bicide was firmly attached to the adsorbent and difficult to remove. Some of the herbicide may also be irreversibly adsorbed by the adsorbent. The significance of a nonsingularity between adsorption and desorption and the persistence of biological activity of the herbicide in the soil has not been studied. The nonsingularity does prevent the herbicide from moving through the soil as a "bell-shaped" pulse and reduces the herbicide solution concentration behind the downward moving herbicide front.

The mobility of dipropetryn, prometryn, and fluometuron in each soil, as determined by soil thin-layer chromatography is shown in Table V. The differences in the mobility of these herbicides in various soils are apparent. Dipropetryn moved the least and fluometuron moved the most. In general, as the cation exchange capacity, organic matter, and clay levels decreased, the mobility of all three herbicides increased. There was a good relationship between the parameters from the adsorption isotherms and herbicide mobility. Soils showing the highest amounts of adsorption showed the least herbicide mobility. The R_f values for prometryn using the soil thin-layer plate technique were similar to the R_f values for prometryn reported by Helling (1971b) and Helling and Turner (1968).

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Action of Weak Bases upon Aflatoxin B_1 in Contact with Macromolecular Reactants

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Radiolabeled aflatoxin B_1 added to corn grain flour, when treated with ammonia at 25–50° for periods of 3–30 days, binds covalently and preferentially to corn protein fractions and water-soluble components. In the presence of base, results indicate that two types of associations can occur between the aflatoxin and macromolecular substrate. A reversible type of association results from opening the lactone ring of the B_1 molecule in basic media and can lead to electrostatic and/or hydrogen bonding interactions with substrate. The irreversible or covalent interaction between B_1 and substrate does not visibly alter the spectral properties of the primary B_1 chromophore (365-nm absorbance). The affixation of this chromophore to much larger molecules in the presence of weak bases implicates the dihydrofurofuran ring system of B_1 as the site of B_1 interaction. A marked reduction or complete loss of toxicity in certain corn fractions containing the primary B_1 chromophore is further evidence indicating the difuran ring system to be the site of interaction.

The problems associated with and proposed methods of detoxifying aflatoxin-contaminated agricultural commodities have been reviewed (Goldblatt, 1969). In a recent review of the toxicity and carcinogenicity of aflatoxins, Wogan (1973) points out that accumulated evidence indicates that aflatoxin B_1 has two functional groups, the dihydrofurofuran segment and the lactone–pentanone ring systems, responsible for B_1 biological activity.

From the chemical standpoint, it was Coomes et al. (1966) who first presented evidence that the lactone ring is quite reactive, readily opening upon refluxing B_1 in water. This reaction in neutral medium is in contrast to that under acidic conditions generally used to add 1 mol of water to the double bond of the terminal furan rings (Andrellos and Reid, 1964). Using ammonium hydroxide at elevated temperatures and pressures, Lee et al. (1974) were able to detoxify a portion of B_1 by removing the carbonyl of the lactone ring, but such drastic treatment did not completely destroy all of the B_1 aflatoxin.

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In the work to be presented, ^{14}C-labeled aflatoxin B_1 preparations were employed to show that low levels of ammonia as ammonium hydroxide (≤2.0 g of NH_3/100 g of flour) caused the irreversible binding of B_1 to corn flour components. Based upon extractability with various solvents after acidification of the basic reaction mixture, in addition to gel permeation chromatography of certain preparations, we established that the binding occurs preferentially with the major protein fractions and water-soluble materials of corn. This conclusion is further supported by the observed low level of interaction between B_1 and starch which is the predominant macromolecular component of corn. We further observed that the reaction between B_1, ammonia, and corn flour could produce water-soluble products which were teratogenic to chick embryos. But, the active products were isolated only from reaction mixtures artificially contaminated at very high levels of toxin (1 mg/g of flour). Water extracts of ammonia-treated whole corn flour (from naturally contaminated corn initially containing 1200 μg of B_1/kg) and corn germ isolate (7000 μg of B_1/kg) contained no substance teratogenic to chick embryos.

With the alkaline form of DEAE-cellulose serving as the source of base, changes occurring in the fluorescence emis-

sion of aflatoxin B_1 in contact with the anion exchange cellulose were noted. These spectral changes were reversed after mild acid treatment of the polymer. The reversible changes in spectral properties for B_1 in contact with a polymer suggested that the B_1 lactone ring opens in a heterogeneous basic medium and could consequently lead to electrostatic and/or hydrogen bonding interactions.

EXPERIMENTAL SECTION

Materials. *Aflatoxin B_1.* Aflatoxin B_1 was produced by fermentation of rice following the method of Shotwell et al. (1966) and purified and crystallized by the procedure of Stubblefield et al. (1968).

