Comparative Studies on the Detoxification of Aflatoxins by Sodium Hypochlorite and Commercial Bleaches

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Cultures of Aspergillus flavus and aflatoxins were destroyed by a commercial bleach (Clorox; active ingredient, NaOCl) or analytical reagent grade NaOCl at $7.0 \times 10^{-3}$ M NaOCl in 5 days. Addition of Clorox or NaOCl at $2.8 \times 10^{-3}$ M to the fungal growth medium prior to inoculation completely inhibited the fungal growth. Aflatoxin production was inversely proportional to the logarithm of NaOCl concentration and time of treatment. Clorox and NaOCl were equally effective on aflatoxins, but fungal cells were lysed more readily by Clorox than by NaOCl. Mycelia older than 8 days lysed more readily than younger ones. Most conidia survived concentrations below $1.4 \times 10^{-3}$ M. The lowest effective concentration for a 2-hr treatment was $8.8 \times 10^{-3}$ M which is well below the Clorox concentration recommended for routine laboratory decontamination of aflatoxins. Mice and rats injected with aflatoxins and aflatoxins incompletely destroyed by Clorox died within 72 hr and had typical liver and kidney damage caused by aflatoxins. However, animals injected with NaOCl or Clorox or Clorox-destroyed aflatoxin extracts survived and showed no obvious liver or kidney damage.

It has been well established that aflatoxins are the most potent naturally occurring mycotoxins that possess carcinogenicity to many warm-blooded animals, and they are considered potential threats to human health (1, 5, 18, 22, 26, 27). The toxigenic strains of Aspergillus flavus, A. parasiticus, and A. fumigatus, etc., have been frequently detected and characterized from many food commodities and agricultural crops (1, 2, 5, 14, 15, 20, 21, 25, 26, 29). Although several chemical agents have been suggested to be effective in destroying aflatoxins (9), in-depth studies about their effectiveness and mechanisms of action against these carcinogens are not yet available. To date, the most common and convenient treatment practiced in laboratories heavily engaged in aflatoxin research is the one that was recommended in 1965 by Fischbach and Campbell (11) and by Stoloff and Trager (24). In this procedure, household bleach, which contains 5 to 6% NaOCl, is applied to decontaminate surfaces of lab equipment, work areas, and personnel. In general, this treatment has proved very effective. However, the high concentration of bleach employed in this procedure often causes difficulties in toxin assays and skin injuries to personnel. Although a one-tenth dilution of bleach was recommended in the original procedure for “general dishwashing” in order to minimize skin irritation, the effectiveness of the diluted bleach in the destruction of aflatoxins as well as its direct effect upon the toxigenic fungi was not investigated. The purposes of the present study were to determine: (i) whether an aflatoxigenic strain of A. flavus was capable of producing aflatoxins after various treatments with bleach; (ii) at what levels of concentration of bleach treatment was no longer effective; and (iii) whether the destructive effect of household bleach was in any way comparable to that of the pure chemical, sodium hypochlorite (NaOCl).

MATERIALS AND METHODS

Culture. Cultures of A. flavus Fc1, an isolate bearing close morphological resemblance to type culture A. flavus NRRL 2999, were used throughout this study. This particular isolate was chosen because it emits, under long ultraviolet (UV) exposure at 365 nm, strong, characteristic fluoroescences, i.e., bluish when grown on Czapek agar and dull green when
grown on yeast extract agar (YES). All cultures were maintained at 4 C on either standard Czapek or YES agar slants.

**Preparation of inoculum.** Cultures were grown on YES containing 2% yeast extract and 5% sucrose (28) for 5 days at 26 C. This is a modification of the YES medium originally developed by Davis, Diener, and Eldridge (7). Spores from mature colonies were aseptically collected into a sterile solution of 0.02% Tween-20 and 1% NaCl. After gentle shaking, samples of spore suspensions were counted with a Coulter type B electron particle counter (23) with upper and lower threshold settings of 50 and 20, respectively, in order to accommodate the small size of the conidia of A. flavus. The trace amounts of contamination from NaCl and Tween-20 inherited from the counting solution had no effect on either fungal growth or subsequent production of aflatoxins. Liquid cultures were started by inoculating 2 ml of spore suspension containing an average of 5.6 x 10^9 conidia/ml into 125-ml long-neck Erlenmeyer flasks which contained 20 ml of sterile YES medium per flask.

