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Cyclopiazonic Acid Production by *Aspergillus flavus* and Its Effects on Broiler Chickens

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Cyclopiazonic acid (CPA) was purified from cultures of *Aspergillus flavus*, and ca. 14 g of the toxin was collected for use in feeding studies. Chicken rations were artificially contaminated with purified CPA at concentrations of 10, 50, and 100 ppm (μg/g) and fed ad libitum to eight groups of chickens for 7 weeks. Chickens receiving feed with 100 ppm of CPA had high mortality, decreased weight gain, and poor feed conversion when compared with birds receiving other doses. Postmortem examination showed that chickens fed the two greatest doses of CPA had proventricular lesions characterized by mucosal erosion and hyperemia (100 ppm) and by thick mucosa and dilated proventricular lumens (50 ppm). Birds given 100 ppm of CPA in feed also had numerous yellow foci in their livers and spleens. Microscopic examination of tissues of birds that received 100 ppm of CPA revealed ulcerative proventriculitis, mucosal necrosis in the gizzard, and hepatic and splenic necrosis and inflammation. Birds given 50 ppm of CPA had hyperplasia of the proventricular mucosal epithelium. Birds given 10 ppm of CPA and control birds had no significant treatment-related lesions.

Cyclopiazonic acid (CPA) is a toxic indoletetramic acid that was first isolated from cultures of *Penicillium cyclopium* Westling in 1968 (5). Subsequently, CPA has been reported to be produced by *Penicillium patulum, P. viridicatum, P. puberulum, P. crustosum, P. camemberti, Aspergillus flavus, A. versicolor*, and *A. oryzae* (8–10, 12, 13, 15). Many of the *Penicillium* species that produce CPA were isolated from meats such as ham, sausage, and frankfurters (9). Other foods and feeds from which CPA-producing fungi have been isolated include cheese, corn, guinea pig ration, turkey ration, mixed feed, walnuts, and peanuts (3, 7, 8, 15). Natural occurrence of the toxin in an agricultural commodity was first reported in 1978, when corn that was contaminated by *A. flavus* was found to contain CPA (3). Lansden and Davidson (6) have recently reported natural contamination of peanuts with CPA.

Gallagher et al. (3) tested 54 strains of *A. flavus* and found that 14 produced both aflatoxins and CPA, 4 produced aflatoxins only, and 14 produced CPA only. Because *A. flavus* is a major constituent of the mycoflora of corn and peanuts in the southern United States, CPA represents a potential mycotoxin problem in these commodities that needs to be thoroughly investigated.

Because the only previous major toxicological study with CPA in animals involved oral and intraperitoneal administration to rats (14), the present study was designed to assess the long-term effects of the consumption of feed contaminated with different concentrations of CPA. Chickens were used in the study because of the economic importance of poultry as well as the likelihood that chickens could be exposed to feed naturally contaminated with CPA. Although purified CPA was used to determine subchronic effects of this toxin, a naturally occurring syndrome involving CPA would likely involve the additive or synergistic effects of other metabolites present (including aflatoxins). The results of this study indicate a possible need to screen commodities, such as corn and peanuts, for contamination with CPA as well as aflatoxins.

MATERIALS AND METHODS

Production and purification of CPA. An isolate of *A. flavus* (CP27) was used for the mass production of CPA. The fungus was grown in 500 Fernbach flasks (2.8 liters), each containing 200 ml of Difco mycological broth plus 15% sucrose and 2% yeast extract (1), for 2 weeks at 25 to 27°C. Cultures were extracted twice with equal volumes of chloroform by homogenization for 5 min. The chloroform layer was withdrawn, filtered through anhydrous sodium sulfate, and con-

centrated under vacuum at 60°C with a rotary evaporator.

