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Mutagenicity of the Tetramic Mycotoxin Cyclopiazonic Acid

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Cyclopiazonic acid was shown to be mutagenic to *Salmonella typhimurium* TA98 and TA100 in the presence of metabolic activation. The activity of cyclopiazonic acid in the presence of aflatoxin B_1 was studied in complete factorial experiments with strain TA98. Both mycotoxins produced significant mutagenic activity singly and in combination. The activity in combination appeared to be additive rather than synergistic. The specific activity of cyclopiazonic acid was estimated to be approximately 140 revertants per μmol in strain TA98.

Cyclopiazonic acid (CPA) is an indole tetramic acid mycotoxin produced by species of *Aspergillus* and *Penicillium* (3–7, 9, 11). Each of the fungi known to produce CPA is widespread in nature or is used commercially (i.e., *Penicillium camemberti*) or both. Le Bars (6) has recently demonstrated CPA production in all of a series of 20 *P. camemberti* strains isolated from different brands of camembert cheese. Gallagher et al. (3) have reported that production of CPA occurs in *Aspergillus flavus* and is common among aflatoxigenic strains. Thus, there is the possibility of dual exposure of individuals to both aflatoxin (AF) and CPA simultaneously. The toxic and genotoxic properties of AF are well established (8, 10, 15). Although much less is known about the toxicity of CPA, Purchase (12) has reported that the 50% lethal dose of CPA in adult male rats is 2.3 mg/kg when administered intraperitoneally. This is comparable with the reported 50% lethal dose of 6.0 mg/kg for AF in the male rat administered by the same route (2).

Wehner et al. (14) included CPA among a group of 17 mycotoxins screened for mutagenic activity to four strains of *Salmonella typhimurium*. Although several of these mycotoxins, including AF B_1 (AFB_1), were mutagenic, CPA was not mutagenic with or without metabolic activation. Preliminary experiments in our laboratory disagreed with these findings. The objective of this report is to study the genotoxic potential of CPA in greater detail.

Mycotoxins. CPA was kindly provided by Richard J. Cole of the National Peanut Research Laboratory, Dawson, Ga. The purity of the CPA was determined to be 99% on the basis of its UV extinction coefficient at 284 nm. AFB_1 was purchased from Aldrich Chemical Co., Milwaukee, Wis. The mycotoxins were dissolved in dimethylsulfoxide.

Test strains. *S. typhimurium* Ames *his*$^-$ testers (TA98 and TA100) were used for detecting reverse mutations from histidine dependence to histidine independence by the plate incorporation test of Ames et al. (1).

Mutation assay. The procedures for the *Salmonella his*$^-$ reversion assay were similar to those described by Ames et al. (1). Briefly, 0.1 ml of overnight culture (TA98 or TA100), 0.1 ml of the mycotoxin solution(s) or dimethyl sulfoxide, and 0.5 ml of S9 mixture were added to molten soft agar. The soft agar was mixed and poured onto a bottom agar. Metabolic activation was accomplished with a 9,000 × *g* supernatant prepared from adult male Syrian golden hamster liver pretreated with Arochlor 1254. The S9 mixture was kindly provided by W.-Z. Whong of our laboratory (National Institute for Occupational Safety and Health).

Statistical analysis. Statistical analysis of the factorial experiments were carried out by analysis of variance and the general linear models procedure of the Statistical Analysis System (13). The Statistical Analysis System was also used to generate linear plots of the effects of AF and CPA alone, as well as plots of observed and expected effects of AF and CPA in combination.

The results of a typical experiment with CPA with *S. typhimurium* TA98 and TA100 are presented in Table 1. The correlation coefficients (Statistical Analysis System) between concentration and the number of revertants per plate were 0.974 and 0.898 with metabolic activation for TA98 and TA100, respectively ($P < 0.0001$ in each case). There was no correlation between concentration and mutagenic response in either strain without metabolic activation.

