Mycotoxins Produced by *Aspergillus fumigatus* Species Isolated from Molded Silage

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Results are presented which strongly suggest that strains of *Aspergillus fumigatus* are one of the predominant fungi in molded corn silage. The clavine alkaloids, fumigaclavine A, a new alkaloid designated fumigaclavine C, and several tremorgens belonging to the fumitremorgen group were produced by *A. fumigatus* strains isolated from molded silage. The LD_{50} of fumigaclavine C was about 150 mg/kg oral dose in day-old cockerels. Calves dosed with crude extracts of *A. fumigatus* cultures experienced severe diarrhea, irritability, loss of appetite, and postmortem examination showed serous enteritis and evidence of interstitial changes in the lungs; abnormal changes were not found in other tissues.

We have investigated a naturally occurring acute toxic syndrome in beef cattle of unknown etiology. The syndrome was characterized by a general deterioration of the herd which was typical of protein deficiency and malnutrition, resulting in some mortalities even though ample pasture and supplemental feed were available. Other clinical signs were diarrhea, irritability, and abnormal behavior. Necropsy of a cow and calf showed that both were emaciated. There were signs of previous ostertagia in the abomasum of the cow. The only other gross abnormality was a large (8-in. diameter) ulcer in the wall of the rumen of the calf. Evaluation of the rumen ulcer indicated that both a fungus and bacterium were involved in its development.

Histopathological examination showed that the hepatic parenchymal cells of both animals had a relatively clear cytoplasm suggestive of protein depletion. The cattle involved were being fed corn silage, from a trench-type silo, that had molded because the packed silage face had been exposed to the atmosphere for 1–2 weeks before emptying and feeding. The silo was too wide for the size of the herd and improper emptying and feeding practices were used. The major fungus isolated from the molded silage was identified as *Aspergillus fumigatus* (AFu-4).

We now report the toxin-producing potential of six isolates of *A. fumigatus* from several sources. Three isolates (AFu-3, AFu-4, AFu-5) represent the dominant fungi from two silos associated with toxic syndromes of cattle and one (AFu-6) was from an unused silo. The isolation and identification of the major toxins produced by these fungi are described. Preliminary data on the effects of crude extracts of one *A. fumigatus* isolate (AFu-3) on test cattle are presented.

MATERIALS AND METHODS

Isolation and Culture of Fungi. Four *A. fumigatus* strains (AFu-3, AFu-4, AFu-5, and AFu-6) representing the predominant fungi in molded silage from three silos were isolated on potato dextrose agar (PDA) plates incubated at 30 and 37 °C. Two *A. fumigatus* isolates (AFu-1 and AFu-2) were provided by Dr. G. P. Lynch, USDA, ARS, Nutrition Institute, Ruminant Nutrition Lab., Beltsville, Md. All six isolates were maintained on PDA slants at 5 °C after 4–7 days growth at 30 °C.

The fungi were cultured on several media to evaluate their toxin-producing potential. Media were: shredded wheat supplemented with Difco mycological broth (adjusted to pH 4.8) plus YES medium (Kirksey and Cole, 1974; Davis et al., 1966); Difco mycological broth supplemented with YES medium (Kirksey and Cole, 1974); sterilized and nonsterilized silage; hydrated rice for solid state shake and static fermentation (Shotwell et al., 1966). Fungi for mass production of toxins were cultured on mycological broth plus YES medium (200 mL/2800 mL fernbach flask) at 35–40 °C.

Extraction of Toxins. For extraction of toxins, fungal cultures were heated with an equal volume of chloroform, then homogenized in a Waring Blendor. The crude chloroform extracts were filtered first through cheesecloth, then by vacuum through anhydrous sodium sulfate.