The fermentative production of radiolabeled aflatoxin B_1 employed the manganese-deficient synthetic culture medium of Detroy and Ciegler (1971). Four Fernbach flasks, each containing 500 ml of medium, were inoculated with approximately 3×10^7 washed spores of *Aspergillus parasiticus* NRRL 2999. After incubating at 28° at 250 rpm on a rotatory shaker for 42 hr, 1 mCi of [^{14}C]-1-acetate was added to each of two flasks and incubation continued for another 24 hr. The contents of the four flasks were separately extracted twice with an equal volume of $CHCl_3$. The combined $CHCl_3$ extracts evaporated to dryness yielded 0.65 g of solids. The aflatoxin B_1 was recovered from the solids by the procedure of Stubblefield et al. (1968) and recrystallized twice to constant radioactivity. By this means, 40–45 mg of B_1 was obtained with activity levels of 1.1 to 2.8×10^5 cpm/mg. The molar absorbance values for B_1 preparation determined in methanol were 13,000 and 22,400 at 264 and 363 nm, respectively. The distribution of ^{14}C atoms in the B_1 molecule is assumed to be the same as that shown by Biollaz et al. (1968).

Macromolecular Reactants. DEAE-cellulose was purchased as Cellex-D from Bio-Rad Laboratories and was washed with 0.5 *M* sodium hydroxide until washings were colorless and then finally with water until washings were neutral. This was stored under nitrogen as a water slurry.

Commercial corn starch was purchased at a local food market. The Engineering Department of this laboratory supplied the following corn preparations: aflatoxin-free white whole corn flour, used as the standard preparation; a dry milled first corn germ fraction (from naturally contaminated corn) containing 7000 μg of B_1/kg; and a sample of whole corn flour prepared by grinding naturally contaminated whole corn after ammonia treatment which reduced the B_1 level from 1200 to 15 μg/kg. This latter preparation was used to obtain extracts which could be compared to similar extracts from artificially contaminated whole corn flour after treatment with ammonia.

Other Reagents. All other chemicals used in these studies are commercially available and of reagent grade quality.

Methods. *Determination of Radioactivity.* Levels of radioactivity were measured by scintillation counting in Bray's scintillation fluid (Bray, 1960) employing a Packard Instruments Model 2002 or Model 3385 Tri-Carb liquid scintillation counter. For counting finely divided solids, the fluid contained 0.5 g of the thixotropic silica gel Cab-O-Sil (trademark of the Cabot Corporation) per 100 ml of fluid. When possible, multiple determinations were made with varying amounts of radioactive substance and activity per unit weight of substance obtained. The logarithm of the activity per unit weight was then plotted against sample weight used for counting and straight lines extrapolated to zero weight as in Figure 1 in order to correct for fluorosphore quenching by solids.

Chromatography. Thin-layer chromatography (TLC) for the determination of aflatoxin B_1 was performed as prescribed by the AOAC Official Methods of Analysis (1970) using $CHCl_3$–acetone (9:1, v/v) as developer. A Schoeffel Instruments Corp. Model SD 3000 spectrodensitometer was used for quantitative TLC determinations.

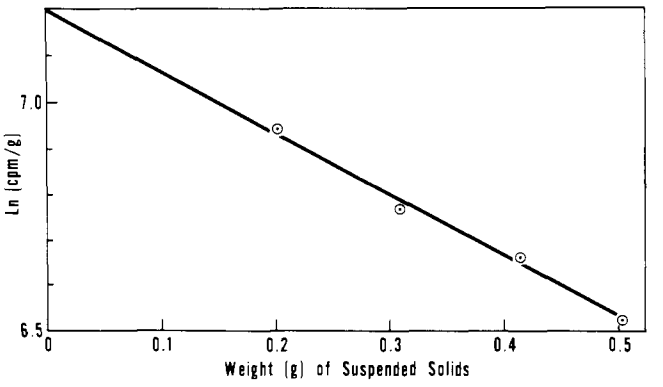

Figure 1. Determination of ^{14}C activity for solids suspended in Bray's scintillation fluid. Line through data of ln (counts per minute per gram) vs. weight of suspended solids is extrapolated to zero weight.

Sephadex G-50 from Pharmacia Fine Chemicals Inc., washed and suspended in distilled water, was used to prepare columns about 75 cm long × 2.5 cm in diameter. Water-soluble isolates were applied at load levels of about 200 mg of solids dissolved in 5–10 ml of water for analytical columns. The columns were eluted with water and the eluate monitored continuously at 365 nm by a Gilford Instruments Model 2000 absorbance recording system. A Buchler Fracto-mette collected 10–12 ml fractions of effluent.

