIG. 4. Difference spectra of zinc-coordinated aflatoxin B_1 in organic solvents and in water. The concentration of aflatoxin B_1 was 2.02×10^{-5} M. The molar ratio of $ZnCl_2$ to aflatoxin B_1 was 50. The curves represent the spectra of aflatoxin B_1–$ZnCl_2$ in water (–○–○–), methanol (–●–●–), chloroform (–△–△–), benzene (–■–■–), toluene (–□–□–), and xylene (–▲–▲–). Except in aqueous and chloroform, all other solvents contained 10% (by volume) of chloroform.

aflatoxin B_1 by $ZnCl_2$ was investigated. In a series of experiments, $ZnCl_2$ (1 *M* in methanol) was added to aflatoxin B_1 in various solvents including chloroform, methanol, water, benzene, toluene, and xylene; the difference spectra of aflatoxin solutions with and without $ZnCl_2$ were then determined. The results of these experiments are shown in Fig. 4. No changes in the spectra were observed in the chloroform, methanol, and water $ZnCl_2$–aflatoxin B_1 systems. However, significant changes were

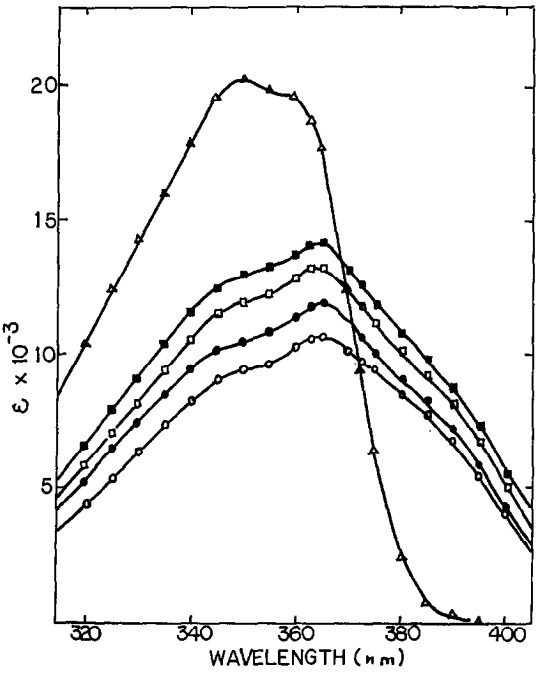

FIG. 5. Concentration dependence of zinc-coordinated aflatoxin B_1 spectra in benzene. The concentrations of aflatoxin B_1 were 1.5×10^{-5} (-■-■-), 2.0×10^{-5} (-□-□-), 3.0×10^{-5} (-●-●-), and 4.0×10^{-5} *M* (-○-○-). The concentration of zinc chloride was 50 times greater than aflatoxin B_1. The open triangles (-△-△-) represent the absorption spectrum of aflatoxin B_1 alone as determined in the above range of aflatoxin B_1 concentrations ($1.5–4.0 \times 10^{-5}$ *M*).

observed in the aromatic hydrocarbon, $ZnCl_2$ and aflatoxin B_1 systems. Zinc chloride induced no spectral change in the corresponding solvent in the absence of aflatoxin B_1. The effect of concentration of Zn-coordinated aflatoxin B_1 on the interaction with benzene is shown in Fig. 5. Due to the complexity of the three component systems, i.e., aflatoxin–Zn, aflatoxin–Zn–benzene, and aflatoxin–benzene, the association constant was not determined.

Interaction of Aflatoxin B_1 with Electron-Donating Amino Acids

Since some aromatic amino acids and related compounds are considered as electron-donating species in some charge-transfer interactions (*22*), histidine, phenylalanine, tryptophan, and imidazole were used in the present study. Figure 6 shows the difference spectra of aflatoxin B_1 in the presence and absence of these compounds. Tryptophan

and imidazole altered the absorption of aflatoxin B_1 significantly, while phenylalanine and histidine induced only minor change. The magnitude of wavelength shifting and absorption changes were in the order of imidazole > tryptophan > histidine and phenylalanine when the experiments were carried out at the same ligand concentration.