Purification of Toxins. The filtered crude chloroform extract was concentrated under vacuum, redissolved in ethyl acetate, and partitioned three times between an aqueous solution adjusted to pH 2.0 with HCl. The acidic phases were combined, made basic (pH 10.0) with sodium carbonate, and partitioned three times with chloroform. The chloroform fractions were pooled and washed three times with distilled water. The extract was dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. The resulting concentrate was chromatographed on a column (2.5 × 10 cm) containing neutral alumina that was deactivated to activity grade IV (ICN Pharmaceuticals, Inc., Cleveland, Ohio). The column was packed as a slurry in benzene, the sample was applied in benzene solution, and the column was eluted with benzene (Scheme I). The column was equipped with an automatic fraction collector that collected 17-mL fractions.

The neutral fraction that contained tremorgenic activity was fractionated on a Florisil column (5 × 50 cm). The column was packed in benzene and eluted with a linear gradient from benzene to ethyl acetate, followed by a second gradient from ethyl acetate to acetone. The column was equipped with a fraction collector and 501 (17 mL) fractions were collected.

Physical and Chemical Analyses. The toxins were analyzed by thin-layer chromatography (TLC) on 20 × 20 cm glass plates coated with silica gel GH-R (0.50 mm thickness). The developing solvent was toluene–ethyl acetate–formic acid, 5:4:1 v/v/v. The toxins were visu-

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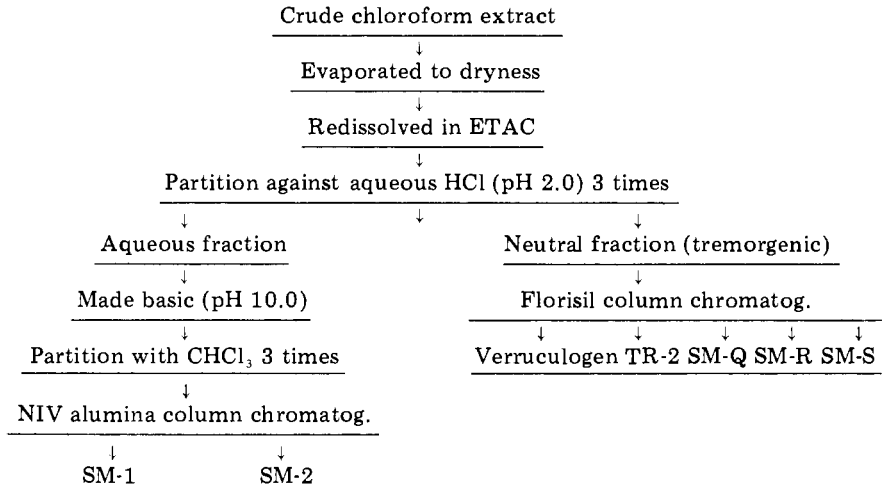
Scheme I. Isolation Technique for *A. fumigatus* Toxins



alized by spraying TLC plates with 50% ethanolic H_2SO_4 and heating them for 5 min at 100 °C.

Melting points were determined on a Kofler micromelting point apparatus and were uncorrected. Ultraviolet spectra (UV) of the toxins were taken with a Beckman Model DB-G recording spectrophotometer in methanol solution. Infrared spectra (IR) were taken with a Perkin-Elmer Model 257 IR spectrophotometer equipped with a 4X beam condenser. Samples were analyzed as a thin film coated onto KBr windows.

High-resolution mass spectral analyses (hrp) were made with an A.E.I. MS-9 mass spectrometer. Samples were introduced into the ion source by the direct-probe method and ionization was effected by electron impact at 70 eV. The ion-source temperature was kept at 200 °C, and high-resolution measurements were made by peak matching with perfluorokerosene as the internal standard.

Proton decoupled, natural abundance ^{13}C NMR spectra were obtained on a JEOL PFT-100 spectrometer equipped with the JEOL EC-100 data system. A pulse angle of 30 with a repetition rate of 3 s was used to collect the data. The FID's were collected into 8K of memory yielding FT spectra of 4K data points. A filter corresponding to 5000 Hz sweep width was used. Single frequency, off-resonance proton decoupled spectra were obtained on each sample. Samples were prepared in $CDCl_3$ solution and chemical shifts are reported in ppm downfield from internal Me_4Si. Proton spectra were obtained on the identical solutions used for ^{13}C spectra with a Varian Associates HA-100 spectrometer. Proton chemical shifts are reported in ppm downfield from internal Me_4Si.