Reaction of Aflatoxin B_1 and DEAE-Cellulose. A portion of the alkaline form of the DEAE-cellulose slurry was cast as a thin coat (ca. 1 mm) on a glass slide (16.7 × 31 mm) and dried under nitrogen with only sufficient heating to firmly affix the cellulose to the slide. With the slide positioned diagonally in the standard cell holder, and the polymer surface facing the incident excitation beam of 250-nm wavelength, the emission spectra of the cellulose layer over the range of 200–800 nm was recorded with an Aminco-Bowman spectrofluorophotometer equipped with standard accessories. After layering a solution of 25 μg of labeled B_1 in 200 μl of $CHCl_3$ over the cellulose surface and removing the $CHCl_3$ under nitrogen, the emission spectra were redetermined periodically for 6 days. When not in the cell holder of the instrument, the slide was stored under nitrogen in a light-proof vial. The spectra were next recorded after exposing the slide to acetic acid vapors. The slide was then soaked overnight in a $CHCl_3$ bath, washed with $CHCl_3$, and soaked again for 4 hr in a methanol–0.01 *N* acetic acid (9:1, v/v) bath. A final emission spectrum of the cellulose layer was recorded prior to removing the layer from the glass for scintillation counting. The bath washes were tested for B_1 by qualitative TLC, and radioactivity level was tested by scintillation counting after reducing the volumes to 10 ml.

Reaction of Aflatoxin B_1 with Ammonia and Corn Preparations. ^{14}C-Labeled aflatoxin B_1 in $CHCl_3$ or benzene–acetonitrile (95:5, v/v) was added with thorough mixing to samples of starch, standard corn flour preparation, or naturally contaminated corn germ flour which were contained in round-bottomed flasks. Sample sizes generally used were 1–5 g but with naturally contaminated flours the sample size was 100 g. Level of B_1 added ranged from 3500 to 2×10^6 μg of B_1/kg. Solvent (0.5–1 ml) used in adding the toxin was removed at 40–50° either at reduced pressure or under a stream of nitrogen. In several experiments with spiked standard flour preparation, oxygen replaced the air in the flasks.

Concentrated ammonium hydroxide was then added to all preparations studied in an amount sufficient to give an ammonia level (as NH_3) equal to approximately 1.5–2.0 g/100 g of solids. The addition of this liquid increased the moisture content by about 4–5 percentage points. To achieve a similar moisture increase, water was added to

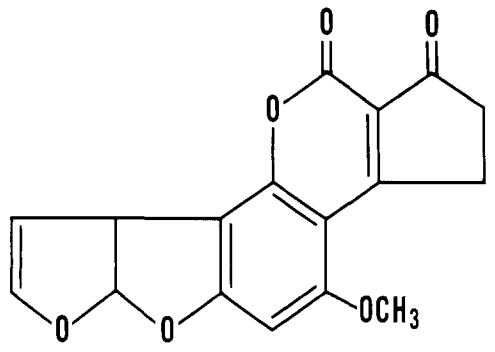

Figure 2. Aflatoxin B_1 structure.

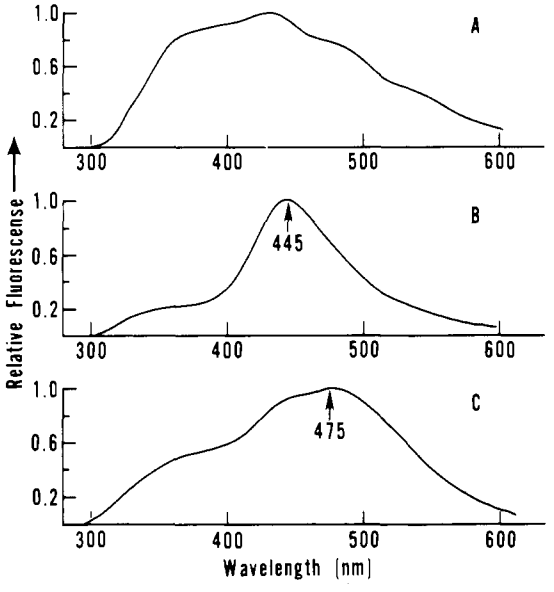

Figure 3. (A) Relative fluorescence from a layer of alkaline DEAE-cellulose when excited with energy at 250 nm. (B) Same as A but after coating with a solution of aflatoxin B_1 at time zero days (see text). (C) Same as B after 6 days.

controls. All flasks were quickly and tightly sealed, then shaken manually to disperse the NH_3 throughout the solid phase. Satisfactory dispersion at this step is indicated by a uniform discoloration of the solid phase. The sealed flasks were maintained at 25 or 50° in a water bath with periodic agitation from 1 to 67 days and were protected from direct light exposure.