The crude extract was divided into four parts, and each part was brought to 300 ml with chloroform. The extract was poured into a 2-liter separatory funnel, and an equal volume of 1 N potassium bicarbonate solution was added. The funnel was shaken, the two layers were allowed to separate, and the aqueous phase was collected. This was done three times, and the combined aqueous fractions were placed in a 6-liter separatory funnel and adjusted to pH 1 to 2 with 5 N HCl. The acidified aqueous phase was then extracted three times with chloroform. The chloroform layers were combined, filtered through anhydrous sodium sulfate, and concentrated at 60°C with a rotary evaporator. The residue was dissolved in a minimal amount of chloroform, and excess methanol was added. Upon further concentration, CPA was precipitated from the solution and collected.

Physical and chemical analyses. Thin-layer chromatographic analyses were performed on precoated silica gel 60 F-254 plates (5 by 10 cm; EM Laboratories, Inc., Elmsford, N.Y.) and also on similar plates that had been pretreated by dipping in 2% aqueous oxalic acid and drying for 1 h at 100°C. The developing solvent system was toluene-ethyl acetate-formic acid (5:4:1, vol/vol/vol), and the developed plates were sprayed first with 1% *p*-dimethylaminobenzaldehyde in ethanol and then with 50% ethanolic sulfuric acid.

UV spectra of CPA in methanol solution were recorded with a Perkin-Elmer model 552A spectrophotometer. Determination of the purity of CPA was based on its extinction coefficient at 284 nm with a 10^{-5} M solution of the toxin in methanol.

Infrared spectra of samples prepared as thin films on KBr windows were obtained with a Perkin-Elmer model 1310 recording spectrophotometer. Low-resolution mass spectra were obtained with a Hewlett-Packard model 5985 spectrometer. Samples were introduced into the spectrometer by the direct probe method. Melting points were determined on a Kofler micro-melting point apparatus and were uncorrected.

Feeding trials. All feed used in the study was analyzed for aflatoxin by the minicolumn method described previously by Holaday and Lansden (4) and by high-pressure liquid chromatography (R. J. Cole, J. W. Kirksey, T. H. Sanders, J. I. Davidson, and D. M. Wilson, unpublished data). Rations were formulated by first dissolving the appropriate amount of CPA in methanol. The solution was then added to the feed in a 2.5-cubic-ft (70.8-liter)-capacity cement mixer at a rate of 200 ml of solution per kg of feed. Mixing continued until evaporation of the methanol was complete.

The feeding trial lasted 7 weeks and consisted of eight groups of 10 chickens each that received feed and water ad libitum. Purina Chick Starter ration was used for the first 3 weeks of the trial and was followed by Purina Chick Grower for the remaining 4 weeks. One-day-old chickens were weighed and randomly assigned to experimental groups receiving feed contaminated with purified CPA at the following levels: groups 1 and 2, 100 ppm (100 μg/g); groups 3 and 4, 50 ppm; groups 5 and 6, 10 ppm; groups 7 and 8, controls. Chickens were numbered with wing bands, rations were weighed daily, and chickens were weighed weekly.

After 7 weeks, the surviving birds were killed, and samples of the proventriculus, gizzard, small intestine, pancreas, bursa, spleen, kidney, liver, heart, lung, and brain were fixed in 10% phosphate-buffered Formalin at pH 7.3. Gross changes in tissues were observed during necropsy, and Formalin-fixed tissues were sectioned at 5 μm and stained with hematoxylin and eosin for microscopic evaluation.

To ensure that the results obtained from the feeding study were valid and accurate, the experiment was repeated with the same format except that all chickens were given the control ration for 3 days before the trial was initiated.

Statistical analyses of data for feed conversion and treatment group differences were made with least-squares analysis of variance.

