Ammoniation of Aflatoxin-Containing Corn: Distribution, in Vivo Covalent Deoxyribonucleic Acid Binding, and Mutagenicity of Reaction Products

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[^{3}H]Aflatoxin B_1 (AFB_1) was ammoniated at 100 °C and the isolated aflatoxin D_1 (AFD_1) characterized by ^{1}H NMR and mass spectrometry. AFD_1 was found to be 130-fold less mutagenic than AFB_1 in the Ames test. In vivo covalent binding to rat liver DNA expressed in the units of a covalent binding index (CBI, a quantitative indicator for genotoxicity) of [^{14}C]AFD_1 was below the detection limit of 71 and at least 280-times lower than for AFB_1, although AFD_1 still has the 8,9a double bond. After ammoniation of [^{14}C]AFB_1-spiked corn grits for 34 days (18.8% H_2O/2.02% NH_3, 25 °C), ^{14}C distribution and CBI were measured: 7% of the radioactivity was CH_2Cl_2 extractable but 14% was extractable when acidified prior to extraction. The H_2O phase contained 42% ^{14}C with a CBI of 1000 but only 31% ^{14}C with a CBI of below the detection limit of 145 when acidified prior to extraction. The extracted corn had still a radioactivity of 33% (38% respectively) with a CBI of <126. More than 85% of the H_2O fraction pass a dialysis membrane with an exclusion limit of 14000 daltons. The results indicate that ammoniation reduces the genotoxicity of AFB_1-containing corn at least 20 times. Besides unreacted AFB_1 the aqueous phase contained about 7% of a still toxic AFB_1 hydrolysis product, which can rebuild AFB_1 after acidification.

Numerous attempts have been made to detoxify by chemical means natural goods contaminated with aflatoxins (Codifer et al., 1976; Dollear et al., 1968; Dwarakanath et al., 1968; Feuell, 1966; Fischbach and Campbell, 1965; Gardner et al., 1971; Kiermeier and Ruffer, 1974; Mann et al., 1970, 1971; Sreenivasamurthy et al., 1967; Trager and Stoloff, 1967). Only the treatment with volatile bases such as methylamine and ammonia has reached some practical importance in animal nutrition (Bagley, 1979; Brekke et al., 1978, 1979; Koltun et al., 1974). Acute toxicity of the treated products appear to be greatly diminished by these procedures (Boulton et al., 1979; Friot et al., 1975; Hughes et al., 1979; Manabe et al., 1977; McKinney et al., 1973; Norred, 1979; Southern and Clawson, 1980; Vesonder et al., 1975; Waldroup et al., 1976).

The only carcinogenicity study concerning ammoniated, aflatoxin-containing products published before the beginning of the experiments described here showed a marked reduction of heptatoma incidence from 96% to a nondetectable level in rainbow trout (*Salmo gairdneri*) fed with corn ammoniated at 49 °C (Brekke et al., 1977a,b). Questions still remain open (Ferrando et al., 1975; Vesonder et al., 1975) with respect to carcinogenicity of residual aflatoxin and the properties of compounds formed during ammoniation at ambient temperature as proposed by Bagley (1979). Furthermore, a possible carry-over into animal products so far has not been investigated. Although trouts are especially sensitive to aflatoxins, their metabolism is different from mammals and extrapolation to the human situation is difficult. In addition, it is not clear if water-soluble compounds (an important fraction of products derived from aflatoxin B_1 during ammoniation at 25 °C) are lost into water during ad libitum feeding of fish.

Mutagenicity studies with the Ames test have shown a 450-fold decrease in mutagenic activity toward bacterial strains *Salmonella typhimurium* TA100 for AFD_1, a major ammoniation product of aflatoxin B_1, in the absence of plant material (Lee et al., 1981), but little is known about the mutagenicity of ammoniation products formed from aflatoxins in natural goods. It must be kept in mind, however, that mutagenicity and carcinogenicity do not correlate well in the case of aflatoxins (Wong and Hsieh, 1976).

The main experiment described in this paper was designed in order to recognize any strongly DNA alkylating compounds formed from AFB_1 during ammoniation that could threaten domestic animals as well as—by carry-over into animal products—humans. In the mean time a long-term feeding study with rats has proved the safety of ammoniated, aflatoxin-containing corn in animal feeding and the absence of tumor formation (Norred and Morrissey, 1983).

