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The Interaction of Aflatoxins with Purines and Purine Nucleosides

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From measurements of thermal hyperchromicity and the behaviour of an aflatoxin–DNA mixture on a Sephadex column it was concluded that aflatoxin B_1 is capable of weak binding to single-stranded DNA. The interactions of the aflatoxins (B_1, G_1 and G_2) with nucleosides result in difference spectra and suggest that the purine bases and the amino group play a role in the binding of all the aflatoxins to DNA.

When DNA is added to a solution of aflatoxin B_1 the spectrum of the toxin is changed. As shown by a difference spectrum (Clifford & Rees, 1966, 1967) the maximum changes occurred at 355mμ and 385mμ. Aflatoxins G_1 and G_2 have also been shown to interact with DNA but to a smaller extent than aflatoxin B_1 (Clifford, Rees & Stevens, 1967). Actinomycin D gives a similar difference spectrum with DNA to aflatoxin B_1 (Reich & Goldberg, 1964) and shows many similar biochemical effects (Clifford & Rees, 1967). Notwithstanding these similarities in action these agents do produce some different biological effects. In contrast with aflatoxin B_1, actinomycin D inhibits protein synthesis in microsomal preparations isolated from the livers of poisoned rats but does not cause liver necrosis in rats (Schwartz, Sodergren, Garofalo & Sternberg, 1965). It would also appear that DNA synthesis in regenerating rat liver is less sensitive to actinomycin D than aflatoxin B_1 (Schwartz *et al.* 1965; De Recondo, Frayssinet, Lafarge & Le Breton, 1966).

Kersten (1961) found that the spectral changes produced in actinomycin solutions by DNA could be mimicked by several purine derivatives and concluded from these studies that the antibiotic reacted in some way with DNA-guanine. It was decided to investigate whether any particular nucleosides were involved in the interaction of aflatoxins with DNA and to determine whether the helical structure of the DNA was necessary for this interaction. In addition, a preliminary investigation has been made of the strength of the binding of aflatoxin B_1 with DNA.

METHODS

Determination of the thermal hyperchromicity (Tm). As described by Geiduschek (1962), *Tm* values were determined by using the 'i-assay'. Samples were kept at 50–100° in stoppered test tubes containing immersion condensers. After 10min. at the required temperature the samples were plunged into ice to bring them rapidly to room temperature and the increase in $E_{260m\mu}$ in a Unicam SP.800 spectrophotometer was measured against unheated samples. The control sample was 60μg.atoms of DNA P/l. alone in 0·01M-tris–HCl, pH7·4, containing 0·01M-NaCl. The mixture also contained 64μM-aflatoxin B_1. Increase in extinction was plotted against temperature.

Preparation of single-stranded calf-thymus DNA. A solution of DNA (3·98mg.atoms of DNA P/l.) in 0·01M-tris–HCl, pH7·4, containing 0·01M-NaCl was heated as above in a boiling-water bath for 15min. and then rapidly cooled in ice. The sample was brought to room temperature just before use.

Fractionation of DNA–aflatoxin B_1 on Sephadex G-50. A column of Sephadex G-50 (18cm. × 2cm.) was packed in 0·05M-NaCl. The bed volume was determined by running a marker of blue dextran 2000 through the column. A mixture of calf-thymus DNA and aflatoxin B_1 in 0·05M-NaCl was added to the column and 3·2ml. fractions were collected. The fractions were read at 260mμ to measure the DNA peak and at 370mμ to measure the aflatoxin B_1 peak.

Determinations of spectra. The difference spectra were studied with a Cary model 14 (serial no. 273) recording spectrophotometer with a 1cm. optical path length. Solutions of the aflatoxins B_1, G_1 and G_2 were prepared in 0·01M-tris–HCl, pH7·4, containing 0·01M-NaCl. The nucleosides, purine and substituted purine bases were dissolved in the same medium and the pH where necessary was adjusted to pH7·4. Great care has to be taken in this pH adjustment as at pH7·6 a spectral change occurs with aflatoxin. No spectral changes occur between pH6·9 and 7·4.