RESULTS

Physical and chemical properties. The metabolite purified from culture extracts of *A. flavus* had a melting point of 243 to 245°C. It had an R_f of 0.68 on oxalic acid-impregnated thin-layer chromatographic plates and turned blue when sprayed with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) followed by ethanolic sulfuric acid. On plates not treated with oxalic acid, the toxin gave the same color reaction, but there was extensive tailing of the spot. The UV spectrum showed absorptions of λ_{max}^{MeOH} 225, 253, 275(sh), 284, and 292(sh) nm, which were identical to those reported for CPA (5). Major infrared absorptions occurred at 3,300, 1,690, 1,590, and 1,425 cm^{-1}. Low-resolution mass spectral analysis showed a molecular ion at *m/e* 336, which was the same as that originally reported for CPA (5). Taken together, these data established that the metabolite isolated from *A. flavus* cultures was CPA. The purity of the CPA was determined to be 99% on the basis of its UV extinction coefficient at 284 nm. Approximately 14 g of CPA was purified from chloroform extracts of *A. flavus* cultures. This corresponds to a yield of 14 mg of CPA per 100 ml of liquid medium.

Feeding studies. In each of the two feeding trials, the primary clinical signs occurred in those chickens given feed with 100 ppm of CPA. Mortality was greatest at this concentration, and weight gain of these birds was markedly affected when compared with the other treatments. Feed containing 10 or 50 ppm of CPA produced no differences in mortality and weight gain when compared with controls; however, pathological changes in some organs, particularly with 50 ppm of CPA, were observed.

In the first trial, three chickens receiving feed with 100 ppm of CPA (group 2) died within the first 3 days of the experiment as a result of chronic respiratory disease. Essentially no feed was consumed by these birds; therefore, they were not considered in the statistical treatment of the data. Four chickens were withdrawn from the study when they became crippled because of

slipped tendons and were thus unable to obtain feed. Two of these birds were in group 7 (control) and were killed at the end of week 3. One was in group 5 (10 ppm) and was part of the experiment for 5 weeks. The other was in group 4 (50 ppm) and survived for 1 week. These birds were not considered in the mortality data; however, they were included in calculations involving weight gain and feed conversion as long as they were able to consume feed and gain weight.

A graph of mortality data from the first trial is presented in Fig. 1. Of the 17 chickens given feed with 100 ppm of CPA (groups 1 and 2), only 7 (41%) survived to the end of the experiment. By contrast, there were 16 of 18 (89%) survivors among controls, 18 of 19 (95%) that were fed 10 ppm of CPA, and all 19 (100%) that were fed 50 ppm of CPA. Weight gain of chickens given 100 ppm of CPA was greatly reduced compared with controls. A plot of the mean weekly weights appears in Fig. 2. The mean weight of surviving chickens (Table 1) that were fed 100 ppm of CPA was 849 g, compared with 1,905 g for controls, 1,952 g for birds given 10 ppm, and 1,705 g for birds given 50 ppm. This reduction in weight gain with the greatest CPA concentration was noted at the end of week 1 and continued throughout the study. Feed conversion (the ratio of feed consumed to weight gained) was impaired in chickens that received feed contaminated with 100 ppm of CPA. The overall mean feed conversion for the two groups at 100 ppm was 5.09, which was significantly different ($P <$ .05) from the means of 2.69 for the control groups, 2.64 for 10 ppm, and 2.72 for 50 ppm.

Results of the second feeding trial were similar to the results of the first trial. Mortality and weight gain were again greatly affected by the high concentration of CPA in the feed. Of the 20 chickens given feed with 100 ppm of CPA, 14 (70%) survived. However, all birds given feed with 10 or 50 ppm of CPA and all controls survived to the conclusion of the second trial. The mean weight of surviving chickens (Table 1) at 100 ppm of CPA was 496 g, compared with 1,728 g for controls, 1,765 g for birds fed 10 ppm, and 1,634 g for birds fed 50 ppm. The effect of CPA on feed conversion was not as clear in the second trial. Mean feed conversions were 2.50 for controls, 2.26 for 10 ppm, 2.53 for 50 ppm, and 3.0 for 100 ppm. Although the greatest concentration of CPA gave the poorest feed conversion, the difference was not statistically significant.