MATERIALS AND METHODS

Labeling of Aflatoxins. [^{14}C]AFB_1 was biosynthetically prepared according to Hsieh and Mateles (1971) from sodium acetate-*1*-^{14}*C*) in cultures of *Aspergillus parasiticus*. Consecutive TLC separations on silica gel (Merck, Darmstadt; No. 5721) in chloroform–acetone–water, 90:10:0.3, and twice in chloroform–methanol, 97:3, yielded 6.45 μCi of labeled AFB_1 with a specific activity of 32 mCi/mmol (amount of AFB_1 determined from UV-absorption spectrum) and 99% radiochemical purity as determined by TLC in both systems. [^{3}H]AFB_1 was prepared by a similar method from sodium acetate-2-^{3}*H* (The Radiochemical Centre, Ltd., Amersham, England). Modifications consisted of an atmosphere enriched to 40% oxygen and a closed system with magnetic stirring instead of the open shaken culture flask. After lyophilization to remove volatile radioactivity and workup as described above, 0.7 μCi of [^{3}H]AFB_1 was obtained with a specific activity of 3.1 mCi/mmol.

Spiking of Corn. Twenty grams of Yellow Plata corn grits of Argentinian origin (W. Schuler Co., 6422 Steinen, Switzerland) containing 12.09% moisture was spiked with 2.5 ppm of [^{14}C]AFB (5.05 μCi, specific activity 32 mCi/mmol). After the mixture was stirred overnight, the solvent was driven off in a stream of nitrogen. The corn was then dried in vacuo at room temperature. In small corn samples, soaked with 50% methanol overnight, 91.1 ± 4.4% of the added radioactivity was detected in scintillating counting.

Ammoniation of Aflatoxin B_1 in a Sealed Glass

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0021-8561/85/1433-0311$01.50/0

Tube. Ten milligrams of inactive (Senn Chemicals, Dielsdorf, Switzerland; No. 8001) and 0.09 μCi of ^{3}H-labeled AFB_1 were placed in a 20-mL glass tube together with 1 mL of 25% NH_3 (Merck; No. 5432) and heated for 4 h to 100 °C after sealing under vacuum. The contents of the tube were subsequently diluted to 5 mL of total volume and lyophilized. Thirty milliliters of water only partially dissolved the residue. The solution was extracted 3 times with 10 mL of dichloromethane (Merck; No. 6050). The aqueous layer was adjusted to pH 12.5 with 1 M NaOH to dissolve the solids and extracted 3 times with 10 mL of dichloromethane. Aliquots of the different organic layers were analyzed by radio-TLC on silica gel (Merck; No. 5554) with chloroform–acetone, 85:15, 80:20, and 70:30, and the eluant. Spots were visualized with fast blue B (Fluka, Buchs SG, Switzerland; No. 44670) according to Mücke and Kiermeier (1971). Aflatoxin D_1 and the compound yielding a red color with fast blue B were isolated by TLC with chloroform–acetone, 85:15.

Radiochemical purities exceeded 94% in the solvent system mentioned last. Mass spectra of these two compounds were obtained on a Varian-MAT CH-7-A spectrometer with direct inlet. For NMR studies, the dichloromethane fraction was separated on silica gel TLC plates (Merck; No. 5717) in chloroform–acetone, 9:1. The AFD_1 band was scraped off and further purified twice by TLC in the same solvent system. ^{1}H NMR spectra were recorded on a Bruker WH 90 FT spectrometer in deuterated chloroform (Merck). To detect an exchangeable phenolic proton, the sample solution was shaken with deuterated water.

Ammoniation and Workup of Corn Spiked with [^{14}C]Aflatoxin B_1. A procedure similar to the methods proposed by Lee et al. (1974) and Cuculla et al. (1976) was used. The spiked corn grits were placed in a 100-mL round-bottom flask. Concentrated ammonia (Merck; No. 5432; d_{20} = 0.897 corresponding to 24.8% NH_3) was added to yield 2.02% NH_3 (dry base). The flask was then sealed with a well-greased ground-glass stopper and incubated at 25 °C for 34 days in a Gallenkamp IH-290 refrigerated incubator. At the end of the incubation period, the flask was frozen in liquid nitrogen for subsequent lyophilization. Combustion of two ammoniated corn samples yielded a recovery corresponding to 81.2% of the total radioactivity added. After soaking overnight at 0 °C in 50% methanol, 80.3% of the total radioactivity was recovered by direct liquid scintillation counting.

