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Aflatoxin B_1 Uptake by *Flavobacterium aurantiacum* and Resulting Toxic Effects

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ABSTRACT

Removal of aflatoxin B_1 from liquid cultures by resting and growing cells of *Flavobacterium aurantiacum* NRRL B-184 was studied. Spectrophotometic and thin-layer techniques served as aflatoxin assays. Cells grown in the presence of 5 ppm or higher levels of aflatoxin developed aberrant morphological forms. These toxin concentrations partially inhibited growth, and the nature of the inhibition suggested that aflatoxin interfered with cell wall synthesis. Incubation of 1.0×10^{11} resting cells per milliliter with 7.0 μg/ml of aflatoxin B_1 during a 4-hr period facilitated complete toxin removal from a buffered aqueous medium. Autoclaved cells and cell wall preparations could remove a fraction of the aflatoxin of a test system. However, the toxin removed by autoclaved cells and cell walls could be extracted by washing with water but the aflatoxin B_1 removed by intact cells could not be extracted into the liquid phase. The uptake of aflatoxin B_1 by resting cells was sensitive to temperature and *p*H. Ruptured preparations of *F. aurantiacum* were not able to remove or modify the aflatoxin in an aqueous solution.

Aflatoxins are a group of four toxic metabolites produced by certain strains of *Aspergillus flavus* Link ex Fries (1, 18). Their chemical structure has been determined (2, 3, 12). The two major constituents are aflatoxin B_1 ($C_{17}H_{12}O_6$) and aflatoxin G_1 ($C_{17}H_{12}O_7$); each of the secondary compounds, B_2 and G_2, contains one less carbon-carbon double bond. Aflatoxin B_1 is particularly toxic to young turkeys and ducklings and is a potent carcinogenic agent to other animals (13). The toxin inhibits protein synthesis in liver and in *Escherichia coli* (20); it also suppresses mitosis and deoxyribonucleic acid (DNA) synthesis in human lung cells (14). The inhibitory capacity of aflatoxin towards nucleic acid synthesis has been ascribed to its potential as an alkylating agent (15). In addition, aflatoxin has been related to the induction of chromosomal aberrations in root cells of *Vicia faba* (16).

Aflatoxin acts as an inhibitor of gibberellic acid-induced synthesis of α-amylase and lipase in germinating seeds of barley and cotton (6) while inhibiting chlorophyll synthesis in germinating cress seeds (19). A sensitivity survey of 329 microorganisms has shown that growth of 14 was inhibited by the toxin (H. R. Burmeister and C. W. Hesseltine, *unpublished data*). Furthermore, some fungi common in peanut pods and kernels are capable of a partial breakdown of aflatoxin (4). This report describes the effects of aflatoxin on growing cells of *Flavobacterium aurantiacum* NRRL B-184, the quantities of toxin bound to the organism, and some investigation of the nature of the binding.

MATERIALS AND METHODS

Production and harvest of cells. F. aurantiacum was grown in a medium containing (per liter): tryptone (Difco), 2.5 g; yeast extract (Difco), 2.5 g; glucose, 10 g; and KH_2PO_4, 1.0 g. The *p*H of the culture medium remained constant at 6.5 during growth of the organism. Growth studies were made in 300-ml Erlenmeyer flasks containing 50 ml of culture medium inoculated with 6.0×10^8 cells. Production of larger numbers of cells for resting-cell assays was carried out in 500 ml of medium in 2.8-liter Fernbach flasks inoculated with 6.0×10^{10} cells. The inoculated flasks were incubated at 30 C on a rotary shaker for 48 hr. Growth was measured by determining the absorbancy (540 mμ, Beckman B spectrophotometer) of cells from a fixed volume of culture fluid. For the analyses, cells were removed from the growth medium by centrifugation and resuspended in water. Colorimetric assay of DNA, as an estimate of growth, was performed by the method of Burton (7). Cells for studies on binding were harvested by centrifugation at $5,000 \times g$ for 1 hr, followed by washing twice with buffer and recentrifugation.

Aflatoxin determination. Aflatoxin B_1 was added to the aqueous test systems in chloroform solutions. The chloroform was removed from B_1 solutions by autoclaving. Thin-layer chromatography assays of aflatoxin were carried out according to the methods described by De Iongh et al. (9). Spectrophotometric tests were executed by the method of Nabney and Nesbitt (17). Absorbancies were measured in a Beckman DB spectrophotometer.