Reagents. Crude aflatoxin was a gift from the Medical Research Council. The toxins were separated as described by Clifford *et al.* (1967).

DNA type I, sodium salt, highly polymerized from calf thymus, was obtained from the Sigma London Chemical Co. (London, S.W. 6), as were the deoxyguanosine, deoxyadenosine, deoxycytidine–hydrochloride, thymidine, cytosine, thymine, guanine, adenine, purine, 2,6-diaminopurine

sulphate, L-cysteine–hydrochloride, DL-histidine–hydrochloride, 2-aminopurine nitrate and 6-dimethylaminopurine. Acetyl-6-aminopurine was synthesized by the method of Duvall (1951).

RESULTS

In the previous studies on the interaction of aflatoxin B_1 with DNA (Clifford & Rees, 1966, 1967; Clifford *et al.* 1967) the calf-thymus DNA was double-stranded and of helical structure. If this interaction involved both strands of the DNA it would be expected that there would be a difference in the thermal hyperchromicity (*Tm*) of the DNA–aflatoxin B_1 complex compared with that of the DNA alone. Measurements of *Tm* over a temperature range 50–100° did not reveal any significant differences between the DNA and the DNA–aflatoxin B_1 complex (Fig. 1). This would suggest that aflatoxin B_1 binds to a single strand of the DNA. It would therefore be expected that when aflatoxin B_1 was mixed with either the single- or double-stranded DNA the same difference spectrum should be produced. This was found to occur (Fig. 2) and it confirms the finding of Sporn, Dingman, Phelps & Wogan (1966) that aflatoxin B_1 binds with single-stranded DNA.

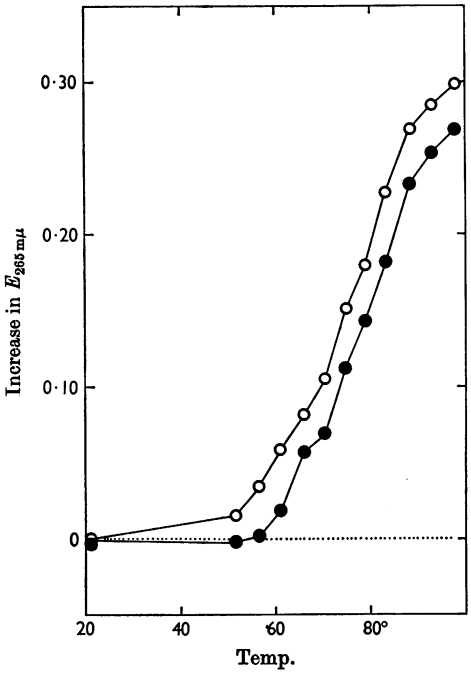

Fig. 1. Thermal hyperchromicity of DNA in the presence (●) and absence (○) of aflatoxin B_1. The concentrations and experimental details are as described in the text.

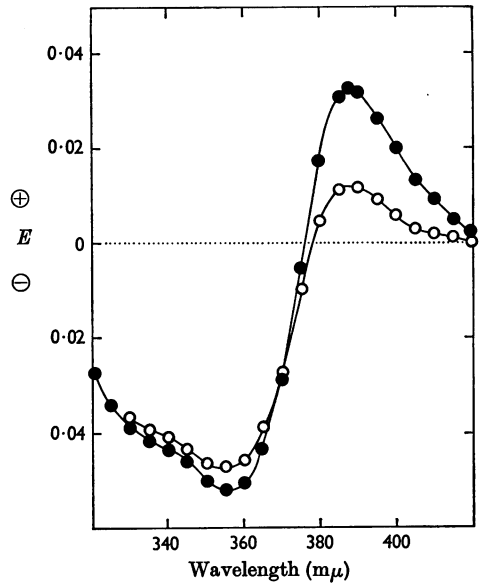

Fig. 2. Difference spectra of aflatoxin B_1 with single- (○) and double-(●) stranded DNA.