Reactions were stopped by adding about 1.1 mol of acetic acid (as glacial acetic acid) per mol of NH_3 added initially. The acid was added to $CHCl_3$ dispersions (25 ml of $CHCl_3$/g of solids) of the reaction solids. The acidified dispersions were stirred 3–4 hr to ensure neutralization of all added NH_3. In one experiment using standard corn flour and 1×10^6 μg of B_1/kg, the reaction was allowed to proceed 3 days at 50° in a flask equipped with septum and gas inlets. A control for this reaction contained flour and B_1 but no NH_3. After acidification of the $CHCl_3$ slurries, nitrogen was passed through the flasks and bubbled into a solution of hydroxide of Hyamine ($1 \times 10^{-3}M$) in methanol–toluene (1:1, v/v) to trap any ^{14}C-labeled CO_2 or other volatiles formed during the reaction (Passmann et al., 1956). Aliquots of the Hyamine solution were subjected to scintillation counting.

Solvent Extraction of Reaction Solids. The slightly acidic $CHCl_3$ slurries obtained in the above section were filtered and the solids washed with additional $CHCl_3$. For corn germ flours, the $CHCl_3$ extracted residues were quite gumlike and cohesive in nature, whereas residues from other corn preparations were not. To ensure that unreacted toxin was not entrapped in the rather cohesive germ residues, the reaction mass of germ flour was slurried in water and reextracted with $CHCl_3$ using 500-ml separatory funnels. A very stable emulsion formed during this extraction process and had to be disrupted by centrifugation at approximately 1000*g* for 10–15 min.

$CHCl_3$ extracts for individual preparations were combined, taken to dryness on a rotatory evaporator, and then redissolved in benzene before the determinations of unreacted B_1 and scintillation counting.

After air drying $CHCl_3$ extracted residues or after freeze drying the aqueous slurries of germ flour, water extracts were prepared by blending the solids with about 5 ml of water per g of solids for 30 min in suitably sized blenders. Standard corn flour reaction mixtures were further extracted with aqueous sodium chloride (1% w/v) and *tert*-butyl alcohol–water (60:40, v/v) by blending as just described. In these extraction procedures, supernatant liquors were decanted from solids after centrifugation at 8000*g* for 20 min at 4°. These extracts and final reaction residues were freeze dried before determining the ^{14}C distribution among the solvent fractions and final residue.

Testing of Toxicity with Chicken Embryos. The procedure of testing chick embryos has been given by Verrett et al. (1964).

RESULTS AND DISCUSSION

Action of DEAE-Cellulose upon B_1. The structure of aflatoxin B_1 as first elucidated by Asao et al. (1963) is shown in Figure 2. The reason for choosing the alkaline form of DEAE-cellulose as a polymer reactant was to gain spectral evidence that the lactone ring of B_1 can be attacked by bases even though the toxin is in a heterogeneous reaction medium.

When excited with 250-nm radiation, the anion exchange cellulose shows the broad fluorescence emission seen in Figure 3A. When B_1 is placed in intimate contact with the polymer, the energy in the neighborhood of 365 nm serves as a secondary excitation source for B_1 molecules which in turn fluoresce with a maximum of about 445 nm (Figure 3B). After about 3 days in contact with the polymer, the emission maximum has shifted to 475 nm (Figure 3C) with no further displacement noted after 6 days. Treatment of the B_1 cellulose complex with acetic acid vapors caused the maximum to shift back to about 445 nm and the spectrum was indistinguishable from that shown in Figure 3B. For aflatoxin B_1 in neutral solvents there is about a 60–70 nm difference between the excitation and emission maxima, but due to background fluorescence from the DEAE-cellulose, an excitation band causing the 475-nm emission of B_1 in contact with polymer could not be detected. However, a solution of B_1 in concentrated ammonium hydroxide (1 mg/ml) was found to have an excitation band with a maximum at 400 nm and the solution emitted a yellowish-green fluorescence with a maximum at 475 nm. The sodium salt of *o*-coumaric acid also gives off a yellowish-green fluorescence when viewed under "Black Light" and the formation of this fluorescent salt is a classical qualitative test for coumarin. Therefore, on the basis of the observed spectral changes for B_1 in concentrated ammonium hydroxide and the known behavior of coumarin treated with base, we infer that when B_1 is in contact with DEAE-cellulose as its alkaline form, ionic B_1 salts involving the lactone carboxyl group are produced leading to at least electrostatic interaction between aflatoxin and polymer.

