Spectral Studies on the Deoxyribonucleic Acid–Aflatoxin B_1 System. Binding Interactions*

W. C. Neely, J. A. Lansden, and J. R. McDuffie

ABSTRACT: Binding in DNA–aflatoxin B_1 mixtures has been investigated using fluorescence polarization and quenching techniques. Quenching of aflatoxin B_1 fluorescence was observed and found to be linearly dependent on the concentration of DNA. From the complete lack of fluorescence polarization it was concluded that bound molecules were totally quenched. From fluorescence titration plots a value of 158 l. mol^{-1} is obtained for Kn, the product of the equilibrium constant and the number of binding sites per DNA-P. Measurements of luminescence intensities and lifetimes indicate that quenching of the aflatoxin singlet leads to a nonradiative dissipation of the excitation energy by the bound system. No involvement of aflatoxin or DNA triplets was found. Mixtures of aflatoxin with calf thymus histone and with bovine serum exhibited no quenching. Studies on light-exposed DNA–aflatoxin mixtures show that photodegradation can easily occur in the course of routine laboratory manipulations and may not be detected by analytical techniques based on absorbance or on fluorescence intensity measurements since the spectral characteristics of the photoproducts differ little from the parent material. The concentration dependence of quenching in the photolyzed system was similar to the unphotolyzed with a value of 400 for Kn. The absolute magnitude of fluorescence from the light-exposed samples was much greater than from equivalent unexposed mixtures.

The carcinogenic and toxic properties of the aflatoxins, metabolic products of certain strains of *Aspergillus flavus*, have led to extensive research on their chemical and physical properties in an effort to determine their mode of action. Since it has been shown in many cases that binding of an active molecule to a substrate greatly enhances the reaction process, various efforts have been made to determine the extent of such binding between aflatoxins and biological materials. In particular, the binding of aflatoxin B_1, the most toxic and carcinogenic member of the group, to DNA has been the object of much study.

Physiochemical investigations include spectral and equilibrium dialysis experiments by Sporn *et al.* (1966), spectral studies by Clifford and Rees (1966), and dialysis measurements by Black and Jirgensons (1967).

The spectral studies were concerned with the occurrence of changes in the intensities and wavelengths of the ultraviolet absorption bands of aflatoxin B_1 caused by the presence of calf thymus DNA. Similar effects were later observed in mixtures of aflatoxin and various nucleosides (Clifford and Rees, 1967). While these shifts are well established it has not been easy to obtain quantitative values of binding constants from the measured shifts. Indeed the data of Clifford and Rees suggest that the relative magnitude of spectral shifts may be opposite to the extent of binding as measured by other methods.

The equilibrium dialysis technique is a direct method which should yield unambigious results. However this technique requires equilibration times on the order of 100 hr and in none of the studies discussed was there any mention of special efforts at the exclusion of ambient light. The photodecomposition of aflatoxin is now known to occur at fairly low light levels (Andrellos *et al.*, 1967; Van Duuren *et al.*, 1968). The unidentified photoproducts have almost the same absorption spectrum as aflatoxin B_1 and a very similar fluorescence spectrum. It is then possible that the measured binding was that of photoproducts not of aflatoxin B_1. Similar arguments apply to the spectral studies although with somewhat less force since the total light exposure would not be expected to be as great in these relatively rapid experiments.

To avoid these difficulties we have designed experiments based on fluorescence measurements which can be carried out under conditions resulting in minimal decomposition of the toxin. The methods used are fluorescence quenching titration and the fluorescence polarization technique. Details of these methods are reviewed briefly in the Experimental Section. For purposes of comparison, difference spectra of the type used by Clifford and Rees were also obtained.