The lyophilized corn was split in two samples, one of which was covered with water, acidified to pH 3.5 with acetic acid (Merck; No. 63), and lyophilized again after stirring at 4 °C for 1 h. Parallel workup of both samples was carried out as follows: 4.5 g of corn was homogenized in 10 mL of 90% methanol at 0 °C to a slurry of finely divided particles by means of a Polytron kinematic high-frequency homogenizer (Kinematica GmbH, CH-6010 Kriens, Switzerland). After addition of 10 mL of 90% methanol, shaking for 5 min, centrifugation, and separation of supernatant, the sample in each tube was reextracted in the same way once more with 20 mL of 90% methanol, twice with 20 mL of 70% methanol, and 3 times with 20 mL of water. Each extract was combined and shaken twice with 30 mL of pentane (Merck; No. 7177). Forty Milliliters of water was added to each of the two 90% methanol extracts and 30 mL to the 70% methanol extracts. All these solutions were then extracted 4 times with 30 mL of dichloromethane (Merck; No. 6050). The dichloromethane layers were reextracted twice with 10 mL of water, dried over anhydrous sodium sulfate, and taken to dryness in a nitrogen stream. From each dichloromethane extract AFB_1 was removed by TLC on silica gel in chloroform–acetone–water, 90:10:0.3. The non-B_1 part of all extracts (acidified and nonacidified) were combined and subjected to TLC in chloroform–methanol, 97:3 (to remove remaining traces of aflatoxin B_1), followed by desorption with dichloromethane–ethanol, 2:1, and evaporation of solvent in a nitrogen stream.

Aqueous phases from partitioning and aqueous extracts were continuously fed into a 8-mm dialysis tubing (Union Carbide, Chicago, IL; No. 13 DM 30 C 205 V 1) for vacuum dialysis over a period of 5 days at 4 °C. The dialysates were lyophilized, and the residues were washed out with methanol and water, consecutively, combined separately for acidificed and nonacidified corn, and lyophilized. The residues were each suspended in 3 mL of water for application to rats. The extraction residues were stored at −20 °C and lyophilized together with the dialysates.

Mutagenicity Testing of Aflatoxin D_1. The mutagenicity of AFD_1 was tested along with that of AFB_1 by using the Ames *Salmonella*/mammalian microsome assay (Ames et al., 1975). The test compounds were dissolved in Me_2SO (50 μL/plate). *S. typhimurium* TA100 was used as the tester strain. Instead of using overnight cultures, the bacteria were incubated for 5 h on a rotary shaker at 37 °C prior to testing, thus yielding cells at the end of the logarithmic growth phase [(2–3) × 10^9 cells/mL). For enzymatic activation 10% rat liver S-9 mix was used (Aroclor 1254 induced 200-g Sprague-Dawley male rats; 4 mg of protein/mL of mix).

DNA-Binding Studies. For determining the covalent binding index (CBI) of the fractionated ammoniation products to rat liver DNA, two male Ivanovas-Sprague-Dawley rats (Dr. Ivanovas, 7964 Kisslegg, GFR) were used for each fraction. A suspension of the combined dichloromethane-soluble fractions (AFB_1 removed by TLC) of both acidified and nonacidified corn in 8.5% (w/w) ethanol and separate suspensions (in 3 mL of H_2O/5% Tween-80) of the lyophilized aqueous extracts were administered to rats intragastrically. The extraction residues were fed to rats with a little water added to form a dew. This prevented loss of the fine powder during feeding. All rats were starved from hour 18 to hour 6 before application. All animals except the ones consuming the extracted corn were then placed back on the regular diet up to intragastric administration of the extracts. Six hours after application (animals with extracted corn 12 h after the start of consumption), the rats were anesthetized with ether and killed by heart puncture. The livers were removed and cut to small pieces. From 10-g aliquots, DNA was isolated according to Sagelsdorff et al. (1983).

Liquid Scintillation Counting. Liquid scintillation counting was carried out in Aquasol (New England Nuclear, Inc., Boston, MA) with a betaszint bf 5001/300 liquid scintillation counter (Berthold, Wildbad, GFR).