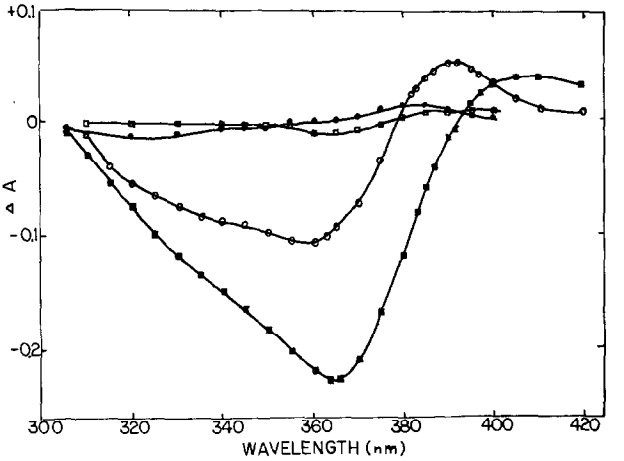

FIG. 6. Difference spectra of aflatoxin B_1–amino acids and imidazole in distilled water. The open circles (–○–○–) represent tryptophan–aflatoxin B_1, closed circles (–●–●–) for phenylalanine–aflatoxin B_1, open squares (–□–□–) for histidine, and closed squares (–■–■–) for imidazole. The concentration for aflatoxin B_1 and amino acids or imidazole were 2.0×10^{-5} M and 2.0×10^{-2} M, respectively.

The effect of tryptophan concentration on the change of aflatoxin B_1 absorption at 390 nm is shown in Fig. 7. The straight line plot shows that a 1 : 1 aflatoxin B_1 : tryptophan complex was formed. The apparent association constant and the molar absorptivity of aflatoxin B_1–tryptophan (1 : 1) were found to be 22.6 (liters mole^{-1}) and 16 600, respectively.

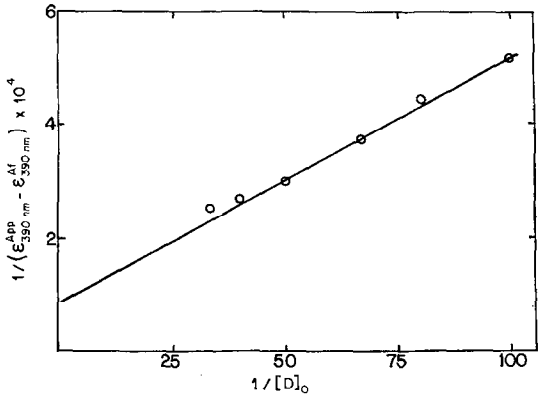

FIG. 7. Determination of apparent dissociation constant (K) and molar absorptivity ($\epsilon^{390\,nm}_{1\,cm}$) for aflatoxin B_1–tryptophan complex. The concentration for aflatoxin B_1 varied from 1.58×10^{-5} to 5.3×10^{-6} M and for tryptophan from 3.0×10^{-2} to 1.0×10^{-2} M. All the measurements were performed at 390 nm.

