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Toxicity to White Mice of Corn Naturally Contaminated with Aflatoxin

Male and female mice of three different ages were fed continuously on balanced corn diets containing naturally occurring aflatoxin. The corn contained 286 ppb of aflatoxin and was fed in diets containing 5, 20, and 40% (14, 57, and 114 ppb) contaminated grain for 6 months. Animals were regularly observed for weight changes, general health, or toxicity symptoms, and at the end of the experiment their livers were examined for total lipid content. Weight gains of mice fed the highest aflatoxin levels were approximately the same as those on rations containing lower levels. No tumors were observed, but lipid accumulation in the liver was significant in animals ingesting the highest level of toxin-contaminated feed.

Aflatoxin has been fed in diets to a number of different animals and a variety of effects have been observed (Wogan, 1972). Carcinogenic activity of aflatoxin has been demonstrated in ducks, rainbow trout, ferrets, rats, mice, and monkeys (Louria et al., 1974; Wogan, 1965, 1972). Apparently, in animals poisoned by aflatoxin, the liver is the main organ affected. Toxin-induced lesions and high lipid accumulations in the organ have been described (Bourgeois et al., 1971; Butler, 1969; Wogan, 1973). Fatty degeneration of the liver has been observed in a number of animal species ingesting aflatoxin (Butler, 1969; Tung et al., 1972). In addition to liver symptoms, growth rates were also affected in ducklings and chickens ingesting aflatoxin (Lynd and Lynd, 1970; Smith and Hamilton, 1970). In feeding experiments with chickens, smaller amounts of the toxin were required to induce lipid accumulation in the liver than were required to cause growth repression (Tung et al., 1972).

Although numerous accounts describe symptoms in animals fed diets artificially contaminated with aflatoxin, few report effects of a diet of grain containing naturally occurring aflatoxin. In our research, we fed mice continuously on a balanced diet containing corn naturally contaminated with aflatoxin. We regularly observed the animals for weight changes, general health, or toxicity symptoms, and at the end of the experiment we examined their livers for size, color, and total lipid content.

EXPERIMENTAL SECTION

Experimental Animals. White mice (CD-1 outbred albino strain) from the Charles River Breeding Laboratories (Wilmington, Mass.) were 3, 5, and 8 weeks old. Half of each age group was male and half female. The animals were divided according to age and sex into groups of 10, and housed in a constant temperature (25°C), constant humidity (60%) room. Water and feed were supplied ad libitum. Mice were observed daily and weighed every other week for 6 months.

Special Diets. The contaminated grain was received from Missouri as whole, shelled white corn. FDA inspectors had found it to contain aflatoxin at levels above acceptable limits ("F.D.A. Recalls Corn Meal, Bread Mix Allegedly Tainted by Toxin", 1971). The particular lot of corn used in the feeding trials contained 286 ppb of aflatoxin B_1 and no aflatoxin G, as determined by thin-layer chromatography (TLC) analysis. B_1 isolated from the corn was confirmed by cochromatography with a sample of the pure compound.

The aflatoxin-containing corn was fed in special diet mixtures of 5, 20, and 40% of the total ration. Total corn content in the various mixtures was maintained at a constant level of 47.1% by addition of ground corn containing no aflatoxin. The corn, blended in a PK Blender to ensure homogeneity, was ground to 40-mesh grade in a Raymond laboratory hammer mill. A protein level of 20% (Mills and Murray, 1960) was achieved through addition of nonfat dry milk (Valley Lea Super Instant, Midwest Producer's Creameries, Inc., South Bend, Ind.). Commercial mixtures of minerals (Mineral Premix, CSM, Mallinckrodt Chemical Works, St. Louis, Mo.) and vitamins (Vitamin Premix, Roche Chemical Division, Hoffman-LaRoche, Inc., Nutley, N.J.) were added for dietary balance (Mills and Murray, 1960). Ingredients were blended, pelletized, and fed to mice in self-feeders. As negative controls, laboratory animal feed ration (Allied Mills, Inc., Chicago, Ill.) was fed to one group of mice in each category, and a diet containing aflatoxin-free corn to another group in each category. Compositions of the special diets are shown in Table I.