Postmortem examination of the chickens in the high-dose group (100 ppm) revealed areas of erosion, ulceration, and hyperemia of the proventricular mucosa (9 of 16 birds). There was also excess mucus overlying the mucosal surface and dilatation of the proventricular lumen. Chickens in this group also had moderately pale livers (11 of 16) and irregular, variable-sized, yellow foci in their livers and spleens (5 of 16). The yellow foci extended into both the hepatic and splenic parenchyma. The bursa of Fabricius was small in all of the birds in this group. Birds

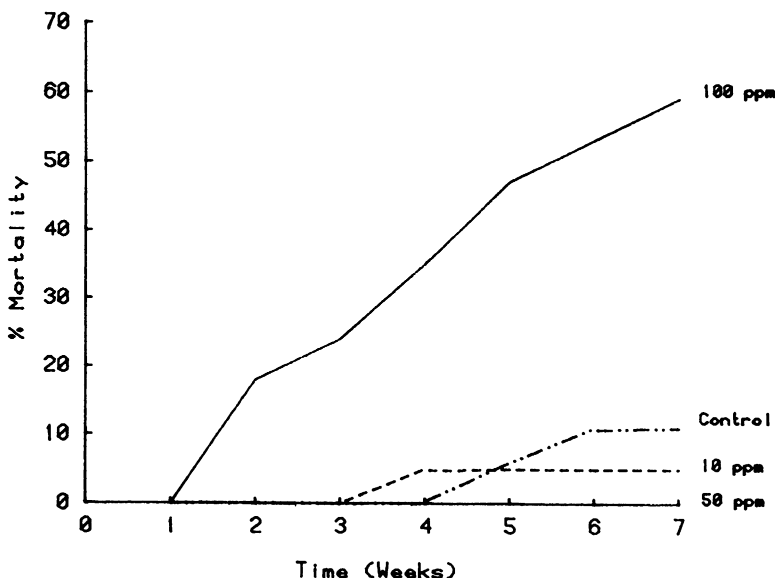

FIG. 1. Effect of CPA-contaminated feed on mortality of broiler chickens.

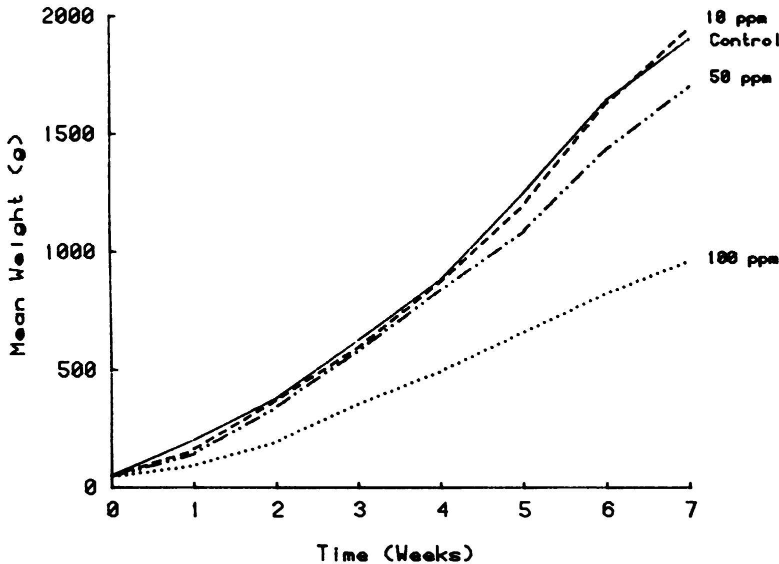

FIG. 2. Effect of CPA-contaminated feed on weight gain of broiler chickens.

given 50 ppm of CPA had dilatation of proventricular lumens and apparent thick proventricular mucosal surfaces (5 of 20). Gross lesions were not observed in the birds given 10 ppm of CPA or in the control birds.