Fragility of cell walls. Fragility was determined by cell susceptibility to rupture by sonic oscillation. Cells were grown in subinhibitory concentrations of aflatoxin B_1 (5 μg ml and below), followed by centrifugation and resuspension of the appropriate number of cells in 20 ml of 0.067 M phosphate buffer (*p*H 6.75). Cells were added to the buffer in quantities necessary to equalize the absorbancy of the test suspensions before sonic treatment. The samples were then exposed to the sonic probe under identical conditions for 4 min. The change in absorbancy between treated and untreated preparations at 540, 260, and 280 mμ was used as an indicator of the relative fragility of the cell walls (10).

Cell wall preparation. Cells were ruptured by 10 consecutive 4-min exposures to a 20-kc, 125-w sonic probe (Branson Instruments, Inc., Stamford, Conn.). The temperature of the sonic preparation did not rise above 40 C during the process. After rupture, the suspension was centrifuged at 600 $\times$ *g* for 20 min, and the supernatant fluid was decanted. Differential centrifugations were repeated until phase-contrast microscopic examination of the supernatant fluid demonstrated that no intact cells remained. The suspended walls were centrifuged at 12,000 $\times$ *g* for 20 min and suspended in an aqueous solution containing 0.5 mg of trypsin per ml. The trypsin solution containing cell walls was adjusted to *p*H 8.0 with dilute sodium hydroxide and incubated for 2 hr at 37 C. The walls were recovered by centrifugation and, after washing, the trypsin treatment was repeated. Amino acid analysis of the hydrolyzed walls served as the criterion of wall purity. Hydrolysis was carried out in 6 N HCl for 20 hr at 100 C, followed by evaporation of the HCl under vacuum. The hydrolyzed material was resuspended in water and assayed by thin-layer chromatography on Kieselgel G-HR (Macherey Nagel and Co., Westbury, N.Y.). The plates were developed in a chloroform-methanol-17% ammonia solution (40:40:20). Amino acids were detected by spraying with 0.3% (w/v) ninhydrin in *n*-butanol plus 3% glacial acetic acid (v/v), followed by heating at 110 C for 10 min. The hydrolysate contained significant quantities of glutamic acid, diaminopimelic acid, and alanine, plus a trace of a ninhydrin-positive material with an R_F greater than glutamic acid. No other amino acids were detected in the hydrolysate. The walls were lyophilized and stored dry before assay.

RESULTS

Aflatoxin and growing cells. Cells of *F. aurantiacum* grown in the presence of aflatoxin developed aberrant forms (Fig. 1). The most striking morphological change was the enhanced length of the cells. In addition, ends of the filamentous cells were swollen and often branched. This response was similar to that reported by others for the action of inhibitors of cell wall synthesis (8, 10, 11). *F. aurantiacum* grown with penicillin (20 units/ml) also produced aberrant forms similar to those observed with aflatoxin. Because the aberrant forms produced in the presence of aflatoxin made cell counts unreliable, DNA assays were employed as the measurement of growth (Fig. 2); 10 and 15 μg/ml of aflatoxin B_1 inhibited DNA production by 73 and 96%, respectively (relative to the control), after 28 hr of growth.

The growth-inhibiting effect of aflatoxin suggested that the toxin was taken up by the sensitive cells. Analysis of the toxin concentration in the culture medium during growth demonstrated that aflatoxin was removed by developing cells.

The aberrancy of the cells grown in the presence of aflatoxin suggested that the cell walls were structurally weakened. Therefore, the relative fragility of cells grown in the presence and in the absence of aflatoxin B_1 was determined (Table 1). The absorbancy at 540 mμ was used to determine the number of cells in a test sample, and the supernatant fluids were examined at 260 and 280 mμ for release of nucleic acid and protein into solution during the sonic treatment. Microscopic examination of the cells before and after treatment showed that approximately the following percentages of cells were ruptured by sonic treatment: control, 15%; 2.5 μg/ml of B_1, 25%; 5.0 μg/ml of B_1, 35%. No aberrant cellular forms were observed in the 2.5 μg/ml concentration of aflatoxin B_1 and only a few in 5.0 μg/ml. The data indicated that the cells grown in toxin were more susceptible to sonic rupture than were the controls. These observations provide evidence that the toxin interferes with the development of a normal cell wall.