Compound SM-1, $C_{23}H_{30}N_2O_2$, crystallized from 95% ethanol as colorless needles (mp 190 °C) in the space group $P4_1$ with A = 1.535 (1) and C = 23.917 (4) Å. Reflection data were collected by use of an automatic four-circle diffractometer with Cu Kα radiation (λ = 1.5418 Å). Of the 1526 unique reflections measured 1351 (89%) were considered observed ($I \geq 3\alpha(I)$) and corrected for Lorentz and polarization effects. Structure solution was carried out routinely by use of a direct methods approach (Germain et al., 1970). Full-matrix least-squares refinements (Busing et al., 1965) lowered the unweighted residual index to 0.044 for the present model including hydrogens.

Biological Studies. The chloroform extracts of *A. fumigatus* cultures were tested for toxicity to day-old cockerels by the method of Kirksey and Cole (1974). The extracts were administered orally, via crop intubation, with corn oil as the inert carrier.

Purification of toxins was monitored by bioassay with day-old cockerels (Kirksey and Cole, 1974). LD_{50} values of purified SM-1 were determined with day-old cockerels dosed orally via crop intubation.

Three Jersey calves (approximately 6 months old) that weighed 66–91 kg were maintained in digestion crates for 8 days of adjustment and assessment of normal feed intake. On day 9, one calf was orally administered several gelatin capsules containing a total of 86 g of a crude chloroform extract from *A. fumigatus* (AFu-3). The chloroform extract had been dried under vacuum and taken up in acetone and dried under vacuum for 24 h; then 86 g of crude extract was mixed with cellulose powder and put into gelatin capsules. The 86 g of crude extract contained approximately 3.25 g of SM-1. Twice daily the calves were given feed allowances of 110% of anticipated intake. Calves were maintained in crates until day 13 when the treated calf was sacrificed and subjected to postmortem examination. Later, three Jersey calves weighing 72–105 kg were maintained in digestion crates for 12 days of adjustment and assessment of normal feed intake. Beginning with day 13, two calves were dosed daily for 5 days, one with gelatin capsules, each containing 5.7 g of crude extract from *A. fumigatus* culture (200 mg of SM-1), and the other with gelatin capsules, each containing 11.4 g of crude extract (400 mg of SM-1). All three calves were maintained in digestion crates through day 27, when the calf that received five daily doses of 11.4 g of crude extract was sacrificed for postmortem examination. Blood samples from all calves, taken before, during, and after treatment, were analyzed for white blood cell count, packed cell volume, hemoglobin, total protein, differential leucocyte count, calcium, magnesium, phosphorous, bilirubin, serum glutamic oxaloacetic transaminase activity, creatinine phosphokinase activity, and sorbitol dehydrogenase activity, and body temperatures were monitored.

RESULTS AND DISCUSSION

Identification and Culture of Fungi. The predominant fungi in molded silage from four silos were *A. fumigatus* isolates. These isolates failed to grow on sterilized silage in the laboratory. However, they were climax microorganisms when cultured on unsterilized silage. This suggested that conditions produced during preliminary fermentation, probably by a succession of other microorganisms, were required before *A. fumigatus* grew on silage or sterilization destroyed the utility of the silage as a fungal substrate.

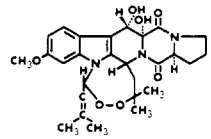
The metabolites (as determined by TLC) produced by five of the six isolates on a given medium were very similar.

The same strains produced different metabolite patterns on the different media. Variation was largest on rice between the shaken and static fermentations.

