Aflatoxin B₁ Binding and Toxic Effects on *Bacillus megaterium*

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SUMMARY

A study was made of the binding of aflatoxin B₁ and its toxic effects on *Bacillus megaterium* NRRL B-1368. After a 12 hr incubation period, 5 µg. aflatoxin B₁/ml. inhibited growth of the test organism 95 %. Viable organisms decreased from 3.8×10^7 /ml. in the controls to 5.0×10^4 /ml. in a growth medium containing 50 µg. aflatoxin B₁/ml., after 4 hr. of incubation. Viability was not significantly decreased, however, when the organisms were incubated with the same concentration of toxin either under nitrogen, or at 5°, or in the presence of bacteriostatic concentrations of tetracycline. Binding experiments showed that 4.0×10^9 organisms/ml. took up approximately 7.0 µg. aflatoxin B₁/ml. After five aqueous washes of the organisms, 20-25 % of the toxin remained bound to them. The tightly bound toxin was removed by ultrasonic treatment of the organisms and chloroform extraction of the macerate. Removal of cell walls by lysozyme following toxin uptake released 23 % of the initially bound toxin, and osmotic rupture of the protoplasts an additional 7 %. Toxin was also taken up by intact and by ruptured protoplasts. Removal of nucleic acids from the membranes did not alter their capacity to bind aflatoxin B₁.

INTRODUCTION

The uptake of aflatoxin B₁ by *Flavobacterium auranticum* NRRL B-184 and the resulting toxic effects have been investigated (Ciegler, Lillehoj, Peterson & Hall, 1966; Lillehoj, Ciegler & Hall, 1967). These studies showed that 10^{11} intact organisms/ml. removed 7.0 µg. aflatoxin B₁/ml. from an aqueous medium. Autoclaved organisms and cell-wall preparations also bound the toxin, which could be recovered from them by washing; toxin taken up by intact organisms could not be entirely extracted. It was concluded that aflatoxin B₁ is initially bound loosely to the cell wall, followed by a tighter binding to sites in the intact organisms.

Aflatoxin B₁ has shown a facility for binding to deoxyribonucleic acid (DNA) (Clifford & Rees, 1966; Sporn, Dingman, Phelps & Wogan, 1966; Black & Jirgensons, 1967). These studies and others have related the cytotoxic aspects of aflatoxin to a DNA binding followed by a perturbation of messenger ribonucleic acid (RNA) synthesis. Binding of aflatoxin B₁ to DNA, however, is not exclusive since it has been observed that it also binds to RNA (Sporn *et al.* 1966) and to histones (Black & Jirgensons, 1967). The nature of the binding of aflatoxin B₁ to DNA has been examined by several investigators. Some workers believe that aflatoxin B₁ functions as an alkylating agent (Legator, Zuffante & Harp, 1965; Wragg, Ross & Legator, 1967). But studies on the stability of the DNA-aflatoxin B₁ complex have shown that the toxin can be separated from the nucleic acid on a Sephadex-50 column (Clifford & Rees, 1967). There is also no effect on the melting profile of the DNA produced by the interaction with the aflatoxin.

The purpose of the present work was to acquire further information about the nature of uptake and binding of aflatoxin B₁ and its toxic effects on a sensitive micro-organism. Since determination of toxic uptake by protoplasts and protoplast membranes seemed desirable, *Bacillus megaterium* NRRL 1368 was chosen as the test organism; it is sensitive to aflatoxin B₁ (Burmeister & Hesselstine, 1966) and readily yields protoplasts by the action of lysozyme.

METHODS

Production and harvest of bacilli. Inhibition studies of *Bacillus megaterium* were done in the malt-extract + yeast-extract medium (MY) described by Haynes, Wickerham & Hesselstine (1955). Ten ml. medium in 300 ml. Erlenmeyer flasks were inoculated with 1.0×10^8 bacilli followed by incubation at 30° on a rotary shaker. Growth was measured by determining the extinction (540 m μ , Beckman B spectrophotometer) of a fixed volume of culture medium. Protoplasts were produced from bacilli grown in a medium containing (w/v): peptone (Difco Laboratories, 920 Henry Street, Detroit, Mich.) 2.0 %, sodium chloride 0.5 % (when necessary solidified with 2 % agar). Five hundred ml. of this medium in 2.8 l. Fernbach flasks were inoculated with 1.5×10^{10} bacilli followed by incubation at 30° for 4 hr on a rotary shaker. Bacilli for protoplast formation were harvested by centrifugation at 5000 g for 1 hr.