Radio Thin-Layer Chromatography. For radio thin-layer chromatography, silica-coated aluminum sheets were cut into 1 by 1 cm pieces after development, each of which was placed in a vial with 1 mL of methanol. After the mixture was gently shaken, 10 mL of scintillation cocktail was added for counting.

RESULTS

Ammoniation of [^{3}H]Aflatoxin B_1 in a Sealed Glass Tube. [^{3}H]AFB_1-labeled biosynthetically according to Hsieh and Mateles (1971) was used as a tracer to obtain quantitative data for the ammonia degradation of pure compound. A procedure similar to the method proposed by Lee et al. (1974) was used. Twenty-four percent of the

Table I. Mutagenicity of Aflatoxins B_1 and D_1 in *S. typhimurium* TA100 [Data Compared to Results of Lee et al. (1981)]

Aflatoxin B_1			
dose applied/ plate, nmol	net revertants[b-d] with S-9/nmol	dose applied[a]/ plate, nmol	net revertants[a] with S-9/nmol
0.005	5200 ± 2800		
0.020	7300 ± 750		
		0.064	6700
0.080	12010 ± 1780		
		0.12	4360
		0.16	7870
		0.21	5500
0.32	11670 ± 1750	0.32	5620
Aflatoxin D_1			
dose applied/ plate, nmol	net revertants/ nmol	dose applied[a]/ plate, nmol	net revertants/ nmol
2.2	93 ± 12.4		
8.8	89 ± 14.6		
35.2	90 ± 7.4	35	20
		70	12
141.0	64 ± 4.7	140	14
		210	12

[a] Data of Lee et al. (1981). [b] Spontaneous revertants per plate: 102 ± 11. [c] Standard deviation $s = (s^2_{\text{rev./plate}} + s^2_{\text{spontaneous rev.}})^{1/2}$. [d] Manual counting. Great numbers in sections of plate ($^1/_{10}$, $^1/_{20}$). Electronic counting yielded lower results by $^1/_2$–$^1/_4$ and therefore was abolished.

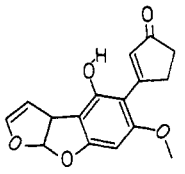

Figure 1. Structural formula of aflatoxin D_1.

total radioactivity was found in the lyophilization cold trap. After the lyophilization residue was partitioned between dichloromethane and water pH 7, the organic layer contained 49.6% of tritium. A white solid not dissolved during this step became soluble under basic conditions. A second dichloromethane extract yielded only 0.6% of tritium, while the residual aqueous phase contained 24.7%, an aliquot of this aqueous phase was shown to contain 13.9% of the total tritium extractable with diethyl ether after reacidification. This is a clear indication for the formation of acidic products other than AFD_1 during ammoniation. Radio thin-layer chromatography of the neutral dichloromethane extract showed AFB_1 to contain only 0.6% of the total recovered radioactivity. AFD_1 is clearly recognized as the main product resulting from ammoniation at elevated temperature and pressure, while the compound with m/e 206 seems to be of minor importance compared to the more polar products under the conditions applied.

Isolation of Aflatoxin D_1 and Molecular Weight 206 Compound. The main ammoniation product, AFD_1 (Figure 1), has previously been described (Lee et al., 1974; Stanley et al., 1975). The substance obtained by desorption from TLC plates showed spectral and chemical properties identical with those described by the abovementioned authors. The proton magnetic resonance spectrum (not published before) fully confirmed their structural proposal. In radio TLC, less than 3% of the total radioactivity were associated with a zone appearing orange after spraying with fast blue B. UV and mass spectra of the desorbed zone were in full agreement with

Table II. In Vivo Covalent Binding of Aflatoxins B_1 and D_1 to Rat Liver DNA

substance	dosage, dpm/kg of body wt	radioact. of isolated DNA, dpm/mg of DNA	CBI
aflatoxin B_1	2.8×10^6	209.7	22800
aflatoxin D_1	1.9×10^6	<0.43	<72 (animal 1)
	1.1×10^6	<0.24	<70 (animal 2)

Table III. Distribution and Recovery of [^{14}C]Aflatoxin-Derived Radioactivity in Spiked Corn after Ammoniation for 34 Days