Removal of aflatoxin by resting and autoclaved cells. Incubation of 10^{11} resting cells per ml with 7.0 μg/ml of aflatoxin B_1 during a 4-hr period facilitated complete toxin removal from the assay system (Table 2). The nature of the toxin removal was examined further by addition of various numbers of resting cells to an aflatoxin solution, followed by toxin assay after 5, 60, and 240 min of incubation at 30 C (Table 3). Addition of cells beyond the level that rapidly removed about 75% of the aflatoxin did not immediately lower further the toxin concentration. However, extended periods of incubation facilitated complete toxin removal by living cells.

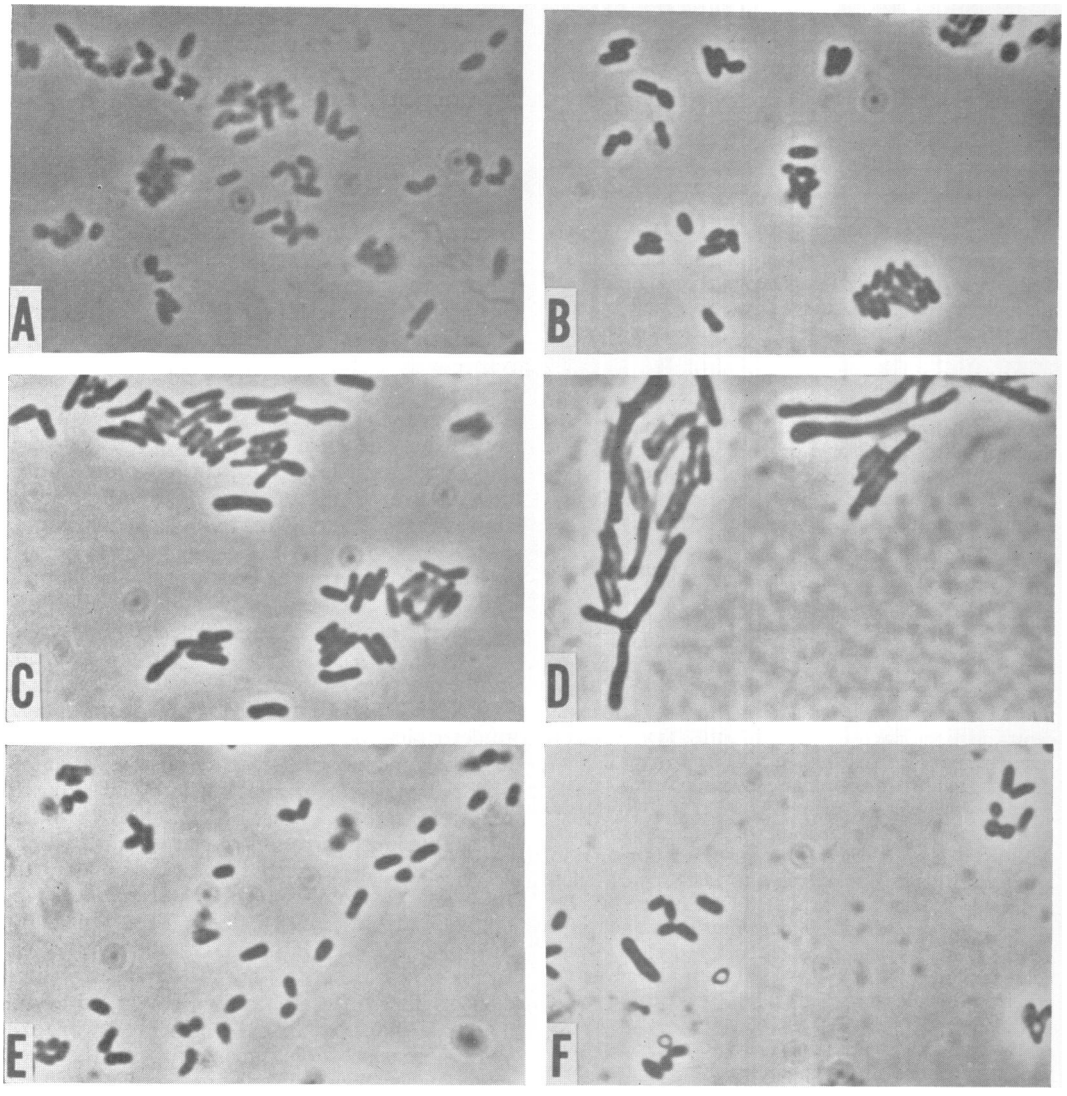
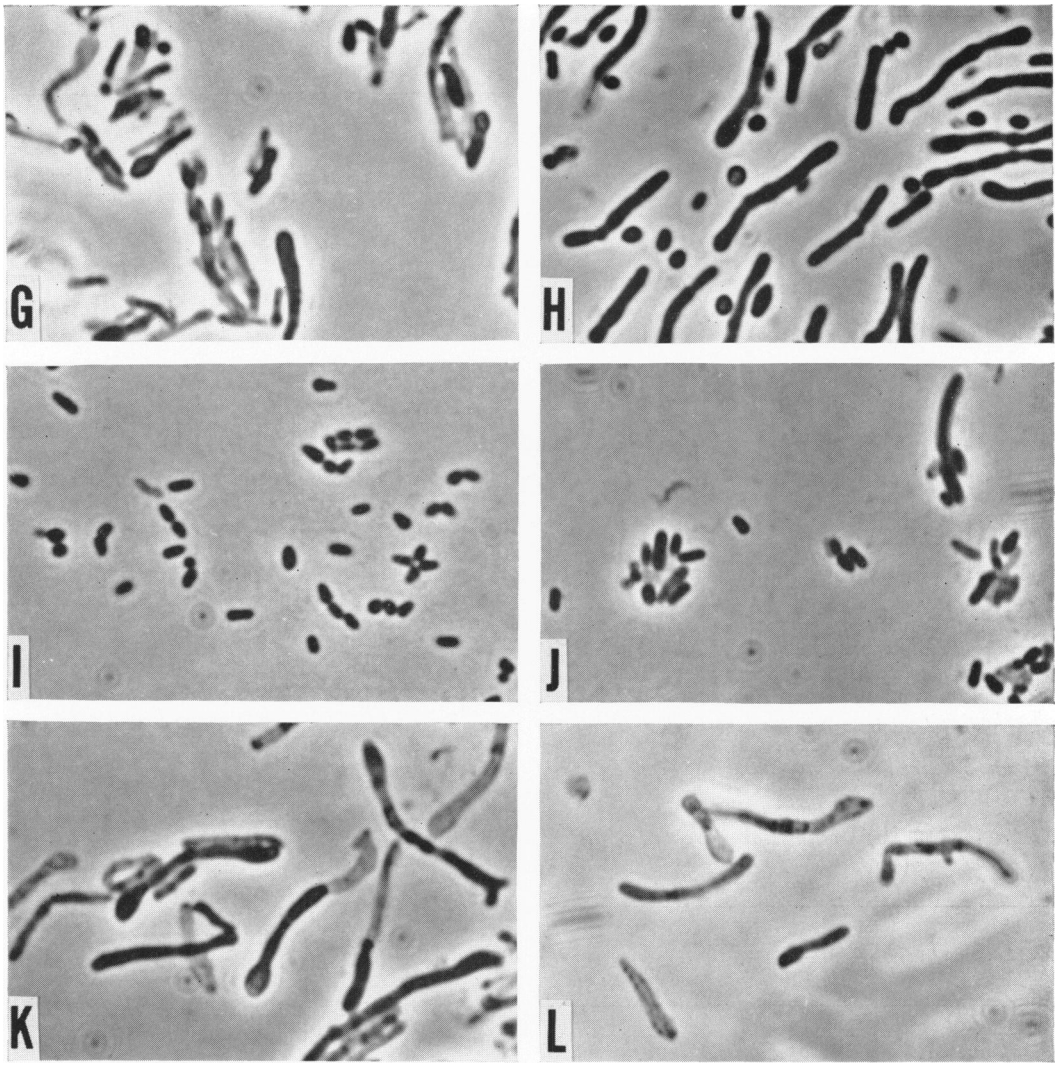


