section (Fig. 1*A*) was quite intense, but the cell did appear damaged. In the thionin-stained section (Fig. 1*B*) the marked cell was surrounded by many glial cells, but it was still easy to distinguish. Cells marked with methyl blue less than 4 to 5 hours before the animal's death were not only relatively undamaged, but often showed a spectacular array of stained dendrites. Figure 2 shows a cell marked 2 hours before death, and dendrites in the unstained section (Fig. 2*A*) can be seen over 500 μ from the cell body. Figure 2*B* shows the same cell after being stained with thionin. Some of the dendritic stain has been lost, this loss being minimized if dioxan rather than alcohols was used for dehydration. The marked cell is of a quite different color from that of the other neurons and can easily be identified in the section. Electrodes containing fast green do not produce quite such intense staining, and we have not found them as satisfactory as electrodes filled with methyl blue; but, in cases where marks of two different colors are desired, fast green is quite acceptable.

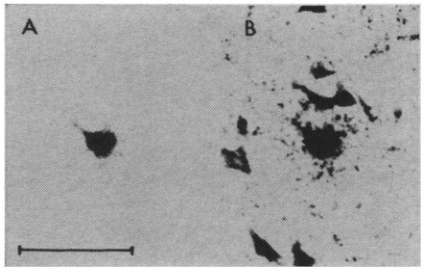

Fig. 1. Motoneuron marked 9 hours before the end of the experiment by passing 2.4 μa for 3 minutes through an electrode containing methyl blue. (*A*) The cell photographed in the unstained section. (*B*) After thionin staining, at the same magnification. Scale, 200 μ.

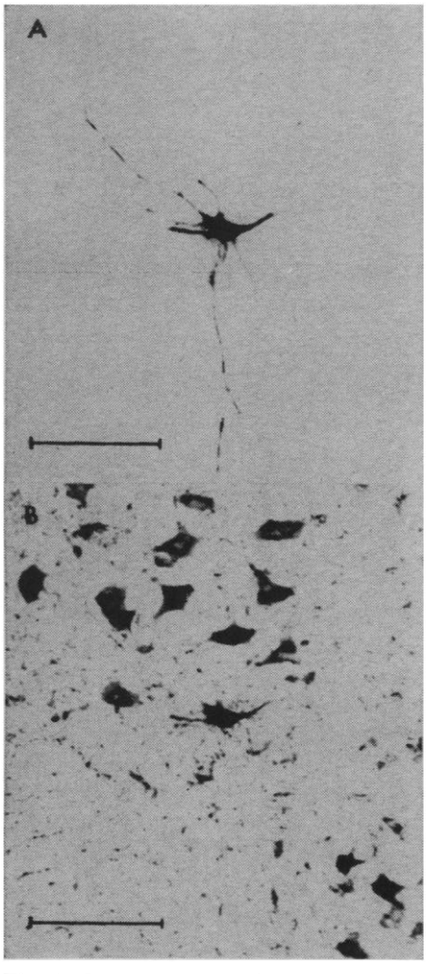

Fig. 2. Motoneuron marked 2 hours before the end of the experiment by passing 1.8 μa for 1 minute through a methyl blue electrode. (*A*) Cell in unstained section; the staining of the dendrites is extensive. (*B*) The same after thionin staining, at the same magnification. Some of the dendritic stain has been lost. Scale, 200 μ.

Currents tested were from 6 to 0.5 μa. However, currents in excess of about 3 μa often caused the dye to spread to neurons near the one impaled. On the other hand, marks made with less than 1 μa have been lost when made more than 2 hours before death. Thus for best results about 2 μa for 3 minutes should be used up to 3 hours before death; less current may be used nearer the end of the experiment. With such currents, we have never found more than one stained cell in the area. We have usually passed the current for 3 minutes, but this does not seem to be necessary in the case of methyl blue, except for marks made many hours before the animal was killed. Using currents in these ranges we have marked over 26 motoneurons with methyl blue and have been able to find all but two of them, probably lost because of bad penetrations. With electrodes filled with fast green we have made 19 marks, with currents ranging from 1.7 to 3 μa for 3 minutes, and, while many of the marks were rather pale, we were able to locate all but one.

Thus the above technique, while not sacrificing the quality of electrodes for intracellular recording, permits the routine marking of single penetrated neurons. The marked cells can easily be located in subsequent histological sections and, in particular with methyl blue, can show some details of the dendritic spread.

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4. Fast green purchased from the National Aniline Division of the Allied Chemical Co.; methyl blue from the Hartman Leddon Co.
5. Supported in part by grant NB-02619 from the USPHS. We thank Mrs. Margaret Moser for technical assistance.