A summary of the histological lesions in chickens from each trial is presented in Table 2. Although the following description of results relates to the initial trial, gross and microscopic lesions in chickens in all dose groups of the second trial were similar to those in birds from trial 1. Histological lesions in birds from the 100-ppm group were characterized by focal to diffuse areas of proventricular mucosal necrosis, chronic inflammation of the submucosa, and focal areas of epithelial necrosis in submucosal glands (10 of 16 birds). Some birds (3 of 16) had focal to diffuse areas of proventricular mucosal epithelial hyperplasia. The hyperplastic response was characterized by crypt elongation, slight tortuosity of crypt lumens, and increased cytoplasmic basophila of epithelial cells. The liver lesions from affected birds in the high-dose group (100 ppm) had focal to diffuse areas of hepatocellular vacuolation in peripheral portions of the hepatic lobules (11 of 16 birds). There were also multifocal areas of nonpatterned hepatocellular coagulative necrosis (4 of 16), multifocal areas of chronic hepatic portal inflammation (12 of 16), and occasional focal hepatic granulomas (5 of 16). In addition, 2 of 16 birds in the high-dose group had multifocal areas of bile duct proliferation. Splenic lesions were characterized by focal to diffuse areas of coagulative necrosis of the splenic parenchyma (4 of 10). Granulomas were observed in the spleens of 2 of 10 birds. There were also focal areas of mucosal necrosis and chronic inflammation in the gizzard (7 of 16 birds), subacute to chronic inflammation of the epicardium (3 of 10), and chronic myocardial inflammation (1 of 10). The bursa of Fabricius from the five birds in the 100-ppm group from which the bursa was obtained had small follicles with marked reduction in lymphoid cells in the cortex and medulla. In addition, 100% of the birds in the high-dose group from trial 2 had multifocal to diffuse areas of necrosis and inflammation of the mucosal surface of the crop. In the 50-ppm group, 9 of 20 birds (45%) had similar but less extensive crop lesions.

Birds given rations containing 50 ppm of CPA had microscopic lesions in the proventriculus, liver, spleen, and heart; however, these lesions were generally more focal and less tissue destructive. The proventricular lesions were characterized by chronic mucosal inflammation (14

TABLE 1. Mean weights (in grams) of chickens fed rations containing CPA for 7 weeks (trials 1 and 2)

CPA level (ppm)	Mean wt (g)	
	Trial 1	Trial 2
Control	1,904 ab	1,761 a
10	1,952 a	1,765 a
50	1,705 b	1,582 a
100	849 c	496 b

[a] Means in a column followed by the same letter are not significantly different (Duncan's New Multiple Range Test, 0.05 level).

TABLE 2. Summary of histological lesions in chickens fed rations containing CPA (trials 1 and 2)[a]

Lesion(s)	100 ppm of CPA		50 ppm of CPA		10 ppm of CPA	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Proventriculus						
Proventriculitis	16/16 (100)	20/20 (100)	14/20 (70)	17/20 (85)	0/20 (0)	0/19 (0)
Hyperplasia of mucosal epithelium	3/16 (18.7)	1/20 (5)	16/20 (80)	10/20 (50)	0/20 (0)	0/19 (0)
Crop (mucosal necrosis and inflammation)	NE[b]	20/20 (100)	NE	9/20 (45)	NE	0/20 (0)
Gizzard (mucosal necrosis and inflammation)	7/16 (43.7)	10/20 (50)	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)
Liver						
Hepatocellular vacuolization	11/16 (68.7)	14/20 (70)	3/20 (15)	3/20 (15)	1/20 (5)	0/20 (0)
Hepatocellular necrosis	4/16 (25)	13/20 (65)	1/20 (5)	2/20 (10)	0/20 (0)	0/20 (0)
Chronic hepatitis	12/16 (75)	18/20 (90)	13/20 (65)	12/20 (60)	4/20 (20)	2/20 (10)
Bile duct proliferation	2/16 (12.5)	9/20 (45)	0/20 (0)	0/20 (0)	0/20 (0)	0/20 (0)
Spleen (necrosis of splenic parenchyma)	4/10 (40)	13/20 (65)	1/20 (5)	7/20 (35)	0/20 (0)	0/20 (0)
Heart (myocardial and epicardial inflammation)	4/10 (40)	16/20 (80)	1/20 (5)	3/20 (15)	0/20 (0)	0/20 (0)