Experimental Section

Materials and General Methods

Aflatoxin B_1. Aflatoxin B_1 (grade B) was obtained from Calbiochem (Anaheim, Calif.) Purity was checked by comparison of the ultraviolet spectrum with that reported in the original literature (Hartley *et al.*, 1963). Further purification was not deemed necessary in the lots which were supplied. Aqueous stock solutions were prepared by lengthy stirring at 5° in the dark. Concentrations obtained in this way were about 2×10^{-5} M and were determined by ultraviolet adsorbance measurements at 224, 265, and 361 mμ where molar extinction coefficients were taken as 22,600, 13,500, and 23,800, respectively.

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DNA, Bovine Serum Albumin, and Calf Thymus Histone. Calf thymus DNA (grade A) was obtained from Calbiochem (Anaheim, Calif.) and used without further purification. Aqueous solutions at room temperature were found free from luminescent impurities when excited at 360 mμ. Crystallized and lyophilized bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo.) was used without repurification. Lysine-rich "fraction F-1" calf thymus histone prepared by the procedure of Johns (1964) and dialyzed to remove trichloroacetic acid was the gift of Miss V. Brinson of the Auburn University Animal Science Department.

Aqueous mixtures of aflatoxin B_1 and DNA were made up in 0.1 M NaCl solution buffered to pH 7.4 with Tris buffer. All necessary precautions were used to prevent exposure to light and to keep temperature below 10° until ready for use. DNA:aflatoxin ratios used were 1:1, 100:1, 200:1, 300:1, 400:1, 500:1, and 600:1.

Fluorescence Quenching Method. Many cases have been observed in which fluorescence of a molecule is greatly altered by adsorption onto a substrate. As shown by Lawrence (1952) these changes can be used to measure the relative number of free and bound molecules. A convenient treatment of the data is a plot of fluorescence intensity of one substance *vs.* concentration of the other component. This "fluorescence titration" curve will approach as a limiting value the fluorescence intensity of the completely bound material.

Flourescence Polarization Method. Early use of the fluorescence polarization method was also made by Weber (1952) and by Lawrence (1952). It is based on the fact that the rotational relaxational time for a small free molecule in a solution of low viscosity (*e.g.*, water at room temperature) is much less than for a large molecule or for a system composed of a small molecule bound to a large one. If the small molecule is fluorescent and the mean lifetime of this fluorescence is long compared with the rotational relaxation time of the free molecule but short compared with that of the bound system then excitation with polarized light will result in polarized emission from the bound molecules but depolarized emission from the freely rotating ones. The ratio of the intensities of polarized and depolarized emission is then a measure of the relative numbers of bound and free molecules. Derivations of the necessary equations are given in detail by Lawrence (1952). It should be noted that a correction for any polarization or depolarization by the optics must be considered in obtaining numerical values of the intensities. As given by Lawrence the most general equation for calculation of the fraction bound is: $(1 - \pi)x = \bar{p}R - R + 1 - \pi$, where x = fraction bound, $\bar{p}$ = ratio of observed polarization of the mixture to that of a system in which the fluorescer is completely bound, R = ratio of fluorescence intensity of the mixture to that of a solution of pure fluorescer of same concentration, and π is defined as polarization of the completely free fluorescer relative to that of the completely bound fluorescer. Polarization has its usual definition of $[I_{||} - I_{\perp}]/[I_{||} + I_{\perp}]$.

Equipment. Fuorescence of aflatoxin and aflatoxin–DNA mixtures was excited at selected wavelengths using a xenon arc lamp and a 0.5-m Jarrell-Ash monochromator. The half-width intensity of the excitation light was 50 Å. For polarization experiments the light could be plane polarized by a dye-film-type polarizer. The analyzing system consisted of a Spex 1700-III monochromator with 1-P-28 photomultiplier detector. A second removable polarizer could be placed in the analyzing light path with its plane of polarization set perpendicular or parallel to that of the excitation polarizer. Using a tungsten filament lamp as a test source of depolarized light it was found that the degree of polarization or depolarization imparted by either of the monochromators at the wavelengths used was negligible. Sample photolysis was minimized by excluding ambient light and by admitting excitation light only during the $^1/_{30}$ sec open time of a normally closed photographic shutter placed in the excitation beam. This brief excitation time necessitated the use of a fast response readout, a Tektronix 549 oscilliscope. By ferrioxalate actinometry the excitation beam was found to deliver a total of 1.05×10^{14} photons to the sample cell during the course of ten replicate measurements. Since the sample cell contained approximately 1.64×10^{16} molecules at the concentrations used and has an absorbance of 0.24, even with a quantum yield for photodecomposition of unity, the photolysis of aflatoxin would be limited to about 0.11%.