DISCUSSION

In the present study, spectrophotometric analyses demonstrate that a number of electron-donating organic compounds, including some amino acids, are capable of forming charge–transfer-type complexes with the carcinogenic mycotoxin, aflatoxin B_1. Since both dimethylaniline and hexamethylbenzene are considered as good π-electron donors (*21*, *26–28*), the formation of complexes from aflatoxin B_1 and these compounds provide new evidence that this potent carcinogen can serve as an electron-accepting species in such interaction. Although no spectral change in aflatoxin absorption has been observed in benzene, toluene, or xylene between 300–400 nm, the possibility of the interaction between aflatoxin B_1 with these π-electron-donating species cannot be ruled out, especially in view of the observation that the molar absorptivity of aflatoxin B_1 is a little lower in these solvents than in chloroform, methanol, or water (*29*) and also the fact that zinc-coordinated aflatoxin B_1 interacts with these solvents (Fig. 4). From the published ionization potentials of benzene, toluene, and xylene [9.24, 8.82, and 8.4–8.6, respectively (*30*)] and the ionization potential of dimethylalanine and hexamethylbenzene [7.3–7.4 and 7.8, respectively (*26–28*)], it could be assumed that if the complex forms between aflatoxin B_1 and benzene, the association constant would be smaller than the Afl-B_1–DMA and Afl-B_1–HMB type; and also the spectra shifting may occur in the shorter wavelength region which is masked by the absorption of the ligand itself, even in very dilute solutions. The enhancement of electron acceptability of aflatoxin B_1 by $ZnCl_2$ is probably due to the coordination of Zn^{2+} to the lone pair electrons of the ketonic carbonyl groups, rendering the conjugated double bond and aromatic nucleus of aflatoxin more electron deficient. It has been well-established that Lewis acids, such as $ZnCl_2$, induce charge–transfer interaction in some weak electron acceptors (*31–33*) such as the interaction between styrene and acrylonitrile.

The spectral changes in aflatoxin B_1 induced by organic electron-donating molecules are qualitatively similar to those induced by amino acids (Fig. 6) as well as by DNA, polynucleotides, purine bases, and proteins (*2–16*). However, there is a marked difference in the magnitude of hypochromicity in the 360-nm region. Since the decrease in absorption of aflatoxin B_1 at 350–360 nm is considered to be the result of the interaction of toxin with ligand, the magnitude of this decrease, thus, reflects the electron-donating property of the ligand. Among the amino acids tested in the present study, tryptophan has the lowest energy coefficient for the highest occupied molecular orbital and, thus, has the highest electron-donating properties (*22*). As a consequence, our results indicate that tryptophan forms complexes with aflatoxin B_1 more readily than does phenylalanine and histidine.

Pullman and Pullman (*22*) suggested that many biologically active compounds fall within the classification of good electron donors or good electron acceptors. Among them, nucleic acid base components, especially guanine and adenine, have been considered as effective π-electron donors. King and Nicholson (*6*) demonstrated that adenine is required for the binding of aflatoxin with polynucleotides since a spectral change resulted only when adenine was present in the polynucleotides examined. This can be interpreted by considering that adenine and guanine are the most powerful electron donors among the nucleic acid bases. The interaction of nucleic acid bases with the strong electron acceptor, chloranil, has been reported (*34*). In a study of the

interaction of aflatoxin B_1 with a series of substituted purines, Cliff and Rees (*4*) found that the greatest spectral change is induced by a dimethylamino group. Our demonstration of the electron-accepting properties of aflatoxin B_1 indicates that their observation can be explained on the basis of a charge–transfer interaction between aflatoxin B_1 and purine bases. The substitution of a dimethylamino group would strongly enhance the electron-donating property, thus rendering a stronger association.

Although we have demonstrated that charge transfer is involved in the interaction of aflatoxin B_1 with electron donating compounds and, possibly, also in the interaction of this toxin with nucleic acids and proteins, it is difficult to assess the biological implication at present. Wogan and his colleagues (*11*) have recently reported no correlation between the biological activity of aflatoxins and the binding of the toxins with DNA. They showed that several aflatoxin B_1 analogs which are less toxic than B_1 also interact with DNA. Furthermore, a biologically inactive, structurally related analog, i.e., 5,7-dimethoxycyclopentenone-(2,3-*c*)-coumarin, has the greatest affinity toward DNA of all the analogs tested. This compound gave a pronounced hypochromicity around 360 nm. Since it has been shown that the terminal dihydrofuran ring determines the biological activity of aflatoxins (*35*), it would be reasonable to speculate that this group is more important than others in regard to charge–transfer interaction, if such reaction is, indeed, involved in the biological systems. In order to clarify these problems, further studies on the interaction of different aflatoxin analogs, especially the biologically inactive ones, with electron-donating compounds, appears warranted.

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