[a] Number of birds with lesions over total number of birds from which tissues were examined microscopically. Percentage follows in parentheses. No control birds had lesions; the crops of trial 1 control birds were not examined.

[b] NE, Not examined.

of 20 birds) and by hyperplasia of mucosal epithelium (16 of 20). The prominent liver lesion was focal mononuclear inflammatory cell infiltration (chronic inflammation) into portal triad regions (13 of 20).

The chickens given 10 ppm of CPA and the control birds did not have proventricular lesions, although 4 of 20 birds at the 10-ppm level had focal areas of chronic inflammation in portal triad regions of the liver.

DISCUSSION

The only previous report of the natural occurrence of CPA in an agricultural commodity was made by Gallagher et al. (3) who reported that aflatoxin-contaminated corn samples were also contaminated with CPA. Their investigation of 54 *A. flavus* isolates showed that whereas 7% of the isolates produced aflatoxin only, 26% produced CPA only, and 26% produced aflatoxin and CPA. Their study indicated that CPA might occur with a frequency similar to or greater than that of aflatoxin. Recently, Lansden and Davidson (6) reported the natural occurrence of CPA in peanuts as well. These findings indicate that CPA might be a more frequent contaminant of agricultural commodities than is currently recognized.

The results of the feeding trials indicated a dose-related response of chickens to CPA-contaminated feed. Birds given 100 ppm of CPA had mucosal epithelial necrosis and inflammation of the crop, proventriculus, and gizzard. The inflammatory changes in the proventriculus also occurred in the birds given 50 ppm of CPA; however, these changes were not usually associated with mucosal necrosis. Proventricular mucosal hyperplasia occurred more frequently in birds given 50 ppm of CPA (26 of 40) than in birds given 100 ppm of CPA (4 of 36). Liver lesions in birds in the 100-ppm group were severe and were consistent with toxic hepatitis. The birds in the 50-ppm group had less-severe liver lesions. Liver lesions in the birds given 10 ppm consisted primarily of mild chronic hepatitis (6 of 40).

These feeding studies were conducted with feed with purified CPA added. The feed contained no aflatoxins before the formulation. Therefore, the effects observed were concluded

to have been the result of the CPA added to the feed. It is not unusual that relatively high toxin levels are required to produce significant changes when the toxin in question is highly purified and artificially incorporated into the ration. Evidence has been presented showing that corn naturally contaminated with *Fusarium roseum* had a greater effect on pigs than did the purified toxin (zearalenone) from *F. roseum* (11). Forsyth et al. (2) found that feed refusal by swine was greater for naturally infected corn samples than for feeds to which equal concentrations of purified toxin had been added. In such a situation, questions must be considered regarding availability of the toxin, changes in solubility, and the absence of the synergistic or additive effects of other metabolites (whether individually toxic or not) that would be present in a naturally contaminated sample. Therefore, it can generally be concluded that feed naturally contaminated with a particular toxin at a certain level is likely to be more toxic than feed artificially contaminated with pure toxin at the same level. Although 50 to 100 ppm of CPA was required in this study to produce significant effects, it is possible that feed naturally contaminated with CPA would produce effects at a lower level of contamination. It would, of course, be useful to compare the results of a feeding study in which feed naturally contaminated with CPA is used with those obtained in this study. The advantages of these results at the present time are that they identify the pathological effects of CPA-contaminated diets fed to chickens and establish that such effects are, in fact, the result of CPA contamination.

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