For low-temperature experiments a quartz dewar fitted with a round 1-cm quartz sample tube was used. Liquid nitrogen was used as the coolant. When necessary a cylindrical phosphoroscope was used to eliminate all but long-lived emission. The solvent used was 1:1 (v/v) mixture of ethylene glycol and water.

Measurements and Techniques. The fluorescence intensity of each sample was measured without polarizers for determination of quenching. Polarization values were then obtained by inserting polarizing optics first mutually perpendicular and then parallel. Finally, ultraviolet absorption spectra were obtained to prepare difference plots of the Clifford and Rees type. These were not obtained directly but by replotting of conventional absorption spectra obtained using solvent as the reference. Plotted data which was a linear function were fitted to a least-squares line.

Results

DNA–Aflatoxin (Light Protected)

Ultraviolet Absorption Spectra. Difference spectra of DNA–aflatoxin mixtures were obtained and are seen in Figure 1 to be similar to those reported by Clifford and Rees (1967).

Fluorescence Polarization Measurements. The degree of fluorescence polarization possible in aflatoxin B_1 at the concentration used was determined by measurements on a series of aqueous aflatoxin solutions containing varying amounts of sucrose to alter the viscosity. Polarization increased with viscosity; at 44 mp it was ~0.2. Aqueous, buffered mixtures of DNA and aflatoxin at ratios between 0:1 and 600:1 were examined but no polarization of the fluorescence was detectable.

Fluorescence Titration Measurements. The relative fluorescence intensities of the DNA–aflatoxin mixtures described above were obtained. Since the binding is apparently weak and reversible (Clifford and Rees, 1966; King and Nicholson, 1967) simple equilibrium considerations apply. Thus, $K = [C]/[N][A_f]$, where [C] = concentration of the DNA–aflatoxin B_1 complex, $[A_f]$ = concentration of free aflatoxin, and [N] is the concentration of binding sites available. Since a very large excess of DNA-P to aflatoxin is required to cause any

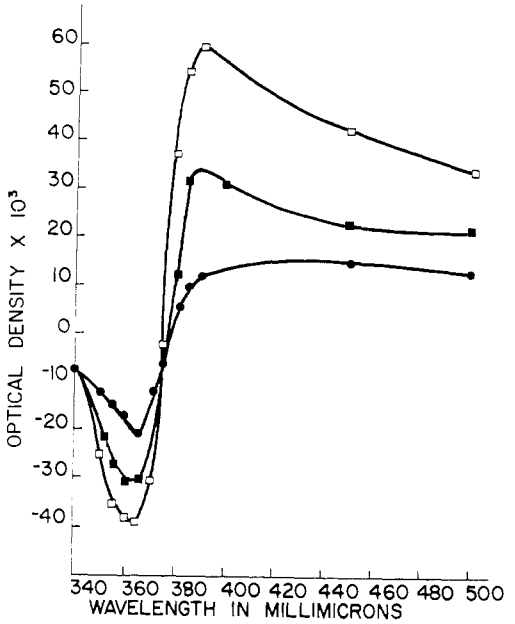

FIGURE 1: Difference spectra of representative light-protected mixtures of DNA and aflatoxin B_1. DNA:aflatoxin ratios: 100:1 (●), 300:1 (■), and 600:1 (□). Aflatoxin concentration in all mixtures = 9.1×10^{-6} M.