Liver Lipid Analysis. At the termination of the feeding trials, the mice were sacrificed for necropsy. Livers were removed, examined visually, pooled according to diet

Table I. Percentage Composition of Diets Containing Naturally Contaminated Aflatoxin[a] Corn

Ration	Diet A	Diet B	Diet C	Diet D
Aflatoxin-containing corn[a]	40.0	20.0	5.0	0.0
Normal corn[b]	7.1	27.1	42.1	47.1
Nonfat dry milk[c]	50.0	50.0	50.0	50.0
Mineral premix[d]	2.6	2.6	2.6	2.6
Vitamin premix[e]	0.3	0.3	0.3	0.3

[a] Aflatoxin B_1 content, 286 ppb. Actual aflatoxin B_1 level for each diet, ppb: A = 114; B = 57; C = 14; D = 0. [b] Aflatoxin B_1 content, none. [c] Nonfat dry milk, 35% protein. [d] Mineral content: $CaCO_3$, 31.58%; $ZnSO_4 \cdot 7H_2O$, 0.21%; ferrous fumarate, 2.42%; NaCl (0.007% I_2), 34.21%; $CaHPO_4 \cdot 2H_2O$, 31.58%. [e] Vitamin content/2 lb: vitamin A palmitate, 15000000 USP units; thiamin mononitrate, 2.5 g; riboflavine, 3.5 g; pyridoxine-HCl, 1.5 g; niacin, 45.0 g; calcium pantothenate, 25.0 g; folic acid, 1.8 g; vitamin B_{12}, 0.036 g; *dl*-α-tocopheryl acetate, 68000 IU; ascorbic acid, 364.0 g; vitamin D_2, 1800000 USP units.

groups, and quickly frozen for later lipid analysis. Total lipid extractions were carried out according to methods of Folch et al. (1957). Total lipids from three 2-g samples of pooled liver from each diet group were measured, and the results analyzed by analysis of variance to determine significance between diet groups. A three-way variance analysis was also computed in order to examine interactions between aflatoxin concentration, sex, and age of animals at the beginning of feeding trials (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

The general health of mice in all groups during the study was good. Weight gains of mice within test groups were generally similar (Table II). In almost all groups, weight gains of animals on the highest aflatoxin levels were approximately the same or, in a few instances, only slightly less than those on corn rations containing lower aflatoxin concentrations. Cumulative weight gains were generally less with the mice on corn rations than those on a commercially formulated feed.

No liver tumors were observed in any test animal, but some livers from mice on the 40% aflatoxin-contaminated corn diet were abnormally light in color. The light-colored livers were seen in all three age groups and in both sexes. Significantly higher levels of total extractable liver lipids were found in animals on the ration containing 40% aflatoxin-contaminated corn, the highest level used in the feeding trials, than in any of the groups on rations containing lesser amounts of the aflatoxin-containing grain (Table II). Total extractable lipids from livers of mice on the 40% ration ranged from 47.9 to 50.3 mg/g wet weight, whereas the range in the remaining groups was from 38.5 to 41.8 mg/g (Table II). A three-way analysis of variance of the results demonstrated that there was no association between total extractable liver lipids and initial age of the test animals, their sex, interactions of age and sex, or interactions of sex and age with aflatoxin levels in the ration.

Mean weights of the animals averaged over the three initial ages are summarized in Table III. A statistical analysis of variance between initial weights, sex, and aflatoxin diets indicated no significant interaction of these factors. However, when final weights were analyzed a significant sex–diet interaction was apparent. The females demonstrated no significant diet-associated weight variation; however, with the males, the controls on commercial laboratory animal feed showed higher weight gains than any of the groups on aflatoxin or corn control diets.

Table II. Average Weight Gains, Total Extractable Liver Lipids, and Mortalities of Mice Fed Aflatoxin-Containing Corn for 6 Months, by Age and Sex[a]

Initial age, weeks	Sex	Aflatoxin-containing corn in diet, %[b]	Av wt gain/mouse, g	Total extractable liver lipids, mg/g wet wt.	Mortality/10 mice
3	M	40	28.5	47.9[c]	0
3	M	20	29.2	41.0	0
3	M	5	28.7	40.5	1
3	M	0	27.3	39.3	0
3	M	CR[d]	30.9	41.0	0
3	F	40	20.1	48.9	0
3	F	20	19.4	39.1	0
3	F	5	19.8	39.3	0
3	F	0	20.7	39.6	0
3	F	CR	20.9	40.5	0
5	M	40	16.4	49.0	0
5	M	20	15.8	40.8	1
5	M	5	16.8	38.5	2
5	M	0	16.2	40.1	1
5	M	CR	18.3	38.9	0
5	F	40	13.9	50.3	0
5	F	20	14.8	41.0	0
5	F	5	14.8	39.0	0
5	F	0	14.6	39.4	2
5	F	CR	15.4	38.7	2
8	M	40	16.8	49.5	1
8	M	20	18.6	41.5	0
8	M	5	15.2	41.3	0
8	M	0	14.7	40.7	0
8	M	CR[c]	21.3	39.5	0
8	F	40	11.9	49.8	0
8	F	20	10.7	39.4	0
8	F	5	12.3	41.8	0
8	F	0	11.1	38.7	1
8	F	CR	12.3	39.7	1

[a] Statistical examination of the variables was carried out in a factorially designed experiment (Snedecor and Cochran, 1967). [b] Aflatoxin B_1 content, 286 ppb. [c] Least significant difference = 2.86. [d] CR, commercial animal feed ration as control.