	9.075 g,[a] 4952400 dpm,[b] for acidified			9.297 g,[a] 5073400 dpm,[b] for nonacidified		
fraction	dpm	% of	total	dpm	% of	total
dichloromethane						
before TLC	690000		13.9	364000		7.1
TLC						
AFB_1	190500	3.8		104000	2.0	
AFD_1	87500	1.8		47000	0.9[c]	
estimate other	221000	4.5		99000	2.0	
recovery	464000	10.1		250000	4.9	
aqueous phase						
dialyzable	1449000		29.3	1901000		37.5
remaining	76000		1.5	251000		4.9
residual corn	1872000		37.8	1661000		32.7
total recovery	4087000		82.5	4177000		82.2

[a] Sample size. [b] Radioactivity determined by extraction of spiked corn before ammoniation. [c] Estimation from two-dimensional TLC of non-AFB_1 fraction desorbed for the DNA-binding experiment.

that reported by Cucullu et al. (1976) for the molecular weight 206 compound.

Biological Activity of Aflatoxin D_1. AFD_1 has previously been recognized as mutagenic to *S. typhimurium* TA100 in the presence of S-9 rat liver homogenate (Lee et al., 1981). Its observed mutagenicity was 450-fold lower than that of its parent compound, AFB_1. Using the same bacterial strain, we obtained comparable results that are compiled in Table I. The mutagenicity of aflatoxin D_1, although lowered to a great extent with respect to B_1, is not neglectable. Since mutagenicity and carcinogenicity do not correlate well in the case of aflatoxins, a DNA-binding experiment was carried out with [^{14}C]AFD_1 prepared by ammoniation of [^{14}C]AFB_1 using the procedure already described. The CBI of AFB_1 was determined in the same experiment as a positive control. Table II shows the CBI values observed.

Ammoniation of [^{14}C]Aflatoxin B_1 in Corn: Tracer Distribution and in Vivo Binding Activity of Decomposition Products to Rat Liver DNA. The experiment was carried out in order to establish the DNA-binding activity of AFB_1 degradation products formed during ammoniation of corn. Since AFB_1 is known to have the greatest CBI of all aflatoxins (Lutz, 1979; Lutz et al., 1980), the ammoniated corn was extracted and fractionated mainly to eliminate AFB_1. Covalent DNA binding was measured separately for the portion soluble in organic solvent, the water-soluble fraction, and the residual corn. The results of the fractionation procedure are given in Table III. Total recoveries are 82.5% of the value obtained in combustion before ammoniation for the acidified and 82.2% for the nonacidified corn. From vacuum dialysis of the aqueous extracts, it becomes clear that most of the radioactivity is attributable to molecular weights smaller than 14000. With methylene chloride, 3.8% of the

Table IV. Genotoxicity of Fractions Derived from Ammoniated Aflatoxin-Containing Corn Expressed in Units of Covalent Binding Index (CBI) after 34 Days of Ammoniation

fraction	dpm/kg of body wt[a]	dpm/mg of DNA ± 2s	CBI ± 2s	detection limit[b]
residual corn (acidified)	2 898 000	0.62 ± 0.56	b.d.l.[c]	117
	3 302 000	0.84 ± 0.345	b.d.l.	103
H_2O dialyzed (acidified)	2 650 000	0.46 ± 0.53	b.d.l.	128
	2 080 000	1.93 ± 0.165	287 ± 25	163
residual corn (nonacidified)	2 686 000	1.03 ± 0.4	b.d.l.	126
H_2O dialyzed (nonacidified)	2 156 000	5.48 ± 1.19	783 ± 170	157
	2 036 000	8.04 ± 0.385	1219 ± 59	167

[a] Male Ivanovas Sprague-Dawley rats. Weight 300.9 ± 23.43 g (2s). [b] Detection limits calculated according to Lutz (1979). [c] b.d.l. = below detection limit.

radioactivity was isolated as AFB_1 from the acidified product compared to 2.0% in the nonacidified portion. The nonacidified aqueous extract contained additional AFB_1 (extractable from an aliquot after acidification), which together with the 2.0% from dichloroethane adds up to a total of 3.5% of the radioactivity in the nonacidified corn. Ammoniation therefore achieved a reduction in AFB_1 between 94.3 (observed 33% loss of AFB_1 in TLC accounted for) and 96.1% (2.5 ppm reduced to 10 ppb). Two-dimensional TLC showed between 14.4 and 23.3% of the total non-B_1 radioactivity in aliquots of the dichloromethane-soluble portion to cochromatograph with added inactive AFD_1. This means that no more than 3% of the total activity was present as AFD_1. AFD_1 was recognized by its very weak, purple fluorescence only when the entire dichloromethane-soluble part of the extract obtained with 70% methanol was separated on one silica gel TLC plate. We have also observed this fluorescence with the pure compound prepared for mutagenicity testing. In the extracts obtained with 90% methanol, a yellow substance quenched the fluorescence of aflatoxin D_1 completely.