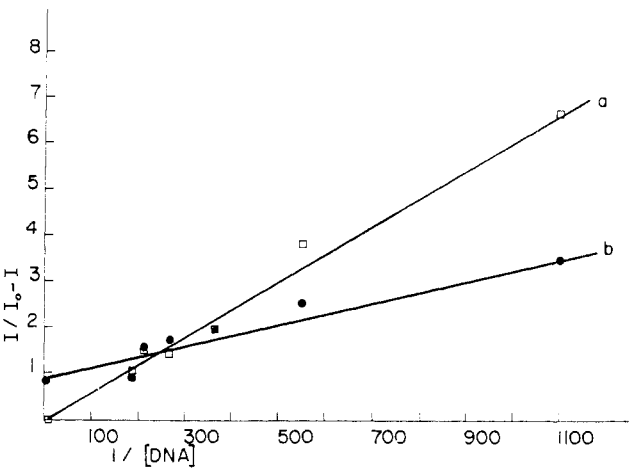

FIGURE 2: Quenching of aflatoxin B_1 fluorescence by DNA. Light-protected samples follow line (a), light exposed mixtures line (b). Concentration of aflatoxin is 9.1×10^{-6} M in both cases.

measurable quenching it is assumed that one complex contains only one aflatoxin molecule and that the bound aflatoxins occupy only a small fraction of the total available sites. Then upon replacing [C] with [A_b], the concentration of bound aflatoxin and [N] with n[DNA], where n is the number of binding sites per DNA-P, one obtains with the foregoing assumptions: $[A_f]/[A_b] = 1/[Kn[DNA]]$. If bound aflatoxin is indeed totally quenched then A_f/A_b is easily shown to be equal to $I_f/(I_{f^0} - I_f)$, where I_f is the fluorescence intensity of a DNA–aflatoxin mixture and I_{f^0} is the fluorescence intensity of a pure aflatoxin solution of the same concentration. It follows that a plot of the left-hand side of the above equation *vs.* 1/[DNA] will be linear with slope of $1/Kn$. A plot of this type is seen in Figure 2a. The value of Kn so obtained is 158 l. mole^{-1}.

Low-Temperature Experiment. In an effort to identify the mechanism by which DNA quenches the luminescence of bound aflatoxin, low-temperature (77°K) emission studies were made. In these it was found that: (1) Excitation of DNA–aflatoxin mixtures at 363 mμ (where aflatoxin absorbs but DNA does not) does not lead to any sensitized emission from DNA. (2) Excitation of these mixtures at 279 mμ where both components absorb does not result in any greater emission from DNA than from solutions of pure DNA of the same concentration. (3) Phosphorescence lifetimes of aflatoxin in pure solution and in 100:1 and 600:1 DNA–aflatoxin mixtures are all identical. All could be fitted to the same exponential decay curve with a correlation coefficient of greater than 0.999. The mean lifetime was 1.27 sec using an unfiltered xenon source for excitation and observing the emission at 467 mμ.

DNA–Aflatoxin (Light Exposed)

The possibility of an effect of light decomposition on the binding interaction was investigated by repeating some of the experiments described in the foregoing section using DNA–aflatoxin mixtures which had been exposed to ambient light for 100 hr. No polarization of fluorescence from these mixtures could be observed.

The fluorescence intensity of these samples was considerably greater than from corresponding unexposed samples. Using the data treatment previously discussed a value of 400 l. mole^{-1} for Kn was obtained. Absorption spectra obtained were similar to those of unphotolyzed mixtures except for the region 220–300 mμ. Here there was an increased absorbance and an obscuring of the 224 maximum. The strong band near 366 mμ was completely unchanged, the difference spectra (Figure 3) between 300 and 500 mμ being almost identical to that of the original mixtures.

Bovine Serum Albumin–Aflatoxin and Calf Thymus Histone–Aflatoxin. Both as a matter of intrinsic interest and because they are possible impurities in commercially supplied DNA, the effects of these on fluorescence polarization and quenching of aflatoxin were determined. A small amount of polarization was observed in both cases but was not sufficient to enable a reliable estimate of the amount of binding. No evidence of fluorescence quenching was found.