Table III. Mean Initial and Final Weights by Sex and Diet[a]

Aflatoxin-containing corn in diet, %[b]	Initial weights		Final weights	
	Males	Females	Males	Females
40	21.43[c]	20.20	42.00	35.50
20	22.07	20.57	43.27	34.53
5	22.40	20.83	41.63	36.46
0	22.23	20.07	41.63	35.53
CR[d]	22.37	20.03	45.87	36.23

[a] Least significant difference (5% level), initial weight = 0.90, final weight = 2.00. [b] Aflatoxin B_1 content = 286 ppb. [c] Mean weight of 3-, 5-, and 8-week animals. [d] Commercial animal feed ration as control.

Our results show that feeding mice corn naturally contaminated with aflatoxin had little effect on growth rate and general health, but a significant increase in liver lipid accumulation was observed in mice ingesting the highest level of toxin-contaminated feed (aflatoxin B_1 content = 114 ppb). Differences in lipid increase were not attributable either to sex or initial age of the mice.

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Peroxidase Activity in Golden Delicious Apples as a Possible Parameter of Ripening and Senescence

Total peroxidase activity was estimated in Golden Delicious apples during storage at 3–4°C, under a controlled atmosphere. As a function of storage time, peroxidase gave two peaks, the first corresponding to climacterium and the second to the start of senescence.

Ribonuclease, protease, and peroxidase have often been associated with senescence (Sacher, 1973). However, Sacher and other authors do not clearly differentiate between ripening and senescence (e.g., Hulme, 1970, 1971). Rate of respiration is a parameter of aging for various kinds of apples stored at 12°C or above. At 3–4°C, the rate of respiration is practically constant (Fidler, 1973). For Golden Delicious apples, Struklec (1970) came to the same conclusion. Biale (1964) and Fidler (1973) have attempted to distinguish ripening and senescence, but only schematically. We investigated whether peroxidase could be used as a parameter to distinguish ripening and senescence in Golden Delicious apples stored at 3–4°C in a controlled atmosphere.

MATERIALS AND METHODS

Golden Delicious apples (about 280 kg), harvest 1973, of uniform color and size (about 70–80 mm diameter) were purchased from a fruit grower at Puiflyk, Netherlands, and stored at 3–4°C under controlled atmosphere (3–4% O_2:7–8% CO_2) (Gorin, 1973; Gorin et al., 1975). Apples were sampled at various intervals (storage samples, Table I) (Gorin et al., 1975).

Acetone (Clements') powders were prepared as before (Gorin, 1973), except that they were kept at −40°C and under nitrogen to avoid loss of peroxidase activity.

Peroxidase was estimated during September–October 1974. Thus, the powder from the first sampling of fruits was stored for about a year and the last about 4 months.

The moisture content of the acetone powders (Table I) was estimated as before (Gorin, 1973).

The nitrogen content of each powder (Table I) was estimated by the Element-Analytical Department of the Institute for Organic Chemistry TNO (Utrecht) using a Heraus Rapid N combustion apparatus. This is a modified Dumas method developed by Merz (1968) and the maximum deviation is 0.2% (w/w).

The nitrogen content of the powder was not corrected for differences in moisture, since their effect on the results was negligible.

Table I. Nitrogen (% w/w) and Moisture (% w/w) Content of Acetone Powders from Fruits Stored for Different Periods[a]

Storage time, days	Moisture[b] $\bar{x}$	Moisture[b] s (±)	Nitrogen
0	0.78*		1.09
26	0.95	0.06	1.00
54	0.18	0.05	1.02
96	0.46	0.12	0.99
124	1.47	0.13	1.01
152	0.77	0.02	1.09
180	0.43	0.08	0.78
208	0.48	0.04	1.01
236	0.61	0.06	0.77

[a] Fruit was put into storage on Oct. 3, 1973. [b] $\bar{x}$ = mean; $s = (\Sigma d^2/n - 1)^{1/2}$, n = number of estimates, which was 3 except for the asterisked entry where it was 2.

The enzyme solution was prepared by stirring in an ice bath a suspension of powder (1.5 g) with 6.7 mmol/l. phosphate buffer (pH 7) (15 ml). After 120 min, it was centrifuged at 2°C for 1 hr at 49000*g*. The supernatant constituted enzyme solution 1.

The residue was resuspended, stirred, and centrifuged as before. The supernatant was enzyme solution 2. The process was repeated to obtain enzyme solution 3. Solution 4 contained insignificant amounts of protein, and was therefore ignored.

The protein content of each solution was estimated by a modified method of Lowry et al. (1951) as described by Bailey (1967).

Peroxidase activity was estimated as described by Lück (1965), slightly modified. The amounts of reagents were decided after preliminary trials.

The sample cuvette contained enzyme solution (0.2 ml), the phosphate buffer (pH 7) (1.8 ml), an aqueous solution