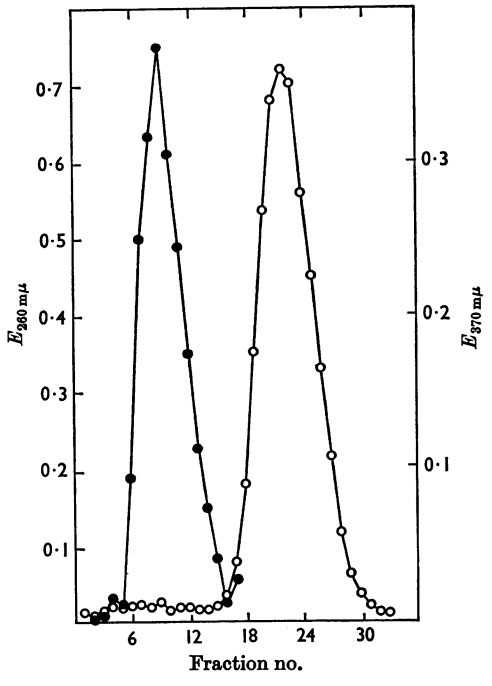

Fig. 3. Separation of a mixture of DNA and aflatoxin B_1 on a Sephadex column. Details of the column are given in the Methods section; 1 mg. of calf-thymus DNA + 200 μg. of aflatoxin B_1 in 1 ml. of 0·05 M-NaCl was applied to the column. ●, $E_{260\,m\mu}$; ○, $E_{370\,m\mu}$.

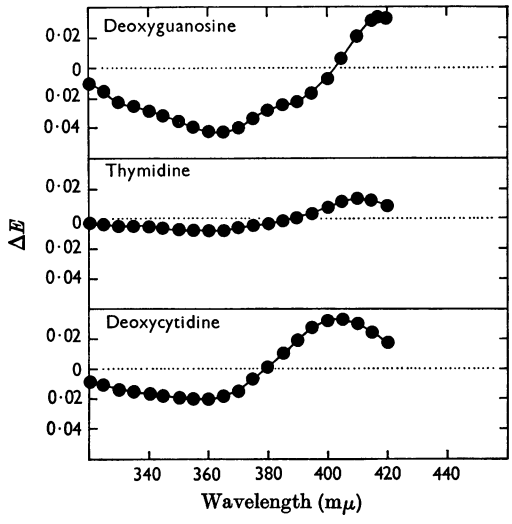

Fig. 4. Difference spectra of aflatoxin B_1 (64 μM) with 5·0 mM-deoxyguanosine, -thymidine and -deoxycytidine.

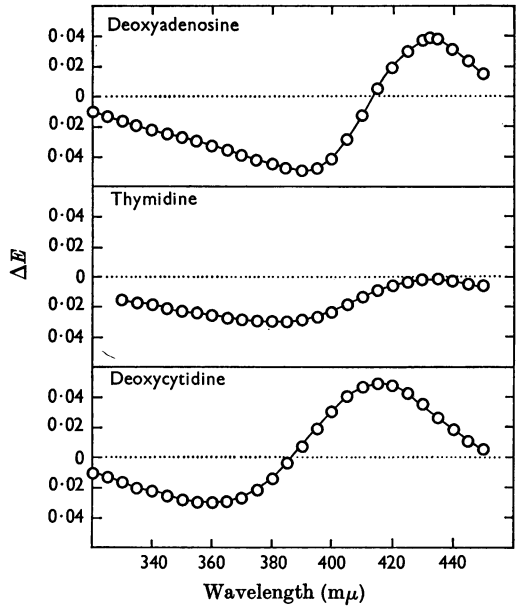

Fig. 5. Difference spectra of aflatoxin G_1 (64 μM) with 5·0 mM-deoxyadenosine, -thymidine and -deoxycytidine.