The $CHCl_3$ wash of the anion exchange cellulose had only 56% of the ^{14}C activity applied to the slide and contained primarily B_1 toxin by qualitative TLC. The methanol–acetic acid wash had no detectable B_1 but it contained

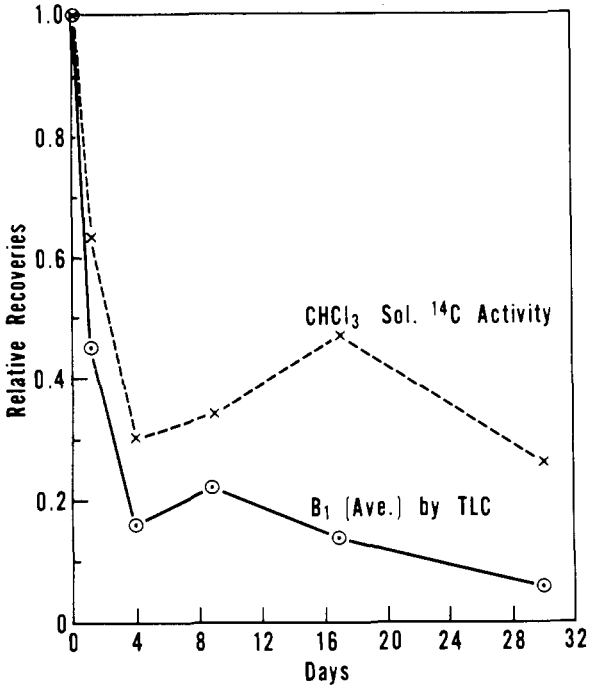

Figure 4. Relative recoveries of $CHCl_3$ soluble aflatoxin B_1 (solid curve) and ^{14}C activity (dashed curve) after treating whole corn flour and aflatoxin B_1 with ammonia in an oxygen atmosphere.

primarily fluorescent material remaining near the origin on TLC plates. This wash contained 38% of the radioactivity and the extracted DEAE-cellulose polymer the remaining 6%. Both photolytic degradation and base-catalyzed reactions probably account for the observed changes in ^{14}C solubility.

Interactions of B_1 Toxin and Corn in Ammonia and Ammonia–Oxygen Atmospheres. The results shown in Figure 1 were obtained with whole corn flour reacted 14 days at 25°, 1.5% NH_3 (w/w) at 17% w/w moisture, and 6×10^3 μg/kg of [^{14}C]aflatoxin B_1. Instead of blending with water after acidification and extraction with $CHCl_3$ the reaction mixture was dialyzed against water overnight at 4°. Both the dialyzed solids and dialysate were freeze dried. From the extrapolated value shown in Figure 1, the final solids contained 76% of the added ^{14}C activity. A methanol–water (9:1, v/v) solution of dialyzable components was used to show that 13% of the radiolabeling was soluble in water but not in $CHCl_3$. Whereas the $CHCl_3$ extract contained 11% of the activity initially added, quantitative TLC indicated that only 6–8% of the aflatoxin remained unmodified. These results demonstrate that the method used in Figure 1 for scintillation counting of solids can be used to quantitatively account for the added activity and provide a very reliable means to show changes in B_1 toxin solubility.

That the biological activity of aflatoxin B_1 entails an epoxidation of the C-1,2 double bond of the terminal furan ring (Figure 2) to form an active intermediate was proposed by Schoental (1970). Garner et al. (1971) have demonstrated the need for oxygen in order to convert B_1 to a very reactive product by liver microsomes. With the potential dependence of B_1 modification upon the presence of oxygen thus demonstrated by the above workers, a sequence of reactions was performed to determine if oxygen enhanced the ammonia-induced binding of B_1 to corn flour components.

The relative amount of $CHCl_3$ extracted, unmodified B_1 as determined by TLC vs. time of reaction in an oxygen atmosphere appears in Figure 4 (solid curve). The relative amount of ^{14}C extracted by the same $CHCl_3$ appears as the dashed curve in Figure 4. The level of B_1 added in these studies was 1 mg of B_1/g of flour. Even at this high B_1 level, the combined moles of base (as NH_3) and moles of water (as moisture) to moles of B_1 are still greater than 1000:1. Aflatoxin B_1 was recovered quantitatively from the control reaction after 29 days as measured by TLC and scintillation counting. TLC determinations were made directly upon $CHCl_3$ extracts since there was no interfering fluorescence in the area of B_1 spots. The difference between the curves in Figure 4 exceeds experimental errors and the noncoincidence of the curves indicates that the toxin has undergone side reactions producing labeled fragments and other compounds. Both curves in Figure 4 appear to have minima at about 4–6 days reaction time. A behavior similar to this was observed by Ciegler et al. (1966) who treated aflatoxin B_1 with peroxide-treated methyl esters of soybean oils. In this instance, their data rapidly reached a minimum value, then passed through a significant increase in B_1 extractability, followed by a more gradual decrease over a 28-day period. The results cited and those shown in Figure 4 might indicate that a toxin–oxygen intermediate retards the direct binding of reaction products to corn constituents.