7 January 1966

Aflatoxin B_1: Binding to DNA in vitro and Alteration of RNA Metabolism in vivo

Abstract. *Aflatoxin B_1 binds to both native and denatured DNA, as shown by spectroscopy and equilibrium dialysis. It also strongly inhibits incorporation of cytidine into rat liver nuclear RNA and lowers the RNA content of the nucleus. The extreme toxicity and carcinogenicity of aflatoxin B_1 may be direct results of the affinity of this agent for DNA.*

Aflatoxins, metabolites produced by some strains of the mold *Aspergillus flavus*, are among the most potent carcinogenic agents for rat liver, if judged by the minimum dose required to induce liver tumors (*1*, *2*). The structure of aflatoxin B_1 (Fig. 1), the most toxic of the four known aflatoxins produced by *A. flavus*, has recently been elucidated (*3*). We report here that aflatoxin B_1 binds to DNA in vitro, and that in vivo aflatoxin B_1 rapidly inhibits incorporation of cytidine-H^3 into rat liver nuclear RNA and lowers the ratio of nuclear RNA to DNA. These results suggest that the binding of aflatoxin B_1 to DNA may be of prime importance in the mechanism of action of this compound.

The binding of aflatoxin B_1 to DNA in vitro was demonstrated both by the shift in the absorption spectrum which occurs when aflatoxin B_1 is bound to DNA and by equilibrium dialysis techniques (*4*). Upon binding, there is a shift in the absorption maximum

Table 1. Effect of aflatoxin B_1 on metabolism of liver nuclear RNA. Each group consisted of two rats. Each rat in group A was injected with 0.1 ml of dimethylsulfoxide (DMSO) 70 minutes before it was killed. Each rat in group B was injected with aflatoxin B_1 (5 mg/kg) in 0.1 ml of DMSO 70 minutes before death. Each rat in group C was injected with aflatoxin B_1 (5 mg/kg) in 0.1 ml of DMSO 17 hours before death. All rats were injected with 80 μc of cytidine-H^3 50 minutes before death. Highly purified liver nuclei were isolated (*6*), the ratios of nuclear RNA to DNA were determined (*7*), and radioactivity in nuclear RNA was assayed (*7*). Figures in parentheses are percentages of values in control group.

Group	Incorporation of cytidine-H^3 into nuclear RNA*	RNA/DNA ratios of nuclei†
A	68,500 (100%)	0.18; 0.18 (100%)
B	5,400 (8%)	.14; .14 (78%)
C	11,500 (17%)	.14; .14 (78%)

* Figures expressed as counts per minute per milligram of DNA, at a counting efficiency of 34 percent obtained with the use of Nuclear-Chicago solubilizer and toluene scintillator. Figures are averages of duplicate determinations. † Duplicate determinations on portions from the identical nuclear lysates used for measurement of cytidine incorporation.

(Fig. 2) from 362–364 mμ to 366–368 mμ, accompanied by a marked hypochromism at 362 mμ; the ratio of the molar extinction coefficient (ϵ) of the unbound to that of the bound was 1.3 at 362 mμ. The ratio $\epsilon_{362}/\epsilon_{384}$ was used as a convenient measure of binding to native DNA; for unbound aflatoxin B_1 the ratio $\epsilon_{362}/\epsilon_{384}$ was 2.2, whereas in the fully bound state it was 1.5. Binding to heat-denatured DNA also caused a shift in the absorption spectrum of aflatoxin B_1, but higher concentrations of DNA relative to aflatoxin were required to induce the full hypochromicity at 362 mμ. At a ratio of 2000 moles of DNA-phosphorus to 1 mole of aflatoxin B_1, the hypochromicities at 362 mμ were identical for aflatoxin bound to either native or denatured DNA. However, even at a ratio of denatured DNA to aflatoxin of 2000, the ratio $\epsilon_{362}/\epsilon_{384}$ of aflatoxin B_1 was lowered only to 1.8 instead of to 1.5, the value observed for native DNA. The shift that occurs on binding to native DNA cannot be attributed to protein or to RNA impurities in the DNA. Neither native nor heat-treated (boiling-water bath, 10 minutes) bovine serum albumin nor calf thymus histone at concentrations of 25 μg/ml in 0.01*M* potassium phosphate, *p*H 7.3, induced changes in the absorption spectrum of aflatoxin B_1 (10 μg/ml). *Escherichia coli* RNA (1000 μg/ml in 0.01*M* potassium phosphate, *p*H 7.3) induced a similar, but slightly less marked, change in the spectrum of aflatoxin (10 μg/ml) than an equivalent amount of denatured DNA did. Finally, prior treatment of native DNA with pancreatic deoxyribonuclease abolished the capacity of DNA to induce a major change in the spectrum of aflatoxin B_1.