To examine the stability of the aflatoxin B_1–DNA complex, mixtures of varying relative concentrations of calf-thymus DNA and aflatoxin B_1 were applied to a Sephadex G-50 column. Fig. 3 shows the results of a typical experiment; the column has completely separated the two components without the appearance of a DNA–aflatoxin B_1 complex. It may be concluded that the bonds forming the complex that was present in the mixture were weaker than any interaction that occurred with the Sephadex G-50.

To investigate the interaction of aflatoxin B_1 with DNA in more detail the difference spectra of the toxin, mixed with various nucleosides found in DNA, were recorded. In Fig. 4 the largest difference in absorption occurred with deoxyguanosine. Deoxyadenosine and adenine gave changes identical with those produced by deoxyguanosine, whereas cytosine and thymine gave changes similar to that shown for thymidine. In Fig. 5, as with aflatoxin B_1, the deoxyguanosine change was identical with that of deoxyadenosine, but adenine, although it gave a similar curve, had a greater decrease in absorption at 390 mμ, reaching an extinction equal to −0·07. Cytosine and thymine with aflatoxin G_1 gave a similar change to thymidine with aflatoxin B_1 (Fig. 4). In Fig. 6 the difference spectrum with deoxyadenosine was similar to that obtained with deoxyguanosine, but the magnitude of the changes varied (at 370 mμ − 0·024 and at 435 mμ + 0·0175).

In general it may be concluded that the greatest spectral changes were observed with the purine bases. As the curves were identical for the adenine and guanine compounds it was considered that the amino groups present in the molecules were playing a part in the interaction with the toxin. Therefore the interaction of aflatoxins B_1 and G_1 with purine

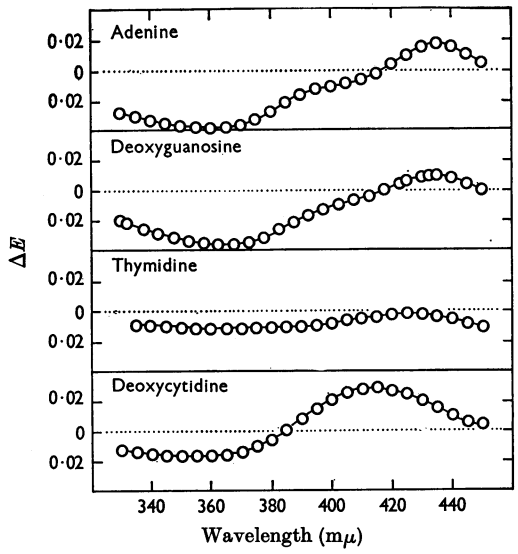

Fig. 6. Difference spectra of aflatoxin G_2 (64 μM) with 5·0 mM-adenine, -deoxyguanosine, -thymidine and -deoxycytidine.

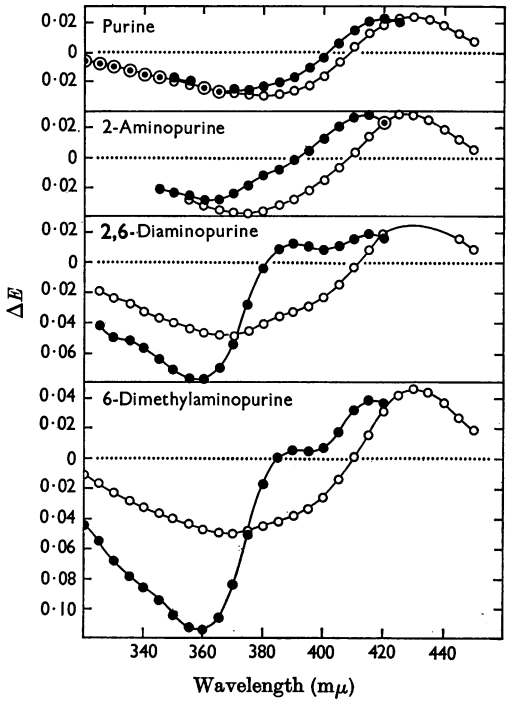

Fig. 7. Difference spectra of aflatoxins B_1 (●) and G_1 (○) (64 μM) with 5·0 mM-purine and -2,6-diaminopurine, and with 2·5 mM-2-aminopurine. Concentration of the 6-dimethylaminopurine with aflatoxin B_1 was 5·0 mM and with aflatoxin G_1 was 2·5 mM.