It is to be further noted from Figure 4 that after 29 days the amount of unreacted B_1 again constitutes about 6–8% of the B_1 added to the system. The formation of metastable B_1 intermediates which react only slowly, in addition to a saturation of active areas on corn solids with B_1 or its products, could explain the repeated 6–8% recovery of unreacted B_1.

Table I. Relative Distribution of Radiolabeling among Corn Flour Extracts and Specific Activity of Fractions

Extracting solvent	% ^{14}C act.	Specific act., (cpm/g) $\times 10^{-6}$
$CHCl_3$	11	0.40
Water	48	2.0
1% NaCl solution (w/v)	6	1.3[a]
tert-Butyl alcohol–water (6:4, v/v)	11	1.1
Final residue	24	0.08

[a] Corrected for NaCl content in freeze-dried fraction.

Distribution of Labeling among Solvent Fractions. The relative distribution of ^{14}C label among various corn flour extracts is presented in Table I. The results of Table I were obtained from flour reacted with [^{14}C]B_1 (1×10^6 μg/kg) and ammonia (2% w/w) for 30 days at 25°. As seen from this table, the water solubles have the highest specific activity (counts per minute per gram). With untreated corn, water would normally extract the albumin proteins, amino acids, and sugars; the material isolated was about 6% of the total flour weight. A more detailed discussion of these water solubles is presented later.

Again using untreated flour as the example, a 1% sodium chloride solution extracted the globulin protein from corn; for the treated flour, this solvent removed slightly more than 1% of the flour solids. Although the amount of material extracted was small, after correcting for inorganic salt (Table I) the specific activity is 65% of that found for the water solubles.

Normally zein, the alcohol-soluble prolamine of corn, is extracted with ethanol–water systems. But to eliminate a dialysis step to remove ethanol, *tert*-butyl alcohol–water was used which can be freeze dried directly after separation from solids. The amount of solids removed by this solvent represented 3% of the initial flour weight used in the reaction. Table I shows that the specific activity for this fraction is still relatively high. Additional information further

characterizing this fraction is unavailable at present; thus, on the basis of solubility only, we assume that this isolate contains primarily B_1–prolamine complexes as well as unmodified zein proteins.

In general, the protein contents of corn range from about 8 to 12% with zein and glutelin protein comprising the major protein fractions. The final residue of an extracted untreated flour would consist chiefly of corn starch but would also contain the high molecular weight glutelin proteins. The final residue in Table I accounted for 82% of the original flour weight. A significant proportion of the radioactivity appears in the residue even though the specific activity is quite low. The specific activity values shown in Table I suggest that corn protein has served as major polymer reactants to covalently bind aflatoxin B_1 or its derivatives. Indeed, when only corn starch was treated in a like manner as the flour but allowed to react 67 instead of 30 days, 50–60% of the added B_1 was recovered as measured by TLC. The $CHCl_3$ extract of the acidified starch reaction removed slightly over 73% of the initially added ^{14}C label. This low level of interaction between starch and toxin indirectly points to the proteins as being the reactive polymers binding B_1 toxin.

Radiolabeled B_1 was added to naturally contaminated germ flour (7000 μg/kg) to determine if B_1 products could be detected in water extracts from an ammoniated moldy corn preparation. One-half and 1 equiv were added to two samples to raise the total B_1 levels to 10,500 and 14,000 μg/kg. The added radioactivity amounted to 382 and 763 cpm/g. These samples as well as a nonspiked sample were allowed to react with ammonia at 50° for 4 days. The water extracts from these reacted samples were highly colored and the solids isolated from the extracts were very hydroscopic. Only in the case of the sample spiked with an equivalent of labeled B_1 could a significant increase in scintillation counts be detected that was above the high background counts obtained with water solubles from the unspiked germ flour. This increase over background indicated that 40–50% of the radioactivity added was to be found in the water extract. This range in percentage is in line with that reported in Table I for the water extract from standard corn flours. The $CHCl_3$ extracts from treated germ flours contained no TLC-detectable B_1.