Discussion

From the observed difference spectra (Figure 1) it is clear that the interaction reported by others between aflatoxin B_1 and DNA is occurring in our mixtures. If aflatoxin indeed binds to DNA then the complete absence of polarization in the aflatoxin fluorescence indicates that one or more of the following must also be true: (a) A depolarization of fluorescence by aflatoxin–aflatoxin excitation energy transfer is occurring. (b) Any binding interaction which is occurring is incapable of preventing rotation of the bound aflatoxin molecules. (c) The binding is broken by the act of excitation of the aflatoxin so that molecule is free to rotate before emitting. (d) There is no emission from bound aflatoxin molecules; the observed fluorescence all originates from the freely rotating ones remaining in solution.

It does not appear that point a is very likely in view of the clearly shown polarization in viscous solutions of the same concentration. The possibility could be raised that the actual intermolecular distance between aflatoxin molecules is less when adsorbed onto DNA than when in solution. We believe that this is not very probable because of the small number of aflatoxin molecules bound per DNA. We have no way to check points b or c directly but it is to be noted that the energy gap between excitation (363 mμ) and emission (423 mμ) of aflatoxin is about 1.5 kcal/mole. This energy is converted into vibrational energy of the excited chromophore. If the excitation occurred in a portion of the molecule which was also the molecular site of binding then the suddenly appearing vibrational energy would be sufficient to break at least two hydrogen bonds.

Although some contribution by factors a, b, and c cannot be ruled out, we feel that the fluorescence quenching data give very strong support to d, *i.e.*, bound molecules cannot fluoresce. The possibility that the observed quenching is the result of random collisions in solution, not actual binding, can be checked by examining the viscosity dependence of the quenching interaction. A reduction in fluorescence quenching proportional to the increase in viscosity of the solvent would be expected for collisional quenching since it is diffusion controlled while there would be little effect on quenching occurring in the bound case (Bowen and Livingston, 1954). Experimentally it was observed, in the measurements previously described using viscous sucrose solutions, that there was an increase in the fluorescence intensity with increasing viscosity but that the increase was proportionally no greater in the DNA–aflatoxin than in solutions of aflatoxin alone. This clearly argues that the well known self-quenching of aflatoxin at these concentrations is a diffusion controlled collision process but that quenching by DNA is not. Lastly, resonance (Foyster type) transfer has an inverse sixth power dependence on intermolecular distance and is inoperative below concentrations of 10^{-4} M. While our DNA concentrations are within this range we do not observe the sixth power dependence on intermolecular distance when mixtures are diluted. Within experimental error, the simple equilibrium relation previously discussed is followed in solutions diluted to 40% of the initial concentration. The small fluorescence intensity made measurements on more dilute solutions impossible with our equipment. In summary, we feel that all the forgoing evidence clearly indicates that binding of aflatoxin B_1 to DNA is necessary for the quenching interaction to occur, therefore quenching can be used as a measure of binding. The arguments against the occurrance of various types of energy transfer refer only to their occurrance in solution; the quenching mechanism occurring in the bound state will be considered later in the paper.

The results of fluorescence polarization experiments with the two materials which are most likely to be found as impurities in calf thymus DNA, calf thymus histone, and bovine serum albumin, indicate that although some degree of binding may exist between them and aflatoxin it is very small compared with that observed with DNA. Additionally, they do not participate at all in any quenching action of aflatoxin fluorescence. Thus, their presence in the DNA samples used would not have affected our results materially.