To study the combined effects of CPA and AF, factorial experiments were performed with five doses of each mycotoxin plus a negative control. Therefore, cells were exposed to each mycotoxin alone over a 100-fold dose range, as well as to all possible combinations of both mycotoxins together. Doses were chosen to fall within the linear portions of the dose-response curves based on preliminary experiments (data not shown). To simplify representation and analysis of data, dose levels are expressed in relative terms, i.e., 1.0,

TABLE 1. Mutagenicity of CPA in *S. typhimurium* TA98 and TA100 with (+S9) and without (−S9) metabolic activation

Concn (μmol/plate)	No. of revertants per plate[a]			
	TA98		TA100	
	+S9	−S9	+S9	−S9
Negative control (DMSO)[b]	25.3	19.7	221.0	147.7
Positive control (2AA)[c]	2,403.0		2,833.0	
0.01	27.7	18.7	156.3	137.0
0.03	26.3	14.7	169.7	143.7
0.1	33.7	18.7	203.7	163.4
0.3	51.0	18.0	256.0	162.7
1.0	137.7	16.0	305.3	134.0

[a] Average of three plates.
[b] DMSO, Dimethyl sulfoxide.
[c] 2-Aminoanthracene, 2.5 μg per plate.

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TABLE 2. Effect of CPA and AFB_1 alone and in combination on mutagenicity in *S. typhimurium* TA98 with metabolic activation

Treatment	No. of revertants per plate (mean ± SE)[a]					
	1.0[b] AF + 2.0 μmol of CPA	0.3 AF + 0.6 μmol of CPA	0.1 AF + 0.2 μmol of CPA	0.03 AF + 0.06 μmol of CPA	0.01 AF + 0.02 μmol of CPA	0.0 μmol of CPA
1.0[b] CPA + 30.0 pmol of AF	524.8 ± 38.0	360.0 ± 36.4	266.0 ± 12.8	482.8 ± 121.9	267.3 ± 6.0	243.0 ± 10.2
0.3 CPA + 9.0 pmol of AF	445.0 ± 4.9	236.0 ± 18.3	139.8 ± 12.1	111.5 ± 5.6	157.0 ± 40.6	100.0 ± 3.3
0.1 CPA + 3.0 pmol of AF	371.8 ± 23.3	280.5 ± 84.8	88.8 ± 7.1	77.5 ± 7.4	81.8 ± 4.9	79.8 ± 4.6
0.03 CPA + 0.9 pmol of AF	394.0 ± 28.2	181.5 ± 11.5	118.8 ± 18.3	74.0 ± 5.9	66.8 ± 3.8	53.5 ± 6.3
0.01 CPA + 0.03 pmol of AF	674.3 ± 169.3	138.3 ± 1.6	98.5 ± 3.3	63.3 ± 5.9	57.3 ± 5.0	60.8 ± 5.7
0.0 pmol of AF	336.8 ± 14.5	167.3 ± 7.6	74.0 ± 6.1	61.8 ± 6.2	67.5 ± 4.7	52.8 ± 5.4

[a] Four replicate plates per treatment.
[b] Relative amount of mycotoxin per plate.

0.3, 0.1, 0.03, and 0.01, where 1.0 is equal to 3 pmol per plate for AF and 2.0 μmol per plate for CPA.

