Rumen motility, tissue and fluid concentrations of aflatoxins B₁ and M₁ and clinical and pathologic changes in acute bovine aflatoxicosis

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Rumen motility, tissue and fluid concentrations of aflatoxins B$_1$ and M$_1$ and clinical and pathologic changes in acute bovine aflatoxicosis

by

William O. Cook

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Department: Veterinary Pathology
Major: Veterinary Pathology
(Veterinary Toxicology)

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa
1985
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I. INTRODUCTION

Studies of bovine aflatoxicosis have demonstrated or suggested interference with rumen function.

Steers fed a diet containing approximately 450 ng of aflatoxin/g for 15 weeks and then given a noncontaminated ration contained aflatoxin concentrations in the rumen 18 days later that were greater than expected based on normal rumen turnover time (Richard et al., 1983). Impaired rumen function could have caused the delay observed in aflatoxin elimination from the rumen. Even though no detectable amount of aflatoxin was found in tissues from these animals, the detection of aflatoxin in rumen contents 18 days after exposure suggested that a withdrawal time greater than 18 days prior to slaughter may be warranted for cattle on aflatoxin contaminated rations.

A lactating Holstein cow administered a single oral dose of aflatoxin at 0.5 mg/kg body weight (BW) was observed clinically to have little or no rumen motility on the second or early part of the third day after dosing, but the animal appeared to have regained normal rumen motility by the end of the third day (Trucksess et al., 1983). Another cow died within this time period following a 24 hour absence of rumen motility (Richard, J. L., NADC, Ames, Iowa, personal communication, 1985). In this study, varied concentrations of aflatoxin in blood and milk suggested altered rumen motility that produced uneven toxin absorption and consequently uneven elimination. The rapid appearance of aflatoxin B₁ and M₁ in urine and milk after oral exposure was evidence of absorption of aflatoxin directly across rumen mucosa, which has been suggested in other studies (Polan et al., 1974).
Cessation or decrease in rumen function was thought to be the cause for uneven elimination of aflatoxin B$_1$ and M$_1$ in urine and from rumen contents after Holstein cows were fed 0.35 mg of aflatoxin/kg BW for 3 days (Stubblefield et al., 1983).

Bovine rumen metabolism of tryptophan and indole-3-acetic acid (IAA) have been extensively studied because of their capability to produce acute bovine pulmonary edema and emphysema. Incorporation of intraruminally infused IAA into tryptophan in rumen microorganisms has suggested that IAA may increase bacterial cell proliferation and fermentation of rumen products (Allison et al., 1974, Kapoor et al., 1983, Yokoyama and Carlson, 1974). Alterations in rumen motility were observed after intraruminal infusion of IAA and a similar compound, indole-3-butyric acid (IBB), in buffaloes (Kapoor et al., 1983). Decreased frequency and amplitude of rumen contractions were observed after the adult buffaloes were infused intraruminally with IAA. Alterations were attributed to a possible increase in rumen concentration of acetic acid. This conclusion was based on the results of another study in sheep that demonstrated intraruminal infusion of acetic acid caused a cessation in reticulorumen contractions (Hamasaki et al., 1974). In the same study in sheep, cessation of motility was attributed to rumen and systemic circulatory acidosis. Intraruminal infusion of IBB in buffaloes increased amplitude of rumen contractions, and the increased amplitude of rumen contractions was postulated to be a result of increased production of fermentation products required for rumen contractions.

Various approaches including clinical observation, direct palpation through a rumen fistula, radiographic techniques using barium sulphate and
measurement of pressure changes accompanying movement have been used to evaluate rumen-recticular motility. The development of radiotelemetry enabled pressure changes to be transmitted by radio waves which eliminated connecting tubes, wires and animal confinement needed with other pressure recording devices. Development of longer battery life has allowed for long-term investigations of rumen motility such as the present study.

A decrease in total volatile fatty acids-(VFA), altered proportions of individual VFA, and inhibition of cellulose digestion and ammonia formation were observed in an in vitro rumen study with 0.2 µg of aflatoxin per ml of rumen fluid (Fehr and Delage, 1970, cited by Mertens, 1979). In another in vitro rumen study, aflatoxin concentrations as low as 0.1 µg of aflatoxin/g of rumen fluid resulted in a decrease in total VFA, inhibition of cellulose digestion and a decrease in microbial protein, but no change in proportions of individual VFA (Sinha and Arora, 1982). Rumen fluid was incubated for 24 hours at 39°C. In vivo rumen studies with a single dose of 200 mg of aflatoxin B₁ and 80 mg of aflatoxin B₂ given to adult cows via a rumen fistula decreased total VFA and altered ratios of individual VFA in the rumen (Dvorak et al., 1977). Alterations in VFA production are attributed to aflatoxin induced changes in rumen microflora. Certain rumen bacteria have been demonstrated to be inhibited in vitro in the presence of aflatoxin, and several species of bacteria have been completely inhibited at concentrations of less than 10 µg of aflatoxin/ml. These demonstrations suggest that growth and function of rumen microorganisms could be altered by levels of aflatoxin found naturally in feed (Mertens, 1979). The effects of aflatoxin induced alterations of rumen VFA produc-
tion, cellulose digestion, protein metabolism and bacterial populations on rumen motility are unknown.