DNA-Binding Studies. For DNA-binding studies, the amount of AFB_1 present in the combined dialysates was determined by two-dimensional TLC of dichloromethane extracts obtained from diluted aliquots after addition of inactive AFB_1. At total of 3.3% of the activity present in the water-soluble portion of acidified corn was extracted, but less than 0.5% of the total aliquot was recovered from the AFB_1 spot. The dialysates of the nonacidified corn contained 10.1% extractable radioactivity including 4.1% AFB_1 (i.e., 1.5% of the total activity before ammoniation). The results of the DNA-binding experiment are listed in Table IV. The non-B_1 dichloromethane-soluble fraction (due to its limited total radioactivity) yielded a CBI no distinguishable from the detection limit.

DISCUSSION

In vivo DNA binding of a chemical substance or its metabolites is thought to be one important factor among several others involved in chemical carcinogenesis. A good correlation between covalent DNA binding and carcinogenic potency could be found for genotoxic carcinogens, i.e., within 1 order of magnitude (Lutz, 1979; Lutz et al., 1980; Garner, 1980). DNA-binding studies allow distinction between strong, intermediate, and low carcinogenic activity in the target organ under consideration.

In vivo covalent binding is determined by comparing the radioactivity attached to liver DNA with the total amount of labeled substance administered to the corresponding animal. The result is expressed in terms of a "covalent binding index" (CBI), which is defined by

$$\text{CBI} = \frac{\text{damage}}{\text{dose}} = \frac{\mu\text{mol of substance bound}/\mu\text{mol of DNA nucleotide}}{\text{mmol of substance applied/kg of animal}}$$

If mixtures of different, even unknown, radiolabeled compounds are to be assessed for DNA-binding activity, the CBI of the mixture is defined as

$$\text{CBI} = \frac{\text{radioactivity bound}/\mu\text{mol of DNA nucleotide}}{\text{radioactivity applied/kg of animal}}$$

Measuring the CBI of mixtures has the advantage that any synergistic effects are expressed in the result. An example for determining the CBI of a mixture has been published by Jaggi et al. (1980).

The setup for the present paper is a compromise. The quantity really interesting is the reduction of CBI in corn ammoniated, with respect to the CBI of the same batch of corn before ammoniation. Since AFB_1 is one of the most powerful DNA-alkylating agents and was expected to still occur after ammoniation, it would have overshadowed all other alkylation agents. It was therefore decided to split the ammoniation products into several fractions, remove AFB_1, and measure separate CBIs for each. In order to estimate the total CBI of the mixture a "contribution to the CBI of the mixture" is defined for each fraction as the CBI of that fraction multiplied by the relative importance of the fractions activity with respect to the total of the mixture.

It can be shown by simple algebraic conversion of the formulas for the CBIs that the sum of the contributions from all fractions add to the CBI of the mixture, assuming that no synergistic effects between single fractions influence the true total CBI.

In Table V, column 4, the "contributions" are calculated in this manner by using the measured CBI of each fraction. The total of column 4 therefore approximately corresponds

Table V. Quantitative Interpretation of DNA-Binding Results after 34 Days of Ammoniation

fraction	% ^{14}C of total added	CBI	contribution to CBI of total corn: before[a] NH_3	contribution to CBI of total corn: after NH_3
acidified				
CBI known				
H_2O dialysates	13.0	<146	2600	<19
extracted corn	16.7	<110	3340	<18
aflatoxin B_1	2.4	20 000	480	240
CH_2Cl_2 (non-B_1)	3.8	<1200	760	<46
CBI unknown				
macromolecular	0.7	<20000	<140	<140
activity unaccounted for[b]	12.5		1250	
total	49.1		9820	463
nonacidified				
CBI known				
H_2O dialysates	17.0	1000	3400	170
extracted corn	14.8	<110	2960	<19
aflatoxin B_1	1.4	20 000	280	140
CH_2Cl_2 (non-B_1)	1.9	<1200	380	<23
CBI unknown				
macromolecular	2.2	<20 000	<440	<440
activity unaccounted for[b]	13.5		2700	
total	50.8		10160	572

[a] Calculated as a fraction of 20 000 for total aflatoxin B_1. [b] Included in CBI of extracted corn (activity not extracted).

to the CBI of ammoniated corn. For column 3, the same computation was carried out by using the CBI of unchanged AFB_1 (20000) for all fractions. The value obtained thus corresponds to the (hypothetical) contribution of the radioactivity of a fraction existing as AFB_1 prior to ammoniation.