**Clorox and NaOCl treatments.** Cultures were treated with either commercial Clorox or pure NaOCl. These experiments can be conveniently grouped into three categories. (i) **Effect of the concentration of NaOCl.** Five-day-old cultures were treated for a duration of 10 min with eight different concentrations of NaOCl ranging from 3.5 x 10^-2 M to 3.5 x 10^-4 M. (ii) **Effect of length of treatment.** Five-day-old and 12-day-old cultures were treated with 2.1 x 10^-4 M NaOCl and Clorox, respectively, for five different time exposures ranging from 120 to 1 min. (iii) **Effect of treatment commenced at various stages of fungal growth.** As many as seven concentrations of NaOCl or Clorox were used to treat the cultures as follows: (i) the additives were added to the growth media before inoculation, and fungal dry weight and aflatoxins were determined 5 days after inoculation; (ii) the additives were introduced to the cultures 4 days after initiation of growth, and assays of dry weights and toxins were made at the end of an additional 4-day period of growth; and (iii) commercial Clorox was introduced into cultures at the end of 8 days of growth, and assays were made at 24 and 48 hr thereafter.

**Assays of aflatoxins, fungal dry weights, and biological activity.** (i) **Aflatoxins.** The procedure used for the extraction of aflatoxins was that of Lee (17) with a minor modification to suit this study. A chloroform to fungal filtrate, volume ratio of 4 to 1 was adopted for preparation of aflatoxin extracts from the fungal cultures. Both fungal mycelia and culture filtrates were extracted together. UV spectrophotometric and thin-layer chromatographic (TLC) analyses were used for characterization of aflatoxins. Spectrophotometric analysis of UV absorption patterns and approximation of amounts of aflatoxin produced in each of the fungal extracts were made with a Cary model 15 ratio-recording spectrophotometer. In general, the molar extinction coefficient (ε) of aflatoxin B₁ in chloroform, which is 23,015 at a wavelength of 363 nm, and a 1-cm light path were used to approximate total aflatoxin production. This method of approximation for the crude aflatoxin extracts is similar to the quantitative estimation method by applying co-chromatography with aflatoxin standards on TLC gel plates. TLC analyses of the aflatoxin extracts were performed by using a standard Desaga one-dimentional ascending technique with a solvent system of chloroform and acetone at 4 to 1 ratio on Adsorbil-1 gel plates. The gel plates were heated at 115 C for 30 min before spotting to render a good separation of the aflatoxin components. To obtain a 15-cm solvent front, a development time of 45 min was necessary. Detection of the aflatoxin components was made by viewing through a Desaga UV lamp at 366 nm, and the chromatograms were photographed for record keeping.

(ii) **Fungal dry weights.** After each treatment, fungal masses were harvested from the chloroform-extracted cultures and dried at 95 C until constant weights were obtained. All fungal weights reported are the average of three replicates.

(iii) **Biological toxicity.** To determine the toxicity of the treated fungal cultures in comparison with that of aflatoxin controls, mice and rats were injected intraperitoneally with typical and atypical aflatoxin extracts. After the animals were killed by the toxins, their lungs, livers, kidneys, and brains were examined for injury.

**RESULTS**

Because of the close similarity in UV absorption spectra and chemical and biological properties of the individual aflatoxin components, no attempt was made to quantify the separate components of the aflatoxin mixtures obtained from the treatments (Fig. 1). However, the aflatoxin extracts were characterized with UV spectrophotometry and conventional TLC at each step in the experiments.

**Effect of pure NaOCl.** The results presented in Fig. 2 show that fungal mass and aflatoxin production are inversely proportional to the logarithm of NaOCl concentrations. At a NaOCl concentration of 3.5 x 10^-2 M, both aflatoxin and fungal mass were completely destroyed. At lower concentrations of NaOCl, aflatoxins were reduced more effectively than fungal mass.

**Effect of pretreatment of the growth medium.** As shown in Table 1, five days after pretreatment of the medium with either commercial Clorox or pure NaOCl at 2.8 x 10^-2 M, total inhibition of both fungal growth and aflatoxin production was achieved. However, at 2.1 x 10^-3 M, only commercial Clorox completely inhibited both fungal growth and aflatoxin production. Although the inhibitory effects on aflatoxin production by pre-treatment with both agents were similar, there was less
VOL. THAN REDUCTION FLAVUS FC, CULTURES AFLATOXINS OF GROWTH.