Counting of viable organisms. Ten ml. MY growth medium in 300 ml. Erlenmeyer flasks were inoculated with stationary-phase bacilli and incubated on a rotary shaker at 30°. Successive tenfold dilutions in growth medium were made of samples of culture. Three samples were taken from an appropriate dilution and spread on growth-medium agar in Petri plates which were incubated for 14 hr at 28°. Colony counts were only recorded on plates which showed 10 to 100 colonies.

Formation and lysis of protoplasts. Eighty-two ml. of 2 M-sucrose, 4 ml. of M-sodium chloride and 100 mg. lysozyme (Calbiochem, Box 54282, Los Angeles, California) were added to 250 ml. phosphate buffer (pH 7.0) containing 2.0×10^{11} *Bacillus megaterium* organisms (Weibull & Bergström, 1958). Protoplast formation was followed microscopically and was generally complete in 1 hr. The protoplasts were centrifuged down (15,000 g for 30 min.) and then lysed by suspension in 0.05 M-tris (pH 7.6) + 0.005 M-magnesium chloride, followed by homogenization with a tissue-grinder. Protoplasts were washed in isotonic medium before testing, and membranes were routinely washed twice with tris buffer (pH 7.6) before aflatoxin uptake studies.

Aflatoxin B₁ production, assay and binding procedures. Aflatoxin B₁ was produced and recovered according to the procedures of Shotwell, Hesselstine, Stubblefield & Sorenson (1966). A standard solution of aflatoxin B₁ was prepared in ethanol and diluted as required in the desired medium. In growth studies the ethanol was removed from the medium under vacuum, before inoculation. Only concentrations of aflatoxin B₁ which exhibited a coefficient near that reported at 363 m μ (eM = 22,000) were used (Nabney & Nesbitt, 1965). Toxin concentrations were determined by thin-layer chromatography (Shotwell *et al.* 1966) and by spectrophotometric methods in a Beckman DB spectrophotometer (Nabney & Nesbitt, 1965). Binding of aflatoxin B₁ to organisms and membranes was done by incubating the cellular components with the toxin in a defined medium containing (w/v); yeast nitrogen base (Difco) 0.7 %; glucose, 0.5 % potassium phosphate 0.1 %; medium adjusted to pH 6.8. Following incubation,

the organisms and membranes were centrifuged down at 15,000 g for 30 min., and the concentration of toxin in the supernatant fluid determined. Washing was done by resuspending organisms or membranes in 70 ml. water followed by centrifugation.

Removal of nucleic acid from protoplast membranes. Protoplasts from 2.0×10^{11} bacilli were prepared and lysed, and then washed twice with 70 ml. tris buffer (pH 7.6). Membranes were resuspended in 50 ml. tris buffer (pH 7.6) + 0.5% (w/v) sodium deoxycholate and gently stirred for 1 hr. The membranes were then centrifuged down (15,000 g for 30 min.) and washed twice with 70 ml. tris buffer (pH 7.6) containing 10 µg deoxyribonuclease (DNase)/ml. (Sigma Chemical Co., 3500 DeKalb Street, St Louis, Mo.) and 25 µg. ribonuclease (RNase)/ml. (Sigma Chemical Co.) and gently stirred for 1 hr. The nucleic-acid-cleared membranes were harvested by centrifugation and washed twice in 70 ml. tris buffer (pH 7.6). A sample of protoplasts and membranes was sedimented, washed and extracted with perchloric acid for nucleic acid determination (Berrah & Konetzka, 1962). DNA was determined by the diphenylamine technique (Burton, 1956) and RNA by the orcinol method (Mejbaum, 1939). Weights of protoplasts and membranes were determined after drying in an air oven for 12 hr at 100°.

RESULTS

Aflatoxin inhibition of growing bacilli

Growth of *Bacillus megaterium* was inhibited in the presence of aflatoxin B₁ (Fig. 1). After a 12 hr incubation, aflatoxin B₁ at 10 and 5 µg./ml. inhibited growth 97% and 95%, respectively; at 2.5 and 1.0 µg./ml. there was no inhibition; the bacilli in a 2.5 µg./ml. concentration, however, showed a growth lag. The effects of a range of concentrations of aflatoxin B₁ on viability of growing bacilli are presented in Fig. 2. There was a striking decrease in viability of bacilli in a growth medium containing aflatoxin B₁ at 25 and 50 µg./ml., with less effect at 5.0 and 2.5 µg./ml. During a 4 hr incubation, viable bacilli decreased in the presence of aflatoxin B₁ at 50 µg./ml. from 2.3×10^6 viable bacilli/ml. to 8.0×10^3 /ml. These tests showed that the viability of the bacilli in a growth medium containing aflatoxin at 25 or 50 µg./ml. continued to decrease during the time-course of the experiment.