If, in order to stay on the safe side, the macromolecular fraction (whose CBI is not known) is assumed to have the same CBI as the original aflatoxin, a 20-fold reduction in CBI has been brought about by ammoniation. The greatest single contributor to the overall CBI after ammoniation is residual AFB_1. It probably is also responsible for the higher CBI of the nonacidified dialysates, for this fraction contained 4.1% of radioactivity cochromatographing with AFB_1 in two-dimensional TLC. If AFB_1 could somehow be eliminated after ammoniation, e.g., by extraction, the CBI reduction would be approximately 100-fold. The radioactivity not accounted for can be taken as nonbinding, since it either consists of $[^{14}C]CO_2$ (lost during workup) or is not extractable from corn and therefore is included in the CBI of the latter.

Effectiveness of Ammoniation in Aflatoxin Destruction. Treatment of naturally contaminated corn with gaseous ammonia has been reported to reduce aflatoxin levels from 1000 to 32 ppb at 25 °C within 2 days and to 10 ppb within 14 days (Brekke et al., 1977a,b, 1978). Other authors reported less spectacular decontamination results. In one case, a natural contamination of 2000 ppb was reduced to 510 ppb within 6 days (Southern and Clawson, 1980). Ground corn artificially spiked with radiolabeled AFB_1 was brought from 6000 to 360 to 480 ppb within 14 days and remained at this level up to day 29 (Beckwith et al., 1975). An experiment similar to the one described in the present paper confirmed the latter results: 6300 ppb was reduced to 370 ppb in 14 days (Schroeder al., 1981). The experiment reported here again was in full agreement with these two results for artificially spiked corn: 2500 ppb was reduced to 83–128 ppb (3.3–5.1%) within 34 days at similar conditions.

For four reasons it is surprising that AFB_1 can be present at all together with ammonia and water: (1) Hydrolysis of the lactone ring is known to occur within a few hours at room temperature (Kiermeier and Ruffer, 1984; Vesonder et al., 1975). (2) The equilibrium should be shifted toward hydrolysis products by the excess of water and by deprotonation of the resulting substituted coumaric acid as well as by subsequent degradation. (3) The decarboxylation after hydrolysis is most likely a first-order reaction (March, 1977). It seems to proceed at a rather fast rate at 25 °C (Brekke et al., 1978). (4) Cracking of corn seems to enhance AFB_1 destruction (Brekke et al., 1978). It seems therefore very likely that part of the AFB_1 is protected from degradation by a secondary effect, such as diffusion of AFB_1 into regions not reached by ammonia and water, or by formation of a more stable salt with a different cation, after hydrolysis. It is interesting to note in this context that about 50% of the AFB_1 recovered after ammoniation is present in a water-soluble form, if no acidification occurs prior to extraction, thus emphasizing the latter suggestion.

It cannot be ruled out completely that the solvent used for spiking corn (inevitably necessary for subsequent DNA-binding experiments) transported some aflatoxin B_1 into regions that with natural contamination do not contain aflatoxin and are not accessible to the degradation reagents.

Biological Properties of Aflatoxin D_1 and Importance in Ammoniated Corn. Alkylation of DNA by aflatoxins is known to be an addition of a nucleotide base to the dihydrofuran moiety in the 8-position of the aflatoxin carbon skeleton (Wang and Cerutti, 1980). All strongly alkylating aflatoxins contain the 8,9-dihydrofuran double bond. It is surprising that AFD_1, which also contains this double bond, is lacking the strong in vivo DNA-binding activity. Alkylation of DNA therefore seems to depend on both the dihydrofuran and the lactone ring moieties, but there is no simple explanation for its absence in AFD_1. More information is needed concerning organotropy and metabolism of this compound. In the case of aflatoxins, the correlation between mutagenicity in the Ames test and DNA-binding activity is poor (Wong and Hsieh, 1976). AFD_1 behaves in the reverse manner of AFG_1, which shows high CBI and low mutagenicity.