The results of the factorial experiment are presented in Table 2. Two identical experiments were run with similar results. There was a highly significant linear correlation between the number of revertants per plate and the dose of either CPA or AF at constant doses of the other mycotoxin. An analysis of variance of these data demonstrated that the effect of each mycotoxin was highly significant and that the interaction was also significant. Although there was a significant interaction between AF and CPA, it is not possible to determine by analysis of variance alone whether the interaction is additive, antagonistic, or synergistic, i.e., less than or greater than additive. To determine whether the combination effect is additive, antagonistic, or synergistic, dose-response curves were obtained for AF alone, for CPA alone, and for the AF-CPA combinations with doses at a constant molecular ratio, i.e., the diagonal in Table 2 from 1.0-1.0 to 0.01-0.01. All response values for the dependent variable were corrected for the number of revertants per plate by subtracting the double negative control value (0.0-0.0). If the interaction between AF and CPA were additive, it would be expected that the predicted values based on the sum of the number of revertants per plate for AF alone and CPA alone should equal the experimental values for the AF-CPA combinations. Similarly, experimental values significantly higher or lower than predicted would indicate synergism or antagonism, respectively. Figure 1 shows a family of curves in which AF, CPA, AC, and A + C represent AF alone, CPA alone, the AF-CPA experimental combinations, and the sum of AF plus CPA, respectively. The data indicate that the experimental curve AC closely approximates the values obtained by the sum of the individual AF and CPA curves. General linear model results indicate that the difference between A + C and AC slopes [i.e., (A + C) − AC] is not significantly different from a slope of zero ($P > 0.8$). These results indicate that the individual effects of CPA and AF on strain TA98 are not significantly different from the effects of these two compounds when administered together. The results, therefore, are additive rather than synergistic.

AFB_1 has been well documented to be one of the most potent naturally occurring genotoxic substances known. Our data show that, although AF and CPA have comparable 50% lethal doses for acute toxicity in rats (when administered intraperitoneally), there is an enormous difference in their mutagenic activity in *S. typhimurium*. By using the data in Fig. 1 and converting relative dose values to actual amounts of AF and CPA per plate, the specific activities of 6.24 revertants per pmol for AF and 140.74 revertants per μmol for CPA can be estimated. Thus, AF was approximately 45,000 times more potent than CPA in these experiments.

The data clearly demonstrate that CPA is mutagenic to *S. typhimurium* TA98 with metabolic activation, whereas CPA was weakly mutagenic to TA100. In a previous study by

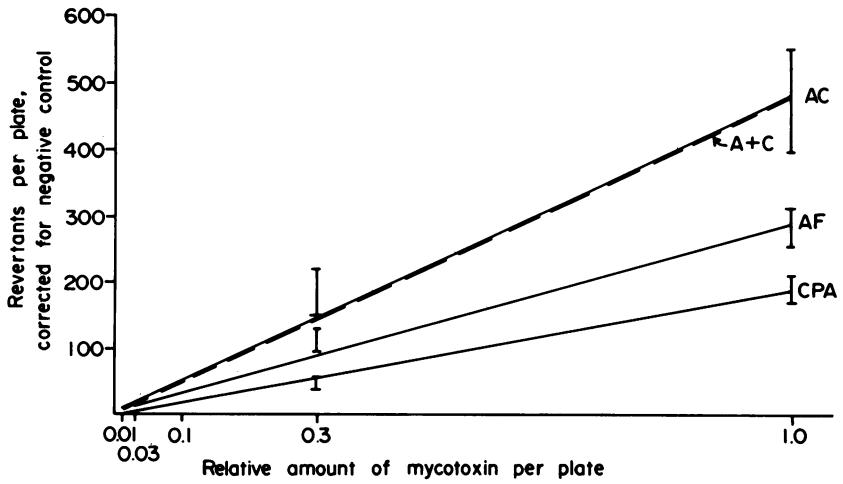

FIG. 1. Dose response to AF, CPA, AF and CPA at a constant molecular ratio (AC), and the expected response if AF and CPA produce an additive response (A + C). Statistical analyses of these lines demonstrated that each slope is significantly greater than zero ($P < 0.0001$) with an associated r^2 value >0.95. Dose levels presented in relative terms are 1.0 equal to 30 pmol for AF and 2.0 μmol for CPA.

Wehner et al. (14), it has been reported that CPA was nonmutagenic, but the highest dose used in that study was approximately 0.75 μmol per plate. Since CPA is produced by *A. flavus* and is commonly produced by aflatoxigenic strains, its presence in AF-contaminated grain contributes to the genotoxic hazard of such grain.

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