D-PURIFIED 0.4 0 0.1 - 0.8 0.2 - 4 0 0.909 WERE 0.7 - PURIFIED 0.6 - 0.3 0.8 0.1 - 0.3 500 5000 0.4 11 200 2000 260 360 0.1 - 0.1 0.0 - 0.0 200 2000 24, 1972 2.1, 2000 U.

FIG. 1. Typical UV absorption spectral patterns of aflatoxin individual components and complex dissolved in chloroform.

FIG. 2. Effect of the addition of different amounts of pure NaOCl to 5-day-old cultures of Aspergillus flavus FC, for a 10-min exposure on the production of aflatoxins and fungal dry mass.

Effect of Clorox or NaOCl on 8-day-old cultures treated 4 days after the initiation of growth. When additions of either Clorox or NaOCl were made 4 days after the initiation of fungal growth, and 4 additional days of growth were allowed, the results were somewhat different from the previous experiment with pretreatment. The data from these treatments are presented in Table 2. Both aflatoxins and fungus were completely destroyed by treatment with Clorox at 2.1 x 10^-3 M. Although treatment with pure NaOCl completely destroyed aflatoxins at 3.5 x 10^-3 M, the fungal mass was 38% of the control. In general, somewhat greater destruction of aflatoxins resulted from treatment with commercial Clorox than from treatment with pure NaOCl.

**Effect on 8-day-old cultures 24 and 48 hr after treatment with commercial Clorox.**

The data obtained from these experiments are shown in Table 3. A 24-hr treatment of Clorox at 3.5 x 10^-4 M on the 8-day-old fungal cultures resulted in a 7% reduction in aflatoxins (Table 3). However, about the same amount of fungal mass was destroyed as in the younger cultures at the same Clorox concentration (Table 1). When the data in Table 3 are compared to those of Tables 1 and 2, it is apparent that aflatoxins formed in the older cultures were more resistant

| Treatment concn (M) | Aflatoxin (µg) | Fungal mass (mg) |
|--------------------|---------------|-----------------|
| Clorox 2.8 x 10^-3 | 0             | 0               |
| Clorox 2.1 x 10^-3 | 0             | 0               |
| Clorox 1.4 x 10^-3 | 400           | 100             |
| Clorox 7.0 x 10^-4 | 3,400         | 400             |
| Clorox 3.5 x 10^-4 | 4,100         | 500             |
| Clorox 0          | 5,600         | 600             |

**TABLE 1. Effects of pretreatment of growth medium with commercial Clorox or NaOCl on aflatoxin production and fungal growth of 5-day-old cultures of Aspergillus flavus**

| Treatment concn (M) | Aflatoxin (µg) | Fungal mass (mg) |
|--------------------|---------------|-----------------|
| Clorox 7.0 x 10^-3 | 0             | 0               |
| Clorox 3.5 x 10^-3 | 0             | 0               |
| Clorox 2.8 x 10^-3 | 0             | 0               |
| Clorox 2.1 x 10^-3 | 0             | 0               |
| Clorox 1.4 x 10^-3 | 3,100         | 400             |
| Clorox 7.0 x 10^-4 | 4,700         | 500             |
| Clorox 3.5 x 10^-4 | 4,900         | 500             |
| Clorox 0          | 5,600         | 500             |

**TABLE 2. The response of aflatoxin yields and fungal growth in 8-day-old cultures of Aspergillus flavus treated with commercial Clorox or NaOCl after 4 days of growth**

| Treatment concn (M) | Aflatoxin (µg) | Fungal mass (mg) |
|--------------------|---------------|-----------------|
| Clorox 7.0 x 10^-3 | 0             | 0               |
| Clorox 3.5 x 10^-3 | 0             | 0               |
| Clorox 2.8 x 10^-3 | 0             | 0               |
| Clorox 2.1 x 10^-3 | 0             | 0               |
| Clorox 1.4 x 10^-3 | 3,100         | 400             |
| Clorox 7.0 x 10^-4 | 4,700         | 500             |
| Clorox 3.5 x 10^-4 | 4,900         | 500             |
| Clorox 0          | 5,600         | 500             |
to destruction by Clorox than those in the younger cultures. However, the fungal cells of the older cultures were more readily lysed than those of the younger cultures. This is particularly evident with treatments at lower concentrations of Clorox. Cultures that were many months old were generally easily lysed by either commercial Clorox or NaOCl.