The quantity of AFD_1 found after ammoniation at 25 °C is of much less importance than originally thought after high-temperature ammoniation of pure AFB_1. A proper analysis for AFD_1 was not worked out for quantification in ammoniated corn, but the maximum amount can be estimated from two-dimensional TLC of the dichloromethane-soluble portion. A total of 1.8% of the total radioactivity in the ammoniated corn could be shown to cochromatograph with inactive AFD_1 added to the dichloromethane extract for TLC.

ACKNOWLEDGMENT

We are grateful to Mr. F. Bangerter, Institute of Technical Chemistry, ETH Zuerich, for recording NMR spectra and to Dres. B. Zimmerli and F. Friedli, Eidg. Bundesamt fuer Gesundheitswesen, Berne, for mass spectral analyses. H. Frohofer, Chemistry Department, University of Zuerich, kindly carried out Microcombustion of radiocarbon.

Registry No. AFB_1, 1162-65-8; AFD_1, 52373-83-8.

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Received for review April 25, 1983. Revised manuscript received July 17, 1984. Accepted November 26, 1984. This work was partially supported by the Swiss National Foundation (fellowship to T. S. for the 13th Postgraduate Course in Experimental Medicine 1980/81 at the University of Zuerich).

An HPLC Method for the Determination of Reactive (Available) Lysine in Milk and Infant Formulas

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A high performance liquid chromatographic procedure for the determination of reactive (available) lysine was developed for the analysis of samples of high carbohydrate content, such as milk or infant formulas. The sample is dialyzed, derivatized with 2,4,6-trinitrobenzenesulfonic acid, acid hydrolyzed, and the ϵ-TNP-lysine quantitated by HPLC. This procedure yielded values similar to those obtained by a conventional spectrophotometric method but did not require the use of reagent and sample blank corrections.

Excessive heat processing, or storage under adverse conditions, of a protein foodstuff of a high carbohydrate content will result in a loss of essential amino acids, particularly lysine (Henry and Kon, 1958; Mauron, 1961). The reaction of the free amino group of lysine in the protein with carbohydrate (Maillard reaction) renders the lysine nutritionally unavailable. Several chemical procedures for the estimation of available lysine (reactive lysine) have been based on the reaction of the free (unbound) ϵ-lysine amino group in the protein with a chromophoric reagent; the treated protein is then hydrolyzed and the concentration of the lysine derivative is determined spectrophotometrically. The reagent used in the classical procedure of Carpenter (1960) is fluorodinitrobenzene (FDNB); the procedure of Kakade and Liener (1969) utilizes trinitrobenzenesulfonic acid (TNBS), which was shown by the latter investigators to yield values similar to the Carpenter method.

In the spectrophotometric procedure, the solution of the hydrolyzed derivatized protein is extracted with organic solvents to remove excess reagent and colored byproducts of the reaction. A reagent blank is required to correct for incomplete extraction. An additional blank, containing the sample without the reagent, corrects for the colored products produced during the hydrolysis of the protein. Corrections are also applied for incompleteness of the reaction induced by high levels of carbohydrate (Posati et al., 1972).

The high performance liquid chromatographic (HPLC) procedure described here was developed for the assay of samples of milks and infant formulas, samples with a high lactose to protein ratio. The samples are dialyzed to remove the interfering lactose (Greenberg et al., 1977), derivatized, and hydrolyzed, as in the Kakade–Liener method, and the aqueous solution is injected directly onto the chromatographic column. Extraction with an organic solvent, or the necessity for reagent or sample blanks are eliminated. This simplified procedure yielded results essentially similar to the spectrophotometric procedure of Kakade and Liener.

EXPERIMENTAL SECTION

Apparatus. The HPLC instrument was a Hewlett Packard 1084B, and the spectrophotometer, a Perkin Elmer Lambda 3A; a laboratory autoclave or household pressure cooker was used for the hydrolysis; the dialysis tubing was Spectrapor, No. 3787-D32, 12 000 MW cutoff.

Purified Proteins. Bovine serum albumin, lysozyme, and ovalbumin were purchased from Sigma, and zinc–

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0021-8561/85/1433-0316$01.50/0