The metabolite pattern on mycological broth plus YES medium cultured at either 30 or 37 °C most nearly resembled the pattern that was produced on unsterilized silage. Therefore, that medium was selected for large scale production of *A. fumigatus* toxins by the isolate designated AFu-3.

Analyses of Extracts. The crude chloroform extracts from all six isolates, cultured on all the media, except hydrated rice by solid-state shake fermentation, contained toxic compounds that caused sustained trembling, convulsions, and/or death of day-old cockerels.

The crude chloroform extract from AFu-3 was divided into basic and neutral fractions, each containing toxic compounds. The tremorgenic activity was found primarily in the neutral fraction, which was chromatographed into five different metabolites that caused sustained tremors in day-old cockerels (Scheme I). Two of these tremorgens were identified as verruculogen (I) (Cole et al., 1975; Fayos et al., 1974) and as TR-2 (II) (Cole et al., 1975) by comparing UV, IR, ^{1}H NMR, ^{13}C NMR, mass spectra and TLC of the unknown with authentic verruculogen and TR-2. The other three tremorgens appear to be related 6-*O*-methyl indole diketopiperazine compounds that are currently under study and will be the topic of a subsequent paper.



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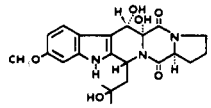


II

The basic partition was fractionated on neutral activity grade IV alumina into three toxic fractions. The eluate in fractions 2–7 contained the compound designated SM-1 with a trace of the companion compound designated SM-2. Fractions 8–11 contained both SM-1 and SM-2; fractions 12–32 contained SM-2 with a trace of SM-1. The impurities present in the basic fraction did not elute from the alumina column with benzene and separation of SM-1 and SM-2 was excellent with this column. The toxin SM-1 was crystallized from methanol as needles, mp 194 °C, from ethanol as needles, mp 190 °C; SM-2 was crystallized from methanol as needles, mp 86 °C. Yields were about 20 mg/2800 mL flask of SM-1 and 2 mg/flask of SM-2 from isolate AFu-3 cultures. Four of the *A. fumigatus* isolates, AFu-2, AFu-3, AFu-4, and AFu-5, produced SM-1 as a major component with lesser amounts of SM-2. AFu-1 produced only SM-2, and AFu-6 produced neither compound.

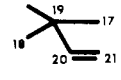
On TLC plates, SM-1 was visualized in normal light as a blue spot at R_f 0.14; SM-2 as a purple spot at R_f 0.05. SM-1 showed λ_{max} MeOH 225, 277, 283, and 292 nm (log ϵ max, 4.54, 4.01, 4.04, and 3.98). The probable assignments of the major IR absorptions of SM-1 are 3395 cm^{-1} (OH and/or indole), 1715 cm^{-1} (acetate ester), 1380 cm^{-1} (methyl), 875 cm^{-1} (terminal methylene), and 755 cm^{-1} (3 adjacent aromatic H's).

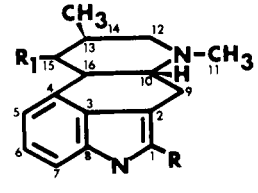
The high-resolution mass spectrum of SM-1 showed a molecular ion peak at measured mass 366.2294 *m/e* consistent with a molecular formula of $C_{23}H_{30}N_2O_2$ (calculated mass for $C_{23}H_{30}N_2O_2$ was 366.2307). The only other prominent feature in the mass spectrum was the base peak at 307.2167 *m/e* due to loss of CH_3COO.