Silica Gel Chromatography. Side reactions, such as fragmentation and condensation reactions of B_1 molecules, were mentioned earlier. To further explore this feature, portions of the $CHCl_3$ extracts (Table I) were subjected to the official AOAC silica gel column cleanup procedure (1970). In addition to the recommended solvents for column elution, methanol was used as a final column extractant. Hexane removed 0.6% and ethyl ether another 12% of the total radioactivity applied to silica gel columns. These solvents removed no B_1 from the columns. The $CHCl_3$–acetone (4:1, v/v) solvent removed 72% of the labeling and contained about 5% of the B_1 initially added to the flour reaction mixture. The final methanol eluent removed an additional 15% of the labeling, but the fluorescent materials in the methanol wash remained at the origin during TLC analysis. While $CHCl_3$ extracts about 7% of the material from the reaction mixture, the hexane and ethyl ether fractions contained 65% of the total material put on silica gel columns. These solvents would remove corn oils and lipids from the silica gel column and the weight of material found in hexane–ether solvents represented about 4% of the initial flour weight.

Formation of Volatile Radioactivity. Flour was treated at 50° for 3 days and tested for volatile radiolabeling as described in the Experimental Section. In brief, the nitrogen carrier gas swept out about 0.5% of the radioactivity both in the reaction with ammonia and in a control of flour and toxin only. The removal of this low level in both control and reaction systems could be due to a carryover of very finely divided matter along with solvent vapors by the nitrogen gas. A very high level of toxin was used for these measurements, 2.0 mg of B_1 and 1 g of flour. Because the B_1 level was so high and the reaction time relatively short, the $CHCl_3$ extract still contained appreciable amounts of unreacted B_1 as indicated by qualitative TLC.

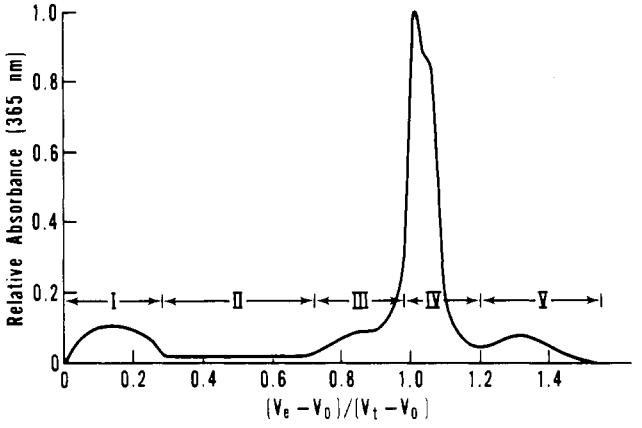

Figure 5. Sephadex G-50 dextran gel chromatography of water-soluble isolates (bed dimensions, 77 cm × 2.5 cm). Flow rate for water effluent was 1 ml/min: V_e, effluent volume; V_0, the bed void volume; and V_t, the total bed volume.

Dextran Gel Chromatography. Even though water extracted but 6% of the solids, Table I shows that it contained almost half of the recovered ^{14}C label. To further characterize the distribution of radiolabeling within this fraction, the water solubles were chromatographed on Sephadex G-50 gel columns. The elution profile appears in Figure 5. Relative absorbance at 365 nm was chosen for the ordinate in Figure 5 because solutions of the water solubles or column fractions gave typical B_1 emission spectra when excited with 365-nm radiation in the spectrofluorophotometer. Thus, Figure 5 shows the absorbance due to the major chromophore or the coumarin pentanone ring system of the B_1 molecule. The retention of B_1 spectral features accompanying a solubility change in chromophore strongly implies that the dihydrofurofuran ring system of B_1 has been modified, and B_1 is covalently bound to water solubles as a result of this modification.

The quantity V_e in Figure 5 represents the effluent volume, V_0, the column void volume, and V_t, the total bed volume. The quantity shown in this figure was chosen for the abscissa since a value greater than one for an eluting solute is evidence that the solute is interacting with the gel. The exclusion limit of Sephadex G-50 ($V_e = V_0$) corresponds to a particle size of approximately 10,000 daltons or greater, while particles smaller than 700 daltons elute at a column bed volume ($V_e = V_t$). Therefore, Figure 5 shows that B_1 chromophore is firmly affixed to some relatively high molecular weight material (fraction I) but the figure also shows that most of the absorbing species elute near a bed volume and beyond. Fraction III would contain amino acids, simple sugars, and low molecular weight reaction products of these compounds as well as the water-soluble salt formed upon neutralization of the basic media.

Because the material in fraction V is definitely interacting with dextran gel, the foregoing relationship between particle size and elution position is not valid. Increasing the load level to about 0.8 g in 20 ml of water did not destroy the salient features of the chromatogram but did decrease the resolution of fractions IV and V. Solids from these two fractions are not soluble in solvents commonly used for ir spectroscopy. In addition, the solids are either too polar or have molecules too large to permit standard mass spectra determinations.