FIG. 1. *Aberrant forms of Flavobacterium aurantiacum grown in 50 ml of culture medium on a rotary shaker at 30 C with the following conditions: (A) 24 hr, control; (B) 24 hr, 10 μg/ml of B_1; (C) 24 hr, 20 μg/ml of B_1; (D) 24 hr, 100 μg/ml of B_1; (E) 48 hr, control; (F) 48 hr, 10 μg/ml of B_1; (G) 48 hr, 20 μg/ml of B_1; (H) 48 hr, 100 μg/ml of B_1; (I) 72 hr, control; (J) 72 hr, 10 μg/ml of B_1; (K) 72 hr, 20 μg/ml of B_1; (L) 72 hr, 100 μg/ml of B_1.*

Concomitant studies with autoclaved cells showed that such preparations were also capable of removing aflatoxin from a liquid medium. Addition of autoclaved cells to an aflatoxin solution removed approximately the same percentage of toxin as did comparable numbers of viable cells (Table 3). However, the autoclaved cells were not able to continue toxin uptake during longer incubation periods.

The nature of the initial fractions of aflatoxin removed by cells was investigated in greater detail. Tests were carried out in which a second sample of aflatoxin was added to an assay system containing an excess number of cells that had previously been exposed for a brief period of time to an initial quantity of aflatoxin (Table 4); approximately the same percentage of toxin was removed from the second sample as from the first. Since both living and autoclaved cells could remove only a fixed amount of aflatoxin without



an extended incubation period, it appeared that an equilibrium was established between the cells and the toxin or that the aflatoxin B_1 might be present in more than one form.

Essentially all of the aflatoxin B_1 removed from solution by autoclaved cells could be recovered by washing the cells several times with water. However, aflatoxin removed by living cells could not be recovered by any of the following procedures: washing the cells with water, sonic treatment and subsequent washing of the ruptured cells with water, or extraction of either intact or ruptured cells with chloroform. In addition, aflatoxin bound by viable cells could not be recovered if the cells were autoclaved after the incubation period and were then washed, ruptured by sonic treatment, and extracted with chloroform. Therefore, nonrecoverable uptake of aflatoxin B_1 appears to require living cells.

Cells that were first starved for 20 hr prior to the assay removed aflatoxin to the same extent as did cells that were freshly harvested from the growth medium. Addition of 1% glucose to the assay systems did not affect B_1 uptake, nor did addition of 0.01 M azide or 1,000 units of penicillin per ml of test solution interfere with toxin removal by resting cells. These tests showed that an exogenous energy source does not affect aflatoxin removal, and it seems unlikely that growth supported by endogenous substrates is involved in toxin removal. Furthermore, since penicillin does not compete with aflatoxin removal, it appears that the two compounds initially bind to the cell at unique loci.

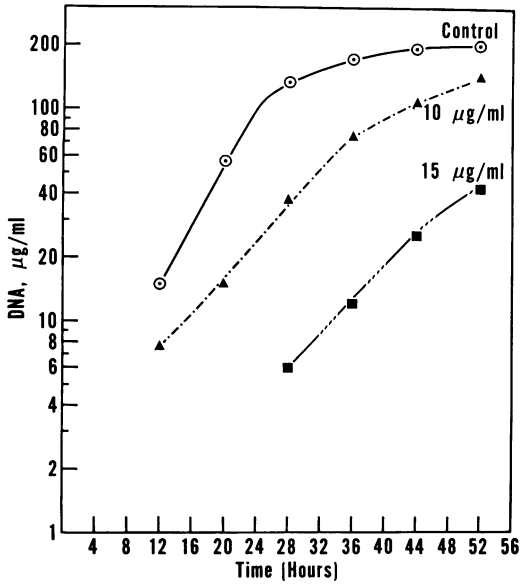


FIG. 2. *Inhibition of growth of Flavobacterium aurantiacum by aflaxotin B_1. Growth determined by measurement of DNA of cells in 1-ml samples of culture medium removed at designated times. The diphenylamine technique was used to quantitate colorimetrically the DNA measured at 600 mμ. A 50-ml amount of growth medium initially inoculated with 6.0 × 10^8 cells.*

TABLE 1. *Effect of subinhibitory levels of aflatoxin B_1 on fragility of cell walls of Flavobacterium aurantiacum*[a]

Aflatoxin B_1	Absorbancy[b]		
	540 mμ	260 mμ	280 mμ
μg/ml			
Control	0.25	0.17	0.12
2.5	0.17	0.26	0.16
5.0	0.11	0.33	0.24

[a] Cells were harvested after 40 hr of culture on a rotary shaker at 30 C in the designated aflatoxin concentrations. Equal numbers of washed cells (4.0 × 10^{10}) from each growth condition were suspended in 20 ml of water and subjected to sonic disintegration for identical periods of time (4 min).

[b] The number of cells was determined by measurements of a 1:10 dilution of the ruptured material at 540 mμ. After centrifugation at 8,000 × *g* for 20 min, a 1:25 dilution of the supernatant liquid from the ruptured preparation was assayed at 260 and 280 mμ for nucleic acid and protein.