Table I

	^{13}C assignments		
	SM-1	Fumigaclavine A (SM-2)	Fumigaclavine B
1	132.1	117.7	117.6
2	106.2	111.6	111.6
3	128.0	126.8	126.9
4	129.1	129.9	129.5
5	112.8	112.9	112.2
6	122.2	123.2	123.0
7	107.6	108.3	108.7
8	136.6	133.6	133.5
9	28.1	26.8	26.2
10	61.6	61.9	60.9
11	43.5	43.6	43.4
12	57.8	57.9	57.4
13	33.1	33.2	34.0
14	16.7	16.8	16.9
15	71.4	71.6	69.0
16	39.4	39.8	41.6
17	27.3		
18	27.4		
19	39.1		
20	145.7		
21	111.8		
C=O	170.8	170.8	
CH_3	21.2	21.2	

Because only the salient features of the structure of SM-1 were obvious from the UV, IR, ^{1}H NMR, ^{13}C NMR, and mass spectral data, the absolute structure was deduced by single crystal x-ray diffraction analysis. SM-1 (IIIa) was shown to be a new clavine alkaloid (fumigaclavine C) closely related to fumigaclavine A (IIIb) and B (IIIc).





IIIa, R = ≈, R_1 = OCOCH$_3$

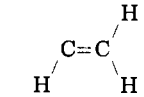
IIIb, R = H, R_1 = OCOCH$_3$

IIIc, R = H, R_1 = OH

SM-1 can be described as fumigaclavine A with a dimethyl allyl moiety derived from mevalonate fused in position 2 of the ergoline ring system. SM-1 appears to be identical with a clavine alkaloid previously isolated from *A. fumigatus* by Yamano et al. (1962) and also designated fumigaclavine C. No chemical structure was proposed for fumigaclavine C by these investigators.

The structure of SM-2 was established by comparison of its ^{13}C and ^{1}H spectra with those of SM-1. The ^{13}C spectrum of SM-2 contains five fewer carbon peaks than that of SM-1. The off-resonance decoupled (ORD) spectrum shows that these five carbons are two methyl carbons, a quaternary carbon, an olefinic methine carbon, and an olefinic methylene carbon (Table I). In addition, comparison of the ^{1}H spectrum of SM-1 with that of SM-2 shows that the spectrum of SM-2 lacks the peaks characteristic of a vinyl group and the six proton singlet assigned to the methyl protons on carbons 17 and 18 (Table II). In addition, the ORD ^{13}C spectrum of SM-2 shows

Table II

^{1}H spectral assignments													
SM-1					Fumigaclavine A					Fumigaclavine B			
1.35	d	CH_3	J = 7 Hz	(C_{14})	1.35	d	CH_3	J = 7 Hz	(C_{14})	1.30		CH_3	(C_{14})
1.56	s	$2CH_3$		(C_{17},C_{18})									
1.90	s	CH_3		CH_3CO	1.90	s	CH_3CO						
2.46	s	CH_3		(C_{11}-N)	2.47	s	N-CH_3		(C_{11})	2.43		N-CH_3	
2.68	m		4 H		2.68	m		4 H		2.62 2.8	m		
3.32	m		1 H		3.4	m		2 H		3.14			
3.54	m		1 H										
										4.54	t		1 H(C_{14})
5.05 5.20													
6.10	→												
5.67	t		1 H	(C_{15})	5.7	t		1 H	(C_{15})				
6.72			1 H	(C_6)	6.76			1 H		6.87			1 H
7.04			1 H	(C_5)	6.86			1 H	(C_1)	6.96			1 H(C_1)
7.08			1 H	(C_7)	7.12			1 H		7.18			1 H
					7.16			1 H		7.21			1 H
7.76		N-H indole			7.94		N-H indole			8.04		N-H	