**Effect of time of treatment.** Effect of time of treatment results are presented in Fig. 3. Although slightly greater reduction of aflatoxins resulted from treatment with Clorox than from treatment with NaOCl, the patterns of decline in the production of aflatoxins were very similar. A 1-min treatment with either agent resulted in almost a 30% reduction in aflatoxins. However, complete destruction of aflatoxins was not obtained with these treatments in less than 2 hr. Again, treatment with Clorox was much more effective in destroying fungal cells than was treatment with NaOCl. Nevertheless, for a 2-hr period, only 60% of the fungal cells were lysed by Clorox. At least 4 hr were required to complete the destruction of aflatoxins, and additional time was necessary to complete the lysis of fungal cells at this concentration. It was also noted that aflatoxins extracted after a 2-hr treatment with either agent did not change appreciably in their characteristics (Fig. 5).

As depicted in Fig. 4, the UV absorption curves of aflatoxins extracted from cultures treated with NaOCl or Clorox were atypical. No absorption spectra were detected with extracts from cultures treated with these agents at concentrations greater than \(8.8 \times 10^{-3}\) M. Curve A represents extracts from cultures treated at \(8.8 \times 10^{-3}\) M. It shows a complete loss of the major peak at 363 nm. However, it still shows a peak near 240 nm. Curve B represents extracts from cultures treated at \(3.5 \times 10^{-3}\) M or \(2.1 \times 10^{-3}\) M for 2 hr or longer. The major peak at 363 nm was drastically reduced, but a pronounced peak near 240 nm occurred. Curve C, which represents extracts from cultures treated at \(1.4 \times 10^{-3}\) M, shows a close similarity in its UV absorption pattern to that of the control, curve D.

When chloroform extracts of the cultures treated with NaOCl or Clorox were co-

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**Table 3. Aflatoxin yields and fungal mass remained in 8-day-old cultures of Aspergillus flavus 24 and 48 hr after commercial Clorox treatments**

| NaOCl (M)   | Aflatoxins after: | Fungal mass after: |
|------------|-------------------|-------------------|
|            | 24 hr (µg) | 48 hr (µg) | 24 hr (mg) | 48 hr (mg) |
| 3.5 × 10^{-2} | 0 | 0 | 0 | 0 |
| 1.8 × 10^{-2} | 100 | 0 | 300 | 200 |
| 8.8 × 10^{-3} | 400 | 300 | 400 | 300 |
| 3.5 × 10^{-3} | 2,400 | 2,000 | 400 | 400 |
| 3.5 × 10^{-4} | 4,900 | 4,100 | 400 | 400 |
| 0 | 5,300 | 5,400 | 500 | 600 |

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**Fig. 3.** Effect of different time exposures of \(2.1 \times 10^{-3}\) M NaOCl or the equivalent of Clorox to 12-day-old cultures of Aspergillus flavus Fc3 on the production of aflatoxins and fungal dry mass.

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**Fig. 4.** UV absorption spectral patterns of NaOCl- or Clorox-treated and untreated aflatoxins dissolved in chloroform. Concentrations of NaOCl or Clorox used for treatments: A, heavily, \(8.8 \times 10^{-3}\) M and above; B, moderately, \(3.5 \times 10^{-3}\) M; C, slightly, \(1.4 \times 10^{-3}\) M; and D, control (untreated).
chromatographed with untreated control extracts of aflatoxins and individually purified aflatoxin mixtures (B<sub>1</sub>; B<sub>2</sub>; G<sub>1</sub>; G<sub>2</sub> mixed at 1:1:1:1), only slight differences were detected among the fluorescent spots on the Adsorbil-1 TLC gel plates. Composite schematic patterns of TLC development are presented as Fig. 5 to illustrate this comparison. All extracts chromatographed, except group B in which complete destruction occurred, developed the four major aflatoxin components, B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>. Group C, extracts from cultures treated with 8.8 × 10<sup>-3</sup> M Clorox, produced a blue fluorescent spot that was not present in the control, group A. It developed close to the solvent front with a R<sub>f</sub> × 100 value of 72. Group D, which includes extracts from cultures that received 3.5 × 10<sup>-2</sup> M or 2.1 × 10<sup>-3</sup> M NaOCl for at least 2 hr, produced a small, purplish fluorescent spot with a R<sub>f</sub> × 100 value close to 5 that was not present in group A. Group E, extracts treated with low concentrations of Clorox or NaOCl (1.4 × 10<sup>-3</sup> M or below), developed two unusual fluorescent spots, one bluish with an R<sub>f</sub> × 100 value close to 22 and the other brownish with an R<sub>f</sub> × 100 value close to 8.0. that were not present in group A. The unusual fluorescent spots detected in these experiments could well be the degradation or reaction products of normal aflatoxins with sodium hypochlorite. However, they occurred in very minute amounts, and further chemical characterization of them was not possible.