The relative resistance of the ruminant to aflatoxin as compared to monogastrics has suggested that rumen microflora may have detoxification mechanisms for aflatoxin. Orally administered aflatoxin in lactating cows was found to be less detrimental to health and milk production than abomasally administered aflatoxin (Wechsler et al., 1980). Ruminal dilution or deactivation of aflatoxin by rumen microflora was suggested as an explanation for the difference. Aflatoxin B₁ has been shown to be poorly degraded by rumen microorganisms in vitro. In one study, aflatoxin B₁ at a concentration of 0.2 mg of aflatoxin/l in rumen fluid from a fistulated sheep was incubated anaerobically under CO₂ at 38°C (Kiessling et al., 1984). Repeated analyses by thin layer chromatography for aflatoxin B₁ in the rumen fluid revealed no significant degradation of aflatoxin B₁. In another study, rumen bacteria did not convert aflatoxin B₁ in rumen fluid at a concentration of 50 µg of aflatoxin/ml of rumen fluid when incubated for 24 hours at 39°C to other fluorescent aflatoxin compounds (Mathur et al., 1976). Degradation of aflatoxin B₁ in this study in rumen fluid was estimated to be less than 10% by visual observation and thin layer chromatography. Different results were obtained in one study that concluded aflatoxin B₁ was significantly metabolized by rumen microorganisms in vitro and in vivo (Engel and Hagemeister, 1978, cited by Bodine and Mertens, 1983). In an artificial rumen system with 0.078-0.392 µg of aflatoxin B₁ per ml of rumen fluid, after incubation over 90% of the aflatoxin B₁ was recovered by thin layer chromatography and fluorodensitometry.
(Bodine et al, 1977, cited by Bodine and Mertens, 1983). This suggested little or no metabolism of aflatoxin B₁. Aflatoxin M₁, a metabolite of aflatoxin B₁, was detected at slaughter in rumen contents from steers fed a diet containing approximately 450 ng of aflatoxin B₁ and B₂ per g of feed for 17.5 weeks (Richard et al., 1983). The presence of aflatoxin M₁ in rumen contents in this study suggested the possibility of rumen metabolism of aflatoxin B₁ to M₁.

The objective of the present study was to determine any changes in rumen motility by radiotelemetry and to correlate any such changes with patterns of aflatoxin absorption in tissues and elimination in fluids. Histopathologic changes of selected tissues, rumen VFA production and selected clinical pathologic changes were also determined. This would extend previous studies of bovine aflatoxicosis to include the evaluation of aflatoxin consumption on rumen motility and any influence of alterations in rumen motility on aflatoxin metabolism.
II. HYPOTHESES

Hypotheses to be examined:

1. Aflatoxin markedly alters amplitude and/or frequency of rumen contractions and delays elimination of intraruminally administered aflatoxin $B_1$ from the rumen.

2. Aflatoxin induced alterations in rumen motility affects absorption of aflatoxin from the rumen and systemic metabolism of aflatoxin.

3. The mechanism of aflatoxin induced alterations in rumen motility could be due to and coincide with decreased feed consumption, severe liver damage and/or changes in volatile fatty acid production.

4. Aflatoxin $M_1$ will be detected in the rumen after intraruminal administration of aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$. Presence of aflatoxin $M_1$ could be a result of rumen metabolism of aflatoxin $B_1$ to $M_1$ or possibly partitioning of aflatoxin $M_1$ from blood into the rumen after liver metabolism of aflatoxin $B_1$ to $M_1$. 
III. MATERIALS AND METHODS

A. Animals

Four steers which weighed 155, 173, 158, and 357 kg were housed in box stalls measuring 3.7 meters x 3.7 meters and fed 0.5 kg of a 16% protein concentrate and 3.0 kg of a 15% protein alfalfa hay cube diet twice daily. They were given water ad libitum.