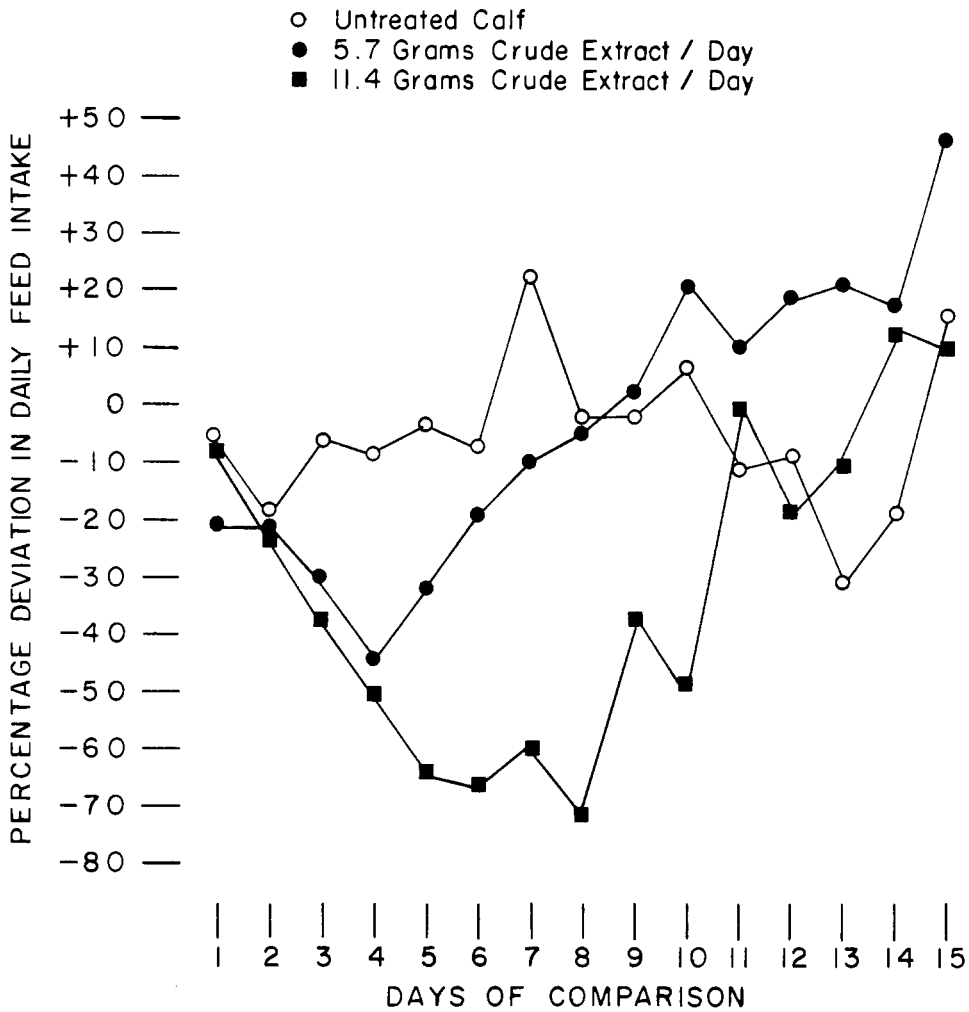


Figure 1. Percent deviation in daily feed intake compared to the mean daily intake during 7 days pretreatment. Comparison includes 5 days of treatment (days 1–5) and 10 days posttreatment (days 6–15).

a doublet characteristic of the peak due to C-1 of a substituted indole ring and the ^{1}H spectrum shows an additional one proton singlet characteristic of the proton attached to C-1 of a substituted indole ring. These data, along with the melting point, firmly establish that the structure of SM-2 is identical with that of fumigaclavine A (IIIb) (Spilsbury and Wilkinson, 1961).

For further confirmation of the structure of SM-2, a sample of SM-2 was hydrolyzed, in base, to fumigaclavine B (IIIc) using the previously reported procedure (Spilsbury and Wilkinson, 1961). Both the ^{13}C and ^{1}H spectra of the hydrolyzed product showed the loss of the acetate group and this, along with the melting point, confirmed that the structure of SM-2 was identical with that reported for fumigaclavine B (244–245 °C) from *A. fumigatus* (Spilsbury and Wilkinson, 1961).

The LD_{50} of SM-1 to day-old cockerels was about 150 mg/kg. Histopathology of surviving cockerels showed

vacuolation of the hepatic parenchymal cells that may have been related to the toxic action of the crude extract or to anorexia.