Comparison of the inhibition of growth (Fig. 1) with the decrease in viability (Fig. 2) showed that aflatoxin B₁ at 5 µg./ml. did not entirely inhibit growth during the first 4 hr, whereas the viability decreased in this concentration of toxin. This difference in sensitivity to toxin was probably due to the use of growing bacilli as the inoculum in growth tests, and of non-multiplying bacilli for the inoculum in viability studies. Further examination of the aflatoxin B₁ effect on the viability of *B. megaterium* was carried out by incubation of the cells in the presence of the toxin in non-growing situations (Table 1). After a 4 hr incubation period, comparisons were made of the viability of bacilli under various conditions. Bacilli incubated at 30° aerobically demonstrated a marked decline in viability with aflatoxin B₁ at 25 and 50 µg./ml. The decrease was similar to that observed in the previous experiments (Fig. 2) and involved approximately three orders of magnitude (3.8×10^7 to 5.0×10^4 bacilli). However, viability of bacilli either in nitrogen, at 5°, or with 5 µg. tetracycline/ml. was decreased less than one order of magnitude (3.8×10^7 to 2.5×10^7). Evidently, the bactericidal action of aflatoxin B₁ was associated with growth of the organism.

Aflatoxin binding by intact bacilli

The nature of the binding of aflatoxin B₁ to *Bacillus megaterium* bacilli was investigated by incubating the organisms with toxin, followed by determining the concentration of toxin associated with the organisms. Of the 950 $\mu\text{g.}$ of aflatoxin B₁ initially present in the incubation mixture, 525 $\mu\text{g.}$ were associated with 3.0×10^{11} bacilli after the first centrifugation (Table 2). After the bacilli were washed 5 times

Fig. 1. Inhibition of growth of *Bacillus megaterium* by aflatoxin B₁. Ten ml. of malt-extract + yeast-extract medium (MY) inoculated with 1.0×10^8 growing bacilli followed by incubation on a rotary shaker at 30°. Growth was measured by determining the extinction of a fixed volume of culture. Points are means of two independent observations.

with 70 ml. water/wash, 118 $\mu\text{g.}$ (22%) of the 525 $\mu\text{g.}$ of originally bound toxin remained associated with the bacilli. Since the decrease in bound aflatoxin B₁ between the fourth and fifth washes was only 3%, it was concluded that the 22% which remained showed a tighter attachment to the organism than did the 78% removed by the five washes. After the fifth wash, the bacilli were broken by ultrasonic treatment and extracted with chloroform. By this procedure the 'tightly bound' toxin was quantitatively recovered from the ruptured organisms.

Recovery of aflatoxin B₁ from protoplasts and protoplast membranes

Since the experiments with whole bacilli showed that tightly bound aflatoxin B₁ could be recovered from broken cells, further tests were made to elucidate the function of the cell wall in toxin-binding. Initial experiments involved measuring uptake of

aflatoxin by intact bacilli, followed by determination of toxin retention after washing, formation of protoplasts, lysing of the protoplasts and extraction of the protoplast membranes with chloroform (Table 3). After two washes, 23 % of the initially bound toxin was released during the formation of protoplasts and another 7 % after lysis of protoplasts. The membranes from ruptured protoplasts retained 11 % of the toxin

Fig. 2. Effect of various concentrations of aflatoxin B_1 on viability of growing *Bacillus megaterium*. Ten ml. of MY medium inoculated with non-multiplying bacilli followed by incubation on a rotary shaker at 30°. Samples from appropriate dilutions spread on MY agar. Points are means of three observations.

originally bound; almost all of this was recovered by chloroform extraction. These experiments showed, in general, that digestion of the cell wall by lysozyme and subsequent lysis of the protoplasts released some of the bound toxin. The 11 % of toxin retained by the bacterial membranes could be largely recovered by chloroform extraction.

Since it had been clearly established that intact bacilli bind aflatoxin B_1 , the question arose whether protoplasts were also capable of taking up toxin. Incubation of intact protoplasts showed that removal of the cell wall had little effect on the capacity of the organism to bind toxin (Table 4). After the protoplasts were washed and the

membranes lysed and washed, 10% of the toxin remained bound. Since washing the membranes removed only 1% of the originally bound toxin, the 10% fraction of bound toxin must have been closely associated with a membrane constituent.

Table 1. *Viability of Bacillus megaterium exposed to aflatoxin B₁*

Bacilli from a 24 hr culture (stationary phase) used as inoculum in 10 ml. MY growth medium. Flasks were incubated on a reciprocal shaker for 4 hr after inoculation. After proper dilutions, organisms plated on three MY plates, incubated for 24 hr at 28° and counted. Values given are means of the three plates for each treatment.