**Biological toxicity.** Young, male, white Swiss-Webster mice injected intraperitoneally with aflatoxins extracted from cultures treated with Clorox showed typical toxic responses similar to those of controls. Injection of mice with single-dose concentrations of aflatoxin extract equivalent to 2.1 and 1.8 mg/kg of B<sub>1</sub> killed the animals in 48 and 72 hr, respectively. Lower doses permitted some of the mice to survive for somewhat longer periods of time. As observed by others (4, 8, 16, 18, 19), liver damage and hemorrhage were the principal effects. Kidney damage was also observed in this study. Lesions induced by control aflatoxins, or by aflatoxins from cultures treated with either NaOCl or Clorox below concentrations of 8.8 × 10<sup>-2</sup> M, showed a periportal zone of necrosis which developed during a 3-day period after dosing. This was accompanied by a marked biliary (bile duct) proliferation.

Also, mice were killed by injections with extracts from cultures treated with NaOCl or Clorox at concentrations greater than 8.8 × 10<sup>-3</sup> M. Neither liver nor kidney damage was observed in this case. Injection with pure NaOCl or Clorox alone did not kill mice even after prolonged periods of dosing. When male, adult Sprague-Dawley rats were tested in the same manner, the results were similar to those obtained with mice. A somewhat higher dosage was required for rats. The acute toxicity of the aflatoxins on the young mice in this study was higher than that reported by other workers from toxin diet feeding experiments (16, 18). This discrepancy might well be due to the difference in the age of animals and route of toxin administration. Comparative studies on the chronic toxicity of sublethal doses of toxin extracts from cultures treated with NaOCl or commercial Clorox in various ways are now in progress.

**DISCUSSION**

The results confirm that high concentrations, 5 to 6% or 0.67 to 0.81 M, of NaOCl will completely destroy aflatoxins in a very short time (11, 24). Most of the fungal cells so treated were lysed within half a day at these concentrations. The results from experiments with additions of either NaOCl or Clorox into cultures at different stages of growth have clearly demonstrated that intact aflatoxins can be produced by A. flavus Fc<sub>5</sub>, especially from those cultures treated at low concentrations, i.e., 3.5 × 10<sup>-4</sup> M and below. Prolonged culturing of the fungus will eventually abolish whatever effect NaOCl or Clorox has on the fungus and aflatoxin production. There was not a great difference in the destruction of aflatoxins by treatment with
either NaOCl or Clorox. However, Clorox proved to be much more effective than NaOCl in destroying the fungus. This is particularly true with older mycelia. Could this be merely due to an overall weakening in fungal mycelia during the process of aging or, rather, is it the result of some synergetic reaction(s) brought about by chemically complexing certain fungal autolytic products with NaOCl in aged culture? Any answer must be merely conjectural at the present. Certainly, the possibility that ingredients in Clorox other than NaOCl which might contribute to greater effect on lysis of fungal cells cannot be ruled out. It is not surprising to find the resistance of aflatoxins exhibited against the low level treatment of NaOCl, because those carcinogens are chemically very stable and well known in their resistance to a host of physical and chemical treatments such as heat, UV and gamma radiation, as well as many other chemicals (6, 9, 10, 13, 28). The changes in UV absorption spectra in relation to concentrations of NaOCl and Clorox indicate the possibility of some kind of stepwise alteration in the molecular structure of the aflatoxins. The lowest effective concentration of Clorox against both fungus and aflatoxins was 3.5 $\times$ $10^{-3}$ M, which is about one-half the concentration of the one-tenth dilution of Clorox that has been recommended for routine use in decontamination. Therefore, a one to nine dilution of the Clorox should provide safe cleanup, provided it is done thoroughly.

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