Daily feed intake of the calf given the single dose of *A. fumigatus* extract (86 g containing 3.25 g SM-1, about 48 mg of SM-1 per kg of body weight) was severely depressed and the calf failed to improve. Intake during the 4 days posttreatment was only 25% of the pretreatment level compared to 97% for the two control calves. The treated calf showed spontaneous irritability and some diarrhea. Postmortem examination revealed hemorrhagic enteritis in the small intestine and, to a lesser extent, in the large intestine. Lung fields contained mild patchy interstitial thickenings of alveolar walls, largely involving nonnuclear cells and some neutrophils. Abnormal changes were not found in other tissues. Body temperatures and calcium, magnesium, phosphorus, total protein, bilirubin, white blood cell count, packed cell volume, hemoglobin, differential leucocyte count, serum glutamic oxaloacetic transaminase activity, creatinine phosphokinase activity, and sorbitol dehydrogenase activity in all calves were within normal ranges. Chloroform extracts of the urine and feces were analyzed for SM-1 by TLC; no SM-1 was detected, but several other differences between the control and treated calves were noted.

Figure 1 shows that five daily doses of 5.7 or 11.4 g of crude extract from *A. fumigatus* cultures (approximately 2.5 and 5.5 mg of SM-1 per kg body weight) depressed feed intake. The degree of depression and time required to effect it were proportional to the dosage. The calf given 11.4 g of crude extract developed severe diarrhea on day 2 of treatment, and it persisted for 7 days posttreatment. The calf getting 5.7 g of crude extract daily developed severe diarrhea on day 4 of treatment, and it persisted until 5 days posttreatment. As with the first group of three calves, body temperatures and blood constituents were not detectably influenced by treatment. Thin-layer chromatograms of $CHCl_3$ extracts of urine and feces of the treated calves were similar to those from the calf given the single dose. Postmortem examination of the calf given the 11.4-g dose showed the small intestine had patchy areas of serous enteritis and its contents were abnormally fluid, but only normal flora were present. The lung had patchy to almost confluent areas of alveolar septal interstitial thickening by cellular elements. Significant changes were not found in other tissues. The calf given 5.7 g of crude extract was maintained on normal husbandry practices and appeared to be free of any permanent damage and grew normally.

Similarities between the clinical signs of the experimentally and naturally affected cattle were irritability, diarrhea, and malnutrition resulting from anorexia. However, the exact role of *A. fumigatus* metabolites in toxic syndromes of cattle ingesting molded silage is not known. Further studies to determine the causes of toxicity associated with ingestion of molded silage and the effects of SM-1 and SM-2 on cattle are in progress.

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Improved Gas Chromatography Method for the Quantitation of Saccharides in Enzyme-Converted Corn Syrups

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A procedure has been developed for the quantitative analysis of sugars in enzyme-converted corn syrups, using *N*-(trimethylsilyl)imidazole to prepare the trimethylsilyl derivatives of glucose and higher glucose oligomers. The method was applied to lyophilized syrups and syrups containing 80% solids. Recoveries of spiked samples of glucose, maltose, and maltotriose were 99%.

Separation and quantitation of trimethylsilyl derivatives of sugars by gas chromatography was first described by Henglein and Scheinost (1956). Since then a number of silylating agents have been developed and their application to sugar analysis reviewed (Birch, 1973).

The standard methods used to produce trimethylsilyl derivatives of sugars in enzyme-converted corn syrups generally involve the use of either hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) (Beadle, 1969), or HMDS and trimethylchlorosilane (TMCS) (Sweeley et al., 1963). In both cases the reaction can be exothermic and can result in splattering of the sample. Furthermore, HMDS, TMCS, and TFA reagents can produce a precipitate which interferes with subsequent sampling.

Derivatives of a number of organic compounds have been prepared with *N*-(trimethylsilyl)imidazole (TSIM). This reagent has been reported to produce trimethylsilyl derivatives of model mono- and disaccharides under mild

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