Expt.	Conditions	Zero time	Aflatoxin B ₁ (μg./ml.)			
			0	10	25	50
			Viable organisms/ml.			
1	30°, air	3.8×10^7	5.6×10^7	5.0×10^6	1.5×10^5	5.0×10^4
	30°, N ₂	3.8×10^7	2.8×10^7	2.7×10^7	2.5×10^7	2.5×10^7
2	30°, air	4.0×10^7	7.6×10^7	6.2×10^6	1.7×10^5	6.0×10^4
	5°, air	4.0×10^7	2.9×10^7	2.1×10^7	2.0×10^7	1.8×10^7
3	30°, air	8.0×10^6	1.2×10^7	1.0×10^6	3.8×10^4	1.4×10^4
	30°, air, and 5 μg./ml. tetracycline	8.0×10^6	5.0×10^6	5.0×10^6	4.0×10^6	3.0×10^6

Table 2. *Bacillus megaterium: recovery of bound aflatoxin B₁ from whole organisms*

3.0×10^{11} bacilli were incubated with 950 μg. aflatoxin B₁ for 30 min. in 75 ml. defined growth medium at 30° on a reciprocal shaker. Values are means of two experiments.

Treatment	Aflatoxin B ₁		
	Associated with bacilli, total (μg.)	μg./bacillus	% retained by bacilli
Cells from incubation	525	1.7×10^{-9}	100
Wash 1	330	1.1×10^{-9}	63
Wash 2	228	0.8×10^{-9}	43
Wash 3	166	0.6×10^{-9}	32
Wash 4	133	0.5×10^{-9}	25
Wash 5	118	0.4×10^{-9}	22
Cells treated ultrasonically and then extracted with chloroform	0	0	0

Effect of removal of nucleic acid from bacterial membranes on aflatoxin B₁ uptake

Although aflatoxin B₁ binds to nucleic acids and to proteins, the biological effects of the toxin are generally attributed to nucleic-acid binding. These observations suggested that binding of toxin to bacilli, protoplasts and membranes of *Bacillus megaterium* might be related to association with the nucleic acids. This question was explored by comparing the aflatoxin binding of control membranes to that of membranes whose nucleic acids had been removed. Table 5 presents results from treatment of the membranes with sodium deoxycholate, DNase and RNase. This procedure essentially freed the membranes of nucleic acid. The control and nucleic-acid-free

membranes did not differ significantly in their toxin-binding capacity (Table 6). It was concluded that nucleic acids are not necessary for binding of the aflatoxin B₁ to *B. megaterium* membranes.

Table 3. *Bacillus megaterium*: recovery of bound aflatoxin B₁ from bacilli following formation of protoplasts

2.0×10^{11} bacilli were incubated with 1090 µg. aflatoxin B₁ for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Values are means of three experiments.

Treatment	Aflatoxin B ₁		
	Associated with bacilli or membranes, total (µg.)	µg./bacillus	% retained by bacilli or membranes
Bacilli from incubation	352	1.8×10^{-9}	100
Wash 1	214	1.1×10^{-9}	61
Wash 2	143	0.7×10^{-9}	41
Protoplasts	63	—	18
Ruptured protoplasts	40	—	11
Ruptured protoplasts chloroform-extracted	3	—	1

Table 4. *Bacillus megaterium*: recovery of bound aflatoxin B₁ from protoplasts and protoplast membranes

Protoplasts from 2.0×10^{11} bacilli were incubated with 900 µg. aflatoxin B₁ for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Protoplasts were washed in 70 ml. 0.5 M-NaCl and membranes washed with 70 ml. water. Values are means of three experiments.

Treatment	Aflatoxin B ₁ bound to protoplasts or membranes	
	Total (µg.)	(%)
Protoplasts from incubation	315	100
Protoplast wash 1	162	51
Protoplast wash 2	96	30
Lysed protoplasts	34	11
Membrane wash	32	10
Membranes chloroform-extracted	1	—

Table 5. *Bacillus megaterium*: nucleic acid composition of protoplast and protoplast membranes

Samples of protoplasts and membranes were sedimented, washed and extracted with perchloric acid followed by determination of DNA (diphenylamine assay) and RNA (orcinol technique). Values are means of three experiments and are based on dry weights of protoplasts and membranes.