Equilibrium dialysis was used to measure the number of moles of aflatoxin B_1 bound per mole of DNA-phosphorus. Aflatoxin B_1 solutions (33 mμmole/ml, in 0.01*M* potassium phosphate, *p*H 7.3), with or without added native or heat-denatured calf thymus DNA (2800 mμmole of DNA-phosphorus per milliliter), were dialyzed for 5 days at 2°C against 15 volumes of 0.01*M* potassium phosphate, *p*H 7.3. The absorption spectra of the dialyzed material and the dialyzates were then read in a 5-cm cell in a Beckman DU spectrophotometer. After dialysis in the presence of native DNA, the spectrum of the aflatoxin in the dialyzate was that of the completely unbound material (Fig. 2), while the spectrum of the aflatoxin within the dialysis sack, when corrected for any freely exchangeable aflatoxin present, was taken to be the spectrum of the completely bound material (Fig. 2). The recovery of aflatoxin (both free and bound) was greater than 99 percent of the material originally added. From the above data, it was calculated that, under these particular conditions, 600 moles of native DNA-phosphorus bound 1 mole of aflatoxin B_1, whereas in the case of denatured DNA 170 moles of DNA-phosphorus bound 1 mole of aflatoxin. The binding affinity of aflatoxin B_1 for native DNA was depressed in 0.6*M* potassium phosphate buffer. The greater binding to denatured DNA apparently does not involve the chromophore absorbing at 362 mμ, since denatured DNA did not induce as much shift in the spectrum as native DNA did.

The results of experiments (*2*) dealing with the effects of aflatoxin B_1 on protein synthesis in rat liver in vivo indicated that the compound markedly inhibits the synthesis of tryptophan pyrrolase and tyrosine transaminase in response to hydrocortisone. One administration of the toxin at a dose of 5 mg/kg (LD_{30}, lethal dose, 30 percent effective) caused essentially complete inhibition of enzyme inducibility, and the inhibition persisted for as long as 10 days after toxin administration. In subsequent experiments (*5*) the characteristics of inhibition caused by aflatoxin during the period of rapid enzyme synthesis were very similar to those caused by actinomycin D. In view of this effect, as well as of the binding of aflatoxin to DNA reported here, the influence of the compound on nuclear RNA metabolism in rat liver was studied as follows. Rats (Fischer-344, male, 100 g, fed ad libitum) were injected intraperitoneally with aflatoxin B_1 in dimethylsulfoxide or with dimethylsulfoxide alone (Table 1). Fifty minutes before they were killed, the rats were injected intraperitoneally with 80 μc of cytidine-H^3. Highly purified liver nuclei were isolated (*6*), the ratios of nuclear RNA to DNA were

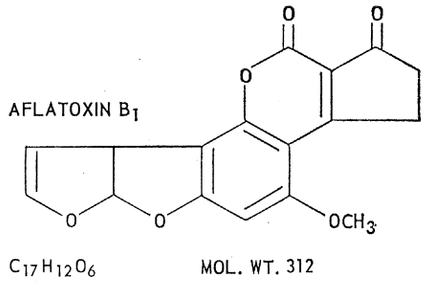

Fig. 1. Structure of aflatoxin B_1.

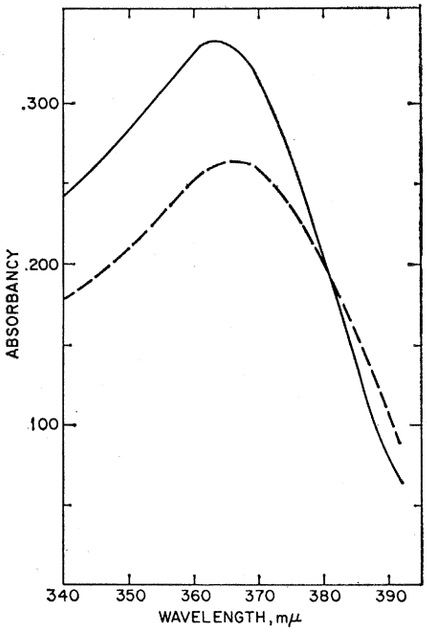

Fig. 2. Absorption spectra of aflatoxin B_1 in the unbound state (solid line) and bound to native DNA (dashed line). Both spectra are recorded at identical concentrations of aflatoxin B_1 (1.55 mμmole/ml) in 0.01*M* potassium phosphate, *p*H 7.3; 10-cm light path cells in a Beckman DU spectrophotometer. The DNA concentration used to obtain the spectrum of the bound aflatoxin was 3.2 μmole of DNA-phosphorus per milliliter.