The uptake of aflatoxin B_1 by resting cells was temperature-sensitive, with maximal removal occurring at 35 C (Fig. 3). The rate of aflatoxin

TABLE 2. *Removal of aflatoxin B_1 from an aqueous solution by resting cells of Flavobacterium aurantiacum*[a]

Incubation time	Aflatoxin B_1[b]	
	Assay solution	Amt removed
hr	*μg/ml*	%
0	7.0	0
1	1.1	84
2	0.5	93
3	0.2	97
4	0.0	100

[a] The tests were carried out in 60 ml of 0.67 M phosphate buffer (*p*H 6.75) containing 1.0 × 10^{11} cells per milliliter in 300-ml Erlenmeyer flasks on a rotary shaker at 30 C.

[b] The values are means of five observations. The 0-hr determination was made prior to addition of cells and was corrected for subsequent dilution. The 1- through 4-hr measurements were made on the supernatant fractions of 5-ml samples of the test suspensions. Cells were sedimented by centrifugation.

TABLE 3. *Removal of aflatoxin B_1 from an aqueous solution by various levels of living and autoclaved Flavobacterium aurantiacum cells*

Incubation time[a]	No. of cells[b] (× 10^{12})	Type of cells	Aflatoxin removed[c]
min			%
5	0.5	Living	49
		Autoclaved	38
5	1.4	Living	61
		Autoclaved	55
5	3.0	Living	71
		Autoclaved	72
5	4.2	Living	73
		Autoclaved	72
60	4.2	Living	85
		Autoclaved	73
240	4.2	Living	100
		Autoclaved	73

[a] The 5-min period is the approximate time required to add the cells to the aflatoxin solution, mix, and sediment the cells by centrifugation. The 60- and 240-min incubations were carried out in 300-ml Erlenmeyer flasks on a rotary shaker at 30 C.

[b] Cells in 10 ml of 0.067 M phosphate buffer (*p*H 6.75) added to 20 ml of 0.067 M phosphate buffer (*p*H 6.75) containing 500 μg of aflatoxin B_1.

[c] Total aflatoxin of the cell-free system measured before and after addition of cells.

TABLE 4. *Removal of aflatoxin B_1 from two consecutive samples by Flavobacterium aurantiacum*

Sample[a]	Aflatoxin B_1[b]		
	Before addition of cells	After addition of cells	Amt reomoved
	μg	μg	%
1	520	150	71
2	300	80	73

[a] Sample 1 consisted of 40 ml of 0.067 M phosphate buffer (*p*H 6.75) containing 4.0×10^{12} cells and aflatoxin B_1. Immediately after adding the cells to the test solution, a sample was removed, the cells were sedimented by centrifugation, and the aflatoxin concentration of the supernatant liquid was measured. The aflatoxin of sample 2 in 20 ml of 0.067 M phosphate buffer (*p*H 6.75) was added to sample 1 immediately after initial sampling followed by cell sedimentation and aflatoxin assay.

[b] The aflatoxin value of sample 2 corrected for the 150-μg quantity carried over from the first sample.

removal by resting cells was also sensitive to the *p*H of the incubation medium, with a maximum rate at *p*H 6.75 (Table 5).

Removal of aflatoxin by cell walls. Addition of increasing amounts of a cell wall preparation to an aflatoxin solution showed that the toxin was bound to the walls (Table 6). Furthermore, about 75% of the B_1 was all that could be removed, regardless of how much cell wall material was added. This action was analogous to the initial removal of toxin by living or autoclaved cells. The binding of toxin by the cell wall preparation was similar to that observed with autoclaved cells, since the toxin could be recovered by successive washings with water.

Effect of ruptured-cell preparations on aflatoxin uptake. Ruptured-cell preparations were tested for their ability to remove or modify aflatoxin. The method for preparation of cell walls was also used for the cell extracts; an ice bath maintained the temperature of the extract below 30 C during the sonic treatment. Microscopic examination of the material revealed that all of the cells had been ruptured. Addition of the entire treated preparation to an aflatoxin solution, followed by sampling and assay every 15 min during a 2-hr incubation period, showed that part of the aflatoxin was removed by the cellular debris associated with ruptured cell walls (the fraction sedimented at 6,000 × *g* for 20 min). However, the toxin removed by the walls could be recovered by washing.