No free aflatoxin could be detected in any column frac-

Table II. ^{14}C Distribution among Column Fractions and Specific Activity of Solids Isolated from Fractions[a]

Fraction	% ^{14}C act. recovered	Specific act., (cpm/g) $\times 10^{-6}$
I	13.8	1.5
II	2.3	1.8
III	4.8	0.18
IV and V	26.3	6.6

[a] Results based on analysis of one column run, using multiple determination with solids obtained by freeze drying fractions.

tion, but all fractions contained radiolabeling as shown in Table II. From this table it is seen that less than 50% of the applied activity was recovered. After washing the column with three additional bed volumes of water an upper portion of gel bed consisting of 140 mg of dried gel was found to have 1.5% of the ^{14}C labeling applied to the column. This small percentage of the total activity found in the top inch of the bed is sufficient to show that the water extracts from standard flour reaction mixtures contain products which can enter into strong interaction with the cross-linked dextran gel bed.

Chromatograms of water extract prepared from moldy corn (1200 μg of B_1/kg initially), treated with ammonia by our Engineering Laboratory at 49°, differed very little from that shown in Figure 5 up to one bed volume of effluent. But in these chromatogram fractions, IV and V were missing even though the column load level was about the same. Effluent beyond a bed volume contained no solids or fluorescence when collected up to two additional bed volumes. The ammonia treatment in this instance was done at an elevated temperature. Therefore, fractions IV and V could represent products not completely reacted with substrate at lower temperatures.

Biological Activity. The LD_{50} dose for aflatoxin B_1 when administered through the air sac to chick embryos has been given as 0.025 μg (Verrett et al., 1964). For the preparations tested here, the radiolabeling was used to estimate the equivalents of bound B_1 and thereby to determine the weight of sample which contained an equivalent LD_{50} dose. This weight of sample and generally a five- to tenfold increase in amount of sample were used for chick embryo tests.

Fractions IV and V (Figure 5) were found to be highly teratogenic after 21 days incubation as this term has been applied by Verrett et al. (1964). This response is not an artifact of gel chromatography. Water extracts prepared after reaction of flour, ammonia, and 1 mg of B_1/g of flour at 25 or 50°, but not chromatographed, also showed high levels of biological activity in chick embryos. When the initial level of B_1 added was reduced from 1×10^6 μg/kg (1 mg/g) to 13,800–27,600 μg/kg, water extracts showed no biological activity to chick embryos exceeding that of controls. Water extracts of treated corn germ flour (7000–14,000 μg of B_1/kg initially) also gave negative test results. Solvent, ammonium acetate solutions, and dispersion of water solubles (10–13% w/v) from standard flour as well as ammonia treated flour were tested in controls. Therefore, the use of exceedingly high levels of B_1 (1 mg/g) before treatment with ammonia appears to be the chief reason that any biological activity with embryos was noted.

A contamination level of 27,000 μg of B_1/kg would be considered extremely high and unlikely to be found in a commercial corn. The results of this section would indicate that as long as the level of B_1 does not greatly exceed 27,000 μg/kg, the action of ammonia is quite likely to destroy the B_1 toxicity without generation of teratogenic by-products.

CONCLUSIONS

This investigation has shown that aflatoxin B_1 binds irreversibly to substrates when treated with weak bases even though the reaction medium is a heterogeneous system. Because the primary chromophore of B_1 appears firmly attached to macromolecular substrates, the authors have inferred that the lactone–pentanone ring moieties have undergone only reversible-type reactions upon treatment with base. It has been further suggested that this portion of the molecule is probably not the point of attachment in B_1 to substrate. The retention of chromophore has indirectly implicated the dihydrofurofuran ring system of B_1 as being the site of modification in B_1 which eventually leads to the uptake of B_1 by substrate. The loss of biological activity when the initial B_1 level is not exceedingly high ($\leq$27,600 μg of B_1/kg) tends to confirm this premise.

If it can be assumed that the hemiacetal of B_1 can form in basic media when B_1 toxin is adsorbed onto a "reaction surface" such as protein molecules, which possess numerous nucleophilic groups, then a reaction sequence proposed by Pohland et al. (1968) to explain their observation is most attractive. These workers confirmed the finding of Büchi et al. (1967) who claimed that the hemiacetal could undergo a reversible opening leading to the formation of a phenolic dialdehyde derivative of B_1. The chemical reaction between aldehyde function of B_1 and amino groups on proteins and amino acids as well as other functional groups would firmly attach the B_1 chromophore to corn constituents. If, indeed, this is the mechanism of detoxification by treatment with ammonia, then the B_1 labeling would most probably be found in protein-rich fractions and water solubles from corn.

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