determined (*7*), and the radioactivity in nuclear RNA was assayed (*7*). Within as short a time as 70 minutes after administration, 0.5 mg of aflatoxin B_1 markedly blocked cytidine incorporation into nuclear RNA and lowered the ratio of nuclear RNA/DNA (Table 1). Although the inhibition of cytidine-H^3 incorporation into nuclear RNA may result merely from inhibition of transport of RNA precursors to intracellular sites of RNA synthesis, the lowering of the ratios of nuclear RNA to DNA shows directly that the RNA content of the nucleus has diminished. The results suggest that a primary effect of aflatoxin B_1 is the blocking of RNA synthesis in the nucleus. Moreover, the alterations in RNA metabolism 17 hours after administration of aflatoxin were essentially the same as those at 70 minutes. The data are very similar to those (*7*) on the effects of actinomycin D on the metabolism of liver nuclear RNA in vivo.

In summary, aflatoxin B_1 itself has been shown to bind to DNA in vitro. The rapid and drastic alteration of nuclear RNA metabolism caused by aflatoxin B_1 in vivo strongly suggests that its ability to bind to DNA is a crucial aspect of its toxic and carcinogenic properties. The inhibitory effects of aflatoxin B_1 on mitotic division in cultured embryonic lung cells (*8*) and the ability of aflatoxin to induce chromosome aberrations in *Vicia faba* (*9*) are also consonant with such a mechanism. It would also appear that aflatoxin B_1 itself, rather than one of its metabolites, may be a proximate carcinogen. Further studies on the possible binding of aflatoxin B_1 to nucleic acids, proteins, and other cellular constituents in vivo will be required before the actual mechanism of the carcinogenesis by aflatoxins can be defined.

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7 January 1966

Actin-Myosin Interaction: Inhibition of the Myosin Adenosine Triphosphatase by Actin

Abstract. *In the absence of magnesium ion, the addition of actin to myosin in a 1 : 4 ratio has a strong inhibitory effect on the adenosine triphosphatase activity, in contrast to the well-known activating effect of actin in the presence of magnesium ion. This finding suggests that both effects result from a conformational change in the active site of the myosin adenosine triphosphatase.*

The contraction of skeletal muscle depends upon a specific interaction between the contractile proteins, actin and myosin (*1*), and the energy for contraction is derived from the hydrolysis of adenosine triphosphate (ATP) (*2*). A striking feature of the actin-myosin interaction in vitro is the transformation of the magnesium-inhibited adenosine triphosphatase of myosin to the magnesium-activated actomyosin adenosine triphosphatase (*3*). This actin activation of the adenosine triphosphatase in the presence of magnesium is maximal when the actin-myosin ratio is about 1 to 4 by weight (*4*), which corresponds to the stoichiometric combining ratio of actin and myosin determined by physical methods (*5*).

It has been proposed that myosin can hydrolyze ATP either directly by simple hydrolysis of the Michaelis enzyme-substrate complex, or through the magnesium-dependent formation of a myosin-phosphate intermediate (*6*), and, furthermore, that actin activates the second pathway of ATP hydrolysis by facilitating the decomposition of this myosin-phosphate intermediate (*7*). If this mechanism alone accounts for the effect of actin on myosin, then in the absence of magnesium ion, when the myosin-phosphate complex cannot be formed, actin should have no effect on the enzymic activity of myosin. In this regard, it has been reported that actin inhibits the calcium-activated adenosine triphosphatase of myosin (*8*) and heavy meromyosin (*9*) in the absence of added magnesium, but this inhibition was evident only at very high ratios of actin to myosin.

We have investigated the effect of actin on the myosin adenosine triphosphatase at extremely low concentrations of both magnesium and calcium ions, and under these conditions we have found that actin has a very marked inhibitory effect. Furthermore, like the actin activation of the adenosine triphosphatase in the presence of magnesium, the inhibition is maximal at a 1 : 4 ratio of actin to myosin.

Myosin was prepared from rabbit muscle by the method of Szent-Györgyi (*1*), and actin was prepared according to Ulbrecht *et al.* (*10*), except that no magnesium was added during the preparation of either protein. The hydrolysis of ATP was recorded by an automatic *p*H-stat at *p*H 7. The ATP concentration at the beginning of the reaction was 2 m*M*, and the reaction was initiated by the addition of 2 mg of myosin per milliliter. The sample temperature was maintained at 25°C. Average reaction rates were determined for the hydrolysis of the first 25 percent of the ATP. To reduce the free magnesium concentration in the reaction mixtures, a chelating agent, 5 m*M* 1,2-diaminocyclohexanetetraacetic acid (DCTA) (*11*), was added. In addition, 1 m*M* ethyleneglycol-bis(aminoethyl-ether)tetraacetic acid (EGTA) (*11*) was included in all the samples to keep