and various substituted purines was examined. It was not possible to use guanine and acetyl-6-aminopurine, as these compounds were insoluble at pH 7·4. In Fig. 7 there is a small but definite trace with purine. The substitution of two methyl groups on the 6-amino group of adenine does not prevent the aflatoxin interacting with this substituted purine. 2-Aminopurine gives a similar trace with aflatoxins B_1 and G_1 to adenine, whereas with 2,6-diaminopurine, aflatoxin B_1, but not aflatoxin G_1, gives an enhanced difference.

No difference spectra were obtained when aflatoxins B_1 and G_1 were mixed with histidine or cysteine.

DISCUSSION

The results indicate that for the binding of aflatoxin to DNA the purine ring is important and that the presence of an amino group on the ring also aids in the interaction. This is supported by the finding that there is an increase in the magnitude of the spectral shifts when the diamine is employed. As would be expected the dimethyl substitution of the amino group did not nullify this effect.

There are many similarities in the difference spectra obtained with aflatoxins B_1, G_1, G_2 and the purine and purine nucleosides. In some reactions aflatoxin G_1 gave spectral shifts of the same magnitude as aflatoxin B_1. This was in contrast with their difference spectra with DNA (Clifford *et al.* 1967), where there are marked differences in the magnitude of the changes. In this instance a correlation was found between the extent of the spectral shift when the toxins were mixed with DNA and the degree to which they inhibited RNA synthesis. It was concluded that the magnitude of these spectral changes were a reflexion of the extent of the binding of the toxins to DNA. Any differences between the binding of the toxin and DNA and its binding in the model systems may lie either in the DNA strand itself imposing special requirements or in that the spectral shift is not, in the latter instances, a measure of the binding.

The results obtained in the present study demonstrate that the interaction of aflatoxin B_1 to DNA is very different from that of actinomycin D (Kersten, 1961). The binding of aflatoxin B_1 is weaker and it will interact with single-stranded DNA. Hartman, Coy & Kniese (1963) were able to separate the actinomycin D–DNA complex from a Sephadex G-50 column. Although the aflatoxins will react with guanosine they react equally well with adenosine. In addition the aflatoxins, unlike actinomycin D, react with the purine base itself.

There are many similarities in the biochemical action of actinomycin D (for references see Reich & Goldberg, 1964) and aflatoxin B_1 (for references see Clifford & Rees, 1967). They both inhibit RNA polymerase, messenger-RNA production and protein synthesis. Both agents also produce similar cytological changes in regenerating rat liver (Bernhard, Frayssinet, Lafarge & Le Breton, 1965, and Schwartz *et al.* 1965). There are, however, differences in action such as the failure of actinomycin D to produce liver necrosis in the intact rat liver (Schwartz *et al.* 1965). If the toxic action of the aflatoxins and actinomycin D depends solely on their interaction with DNA it may be that differences in their toxicity could lie in this difference in their binding to DNA. The antibiotic phleomycin that requires adenine and thymine residues for interaction with DNA, and not guanine as required by actinomycin D, produces the opposite effect to actinomycin D in that it inhibits DNA polymerase more than RNA polymerase (Falaschi & Kornberg, 1964).

The production of a difference spectrum when two molecules are mixed indicates that an interaction has occurred. The magnitude of the spectral shifts, however, is not necessarily a measure of the degree

of interaction but depends on the molar extinction coefficient of the complex and the binding constant. Thus to confirm some of the findings in this present study, the reactions are being studied quantitatively by the determination of the binding constants.

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