Treatment	DNA (mg./g.)	RNA (mg./g.)
Intact protoplasts	12	122
Protoplast membranes treated deoxycholate, DNase and RNase	1	4

Table 6. *Bacillus megaterium*: recovery of bound aflatoxin B₁ from protoplast membranes

Membranes from lysed protoplasts of 2.0×10^{11} bacilli incubated with 900 μ g. aflatoxin B₁ for 15 min. in 50 ml. defined growth medium at 30° on a reciprocal shaker. Membranes washed with 70 ml. water. Values are means of three experiments.

Treatment	Aflatoxin B ₁ bound to protoplast membranes			
	With nucleic acids		Without nucleic acids	
	(μ g.)	(%)	(μ g.)	(%)
Membranes from incubation	301	100	305	100
Membrane wash 1	149	50	146	48
Membrane wash 2	71	24	68	22
Membrane wash 3	27	9	33	11
Membranes chloroform-extracted	0	0	2	1

DISCUSSION

Initial experiments on binding aflatoxin B₁ to intact bacilli of *Bacillus megaterium* showed that 78 % of the toxin initially bound per bacillus could be removed by aqueous washing. The remainder of the toxin associated with the organisms appeared to be more rigorously bound. However, chloroform extraction of the organisms ruptured by ultrasonic treatment gave quantitative recovery of toxin. These tests showed that although there was uptake of aflatoxin B₁ by the bacilli, there was no significant metabolic degradation of the molecule, since all the toxin was recovered. Furthermore, chloroform-extractability of the tightly bound toxin suggested that it was not covalently bound to a cellular constituent. These observations support the hypothesis of Clifford & Rees (1966, 1967) that the primary cytotoxic effect of aflatoxin B₁ involves a weak binding of the toxic molecule to a cellular constituent.

Earlier studies in our laboratory had shown that aflatoxin B₁ binds to bacterial cell walls. When whole bacilli containing toxin were converted to protoplasts 23 % of the toxin bound initially was released during digestion of the cell wall by lysozyme, and 18 % was retained by the intact protoplasts. Lysis of the protoplasts released an additional 7 %, whereas the membranes retained 11 %. These tests showed that removal of the wall did not release all the bound toxin. It was concluded that binding does not depend exclusively on the cell wall. Studies of the permeability of the protoplast to aflatoxin B₁ showed that the removal of cell wall did not prevent toxin uptake. Furthermore, following lysis of the protoplasts containing bound aflatoxin B₁, the water-washed membranes still retained 10 % of the initially bound toxin B₁. This showed that, as in intact bacilli, a portion of the bound toxin was readily dissociated from cellular constituents by aqueous washing. Examination of binding of aflatoxin B₁ by membranes, with or without nucleic acid, showed that association with DNA or RNA was not required for toxin uptake. Since a binding of toxin to protein has been reported, it seems reasonable to assume that the aflatoxin B₁ may be bound to the protein in the membranes of *Bacillus megaterium*.

The multiplying bacilli of *Bacillus megaterium* showed a marked decrease in viability in relation to bacilli in a non-multiplying situation. In some instances, toxicity of this

type has been attributed to 'unbalanced synthesis' (Roberts, McQuillen & Roberts, 1959). A similar imbalance has been observed (Lillehoj & Ciegler, 1967) in macromolecular biosynthesis of *Flavobacterium aurantiacum* grown in the presence of aflatoxin B₁ at 50 µg./ml., which decreased DNA synthesis by 80 %, RNA production by 48 % and protein synthesis by 32 %. It appears that the imbalance in macromolecular synthesis, particularly DNA production, might be responsible for the decrease in viability of multiplying *Bacillus megaterium*.

Flavobacterium aurantiacum grown in the presence of aflatoxin B₁ develops aberrant forms (Lillehoj *et al.* 1967). *Bacillus megaterium* grown in presence of the toxin also showed an inhibition of the cell-division mechanism, and unusually long forms were routinely observed. That the toxin-induced aberrancy of *F. aurantiacum* was a manifestation of cell-wall inhibition was rejected when it was found that growth of an L-form of an aflatoxin-sensitive *Bacillus* strain was inhibited by the toxin (Burmeister & Hesseltine, 1967). Since the mechanism of cytokinesis in bacteria probably involves an interaction between nucleus and cell membrane (Jacob, Brenner & Cuzin, 1963), the mode of aflatoxin action in inducing aberrant forms might be due to an initial toxic event involving nucleic acid synthesis. These observations suggest to us that the intact aflatoxin B₁ molecule interferes with nucleic acid synthesis, without covalent attachment to a cellular component or metabolic degradation. Whether the binding of aflatoxin B₁ to *B. megaterium* is related to the mechanism of toxic action, however, is not clear.

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