The mechanism by which DNA quenches the fluorescence

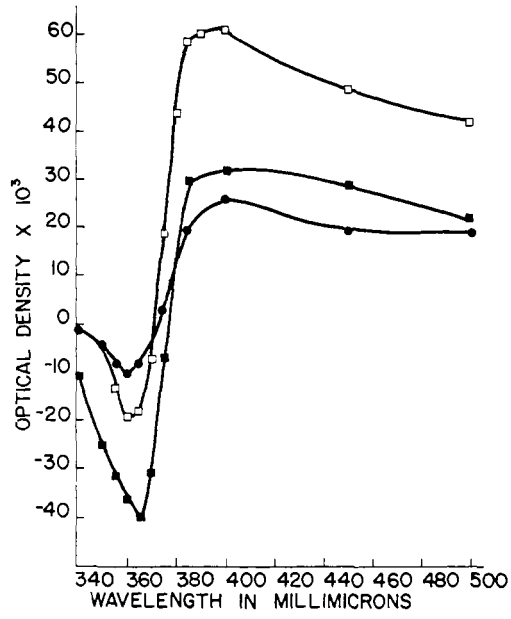

FIGURE 3: Difference spectra of representative light-exposed mixtures of DNA and aflatoxin B_1. DNA:aflatoxin ratios: 100:1 (●), 300:1 (■), 600:1 (□). Aflatoxin concentration in all cases = 9.1×10^{-6} M.

of aflatoxin is of considerable intrinsic interest. The transfer of excitation energy between DNA and various biologically active molecules has been observed (*e.g.*, acridine dyes, Isenberg *et al.*, 1964). Methods for studying energy transfer are well known; a recent review has been given by Windsor (1965). Purely spectral methods usually depend on measuring one or both of the following: (i) A decrease in the luminescence intensity of the donor or an increase in that of the acceptor, in a mixture of the two compared with that of the pure separate components. (ii) A decrease in the mean lifetime of the interacting excited state of the donor or increase in that of the acceptor compared with that of pure components. Since DNA neither fluoresces nor phosphoresces at room temperature in aqueous media, we have examined the low-temperature emission spectra of DNA, of aflatoxin and of mixtures. The measurements made were relative intensities of luminescence (both fluorescence and phosphorescence) and the phosphorescence lifetime of aflatoxin. Observations were limited to these because the weak DNA phosphorescence is obscured by the comparatively strong and longer aflatoxin emission and because we were unable to measure the short ($\sim 10^{-8}$ sec) fluorescence lifetimes with the available apparatus. However, with the data presently available several conclusions can be drawn: (1) The quenching of aflatoxin fluorescence demonstrates a transfer of excitation energy from the singlet of aflatoxin to *some* state of DNA. This may result in an electronically excited state of DNA or it may simply result in a vibrational excitation. (2) The lack of sensitized fluorescence or phosphorescence from DNA when only the aflatoxin in a mixture of DNA and aflatoxin is excited argues against the production of DNA singlet or triplet states by energy transfer from aflatoxin. (3) The complete absence of any change in the phosphorescence lifetime of aflatoxin by the presence of DNA rules out any

participation by the aflatoxin triplet in energy transfer to *any* excited state of DNA, electronic or vibrational. In summary, we feel that it is safe to conclude that quenching of the excited singlet state of aflatoxin leads only to vibrational excitation of DNA and that there is no interaction of the aflatoxin triplet with DNA.

As noted in the introduction there appears to be some possibility that photodegradation of aflatoxin may have occurred in the course of some reported experiments on aflatoxin binding. The longest time required for any of these was a dialysis time of 100 hr. Since samples exposed to ambient laboratory light for this period of time showed marked changes in the ultraviolet absorption spectrum and in the fluorescence intensity we conclude that substantial photodecomposition occurred. The methods employed by us did not show any change in the extent of binding. It is worth noting however that the most commonly used criteria for quantitative determination of aflatoxin B_1 concentration, absorbance at 363 mμ fluorescence intensity, would *both* be in error if relied on to indicate loss of B_1 by photolysis. The photoproducts have the same absorbance at 363 mμ and their fluorescence intensity is actually higher. Efforts to identify these photoproducts are underway; at present the suggestion of Andrellos *et al.* (1967) that a major constituent is hydroxylated aflatovin appears reasonable.

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