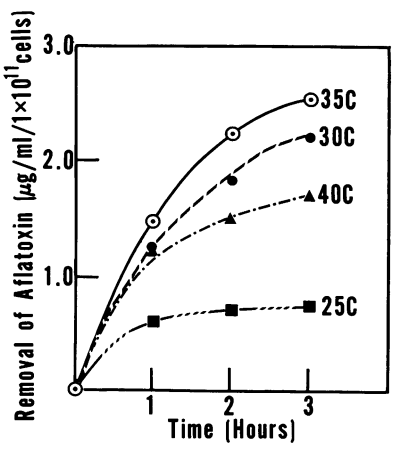


FIG. 3. *Temperature effect on aflatoxin B_1 removal by living cells of Flavobacterium aurantiacum. Tests were carried out in 60 ml of 0.067 M phosphate buffer (pH 6.75) containing 2.5 μg of aflatoxin B_1 per ml and 10^{11} cells per milliliter. The incubations were carried out in 300-ml Erlenmeyer flasks on a rotary shaker. The concentration of aflatoxin at zero-time was based on the amount present in the supernatant fraction after addition of the cells, immediate mixing, and sedimentation of the cells by centrifugation.*

DISCUSSION

Cells of *F. aurantiacum* grown in subinhibitory concentrations of aflatoxin B_1 develop aberrant forms and are more susceptible to sonic rupture than are control cells. We believe the toxin interferes with synthesis of the cell wall. This toxic action can be related to the uptake of B_1 by the cells because the concentration of aflatoxin in the medium decreases during development of the organism. Autoclaved cells and cell wall preparations remove a portion of the B_1, but the toxin can be completely recovered from the dead cells and cell walls, whereas toxin removed by living cells cannot be extracted from the cells. This binding is somewhat similar to that reported by Best and Durham (5) for the binding of vancomycin to *Bacillus subtilis* walls. However, they reported that the initial reversible binding to the walls becomes more irreversible when the antibiotic is incubated with the walls for longer periods of time. This condition was not observed with the binding of aflatoxin to either autoclaved cells or walls of *F. aurantiacum*. Rapid initial uptake of toxin was observed with both living and nonliving cells. However, nonextractable uptake occurred only with viable cells. If the primary reaction of the living cells is identical to that of autoclaved cells or walls, then the adsorbed toxin must subsequently be modified by a process mediated only by viable

TABLE 5. *Effect of pH on initial rate of aflatoxin B_1 removal by resting cells of Flavobacterium aurantiacum*

pH[a]	Initial rate (μg per ml per hr)
3.0	0.2
4.0	0.3
5.0	0.6
6.0	1.1
6.75	1.3
8.0	0.8

[a] Tests were carried out in 60 ml of 0.067 M phosphate citrate buffer which maintained a constant *p*H during the assay. The assay medium contained 2.5 μg of aflatoxin B_1 per ml and 10^{11} cells per milliliter. The incubations were carried out in 300-ml Erlenmeyer flasks on a rotary shaker at 30 C. The concentration of aflatoxin at zero-time was based on the amount present in the supernatant fraction immediately after addition of the cells and sedimentation of the cells by centrifugation.

TABLE 6. *Removal of aflatoxin B_1 from an aqueous solution by cell walls of Flavobacterium aurantiacum*

Cell walls[a] (mg, dry wt)	Aflatoxin B_1 removed	
	Amt	Per cent
	mg	
260	0.77	35
520	1.60	72
780	1.68	76
1,300	1.70	77

[a] Successive addition of 2-ml samples of cell walls to an initial 15-ml volume of water containing 2.2 mg of B_1. Aflatoxin concentration of supernatant liquid was assayed after sedimentation of cell walls by centrifugation.

cells. The nonextractability observed with living cells suggests that the toxin might be metabolically degraded. However, ruptured-cell preparations were not capable of modifying the toxin. These observations imply that aflatoxin is initially loosely bound to the periphery of the cells, possibly by hydrogen bonds or van der Waals forces. The nonextractable uptake of the toxin is somewhat slower and depends on a living cell.

It is also possible that aflatoxin uptake by living cells and dead cells may be an unrelated process. Thus, the toxin initially removed might or might not be nonextractably removed by the living cells at the outset. However, since ruptured cells do not change the toxin, it appears that the nonextractable removal of aflatoxin requires the architecture of the intact cell. The fate of the toxin after uptake is under investigation.

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