B. Aflatoxin

Aflatoxin was produced for this study as follows: Substrate for aflatoxin production was prepared by adding 75 g of polished rice and 35 ml of water to a 1 L Erlenmeyer flask. The preparation was allowed to imbibe for 2 hours before autoclaving and cooling (Shotwell et al., 1966). Substrate was inoculated with Aspergillus parasiticus, NRRL 2999, and grown at 28° C for 1 week on a rotary shaker at 300 revolutions per minute (RPM). Contents were extracted twice with 250 ml of chloroform. Extract was dried under vacuum, rediluted with chloroform and precipitated with 1:4 v/v chloroform:hexane. Precipitate was collected by filtration through a #1 Whatman filter paper on a Buchner funnel, washed with hexane, air-dried and analyzed for aflatoxin by thin layer chromatography. Precipitate consisted of 42% aflatoxin B₁, 27% aflatoxin G₁ and a trace of aflatoxin B₂ and G₂, and was calculated to be a 55.5% aflatoxin B₁ equivalent preparation. In computing toxic equivalents of aflatoxin B₁, aflatoxin G₁ was one-half as toxic as B₁ and aflatoxins B₂ and G₂ were negated, according to the relative toxicities of aflatoxin reported by Carnaghan et al. (1963).
C. Surgical Procedure

For sedation, 10 to 20 mg of xylazine was administered to each steer intramuscularly for sedation prior to halothane gas anesthesia and surgeries. A two-stage rumen fistulation procedure was performed, in the first stage a left paralumbar approach to the rumen was made, and the perimeter of a 5 cm diameter circular area of rumen was sutured to the skin. After one week, the second stage was performed consisting of an incision through the rumen wall and placement of a 4 piece silicone fistula\(^1\) having a 2.5 cm lumen (Fig. 1). All steers were allowed to recover at least one week prior to dosing.

D. Aflatoxin Administration

Steers were dosed one time with 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW in a 000 gelatin capsule via the rumen fistula. All measurements and samples were taken from each steer prior to dosing, therefore each steer served as its own control.

E. Rumen Motility

Rumen motility was monitored by radiotelemetry with modifications of the pressure transducer described by Cook and Riley (1970). A transducer was packaged in an oil filled silicone tube 2.2 cm in outside diameter, 1.6 cm in inside diameter, with a 0.5 mm silicone sheet glued to one end and a lead weight attached to the other end (Fig. 2). The package was inserted into the rumen via the fistula. Pressure changes on the outside of

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\(^1\)Sylgard 186 silicone elastomer kit, Dow Corning Corp., Midland, Mich.
the tube were transmitted through the oil to a LX0503 pressure transducer\(^2\) which converted pressure changes to voltage changes. This circuit bridge was excited with 1.35 V from a Hg battery and the output amplified by a differential amplifier. This signal generated a frequency modulated sub-carrier frequency of 7500 Hz using a battery controlled oscillator. The subcarrier signal frequency modulated a radio frequency of 100 Hz. The signal generated by the transducer was transmitted from the animal to a FM receiver. Output of the receiver was demodulated to give the voltage signal, representing pressure, that was then recorded on a strip chart. Rumen motility was monitored immediately after animals were fed. The motility was recorded prior to dosing steers with aflatoxin and twice daily thereafter for 7 days. A minimum rise of 3 mm above baseline was the basis for counting rumen contractions.

F. Sample Collection

Fifty ml blood samples were collected by jugular venipuncture prior to aflatoxin administration and at 0.5, 1, 2, 4, and 8 hours after dosing steers with aflatoxin on day 1 and twice daily thereafter during the 7 day experiment. Serum was separated from 20 ml of blood after clotting and centrifugation at 3,000 RPM for 5 minutes. Thirty ml of blood was prevented from clotting with calcium EDTA and frozen for later aflatoxin analysis. Rectal temperatures were recorded when calves were bled. Voided urine samples were collected prior to aflatoxin administration, 2 and 8 hours post dosing on day 1 and twice daily thereafter during the 7 day experiment.

\(^2\)National Semiconductor Corp., Santa Clara, Calif.
day experiment. Rumen contents were collected prior to dosing, 2 and 8 hours post dosing on day 1 and twice daily thereafter during the 7 day experiment. Rumen samples of approximately 500 g were collected in a 5 L flask by means of a stomach pump and a 2 cm diameter hose that was inserted into the rumen via the fistula.

Voided feces were collected prior to dosing with aflatoxin and once daily thereafter for 7 days. On day 7 each steer was killed, and liver, kidney and bile were collected at necropsy for aflatoxin analysis. All tissues and fluids were stored at least below -20° C until analyzed. Samples of kidney, liver, lung, heart, skeletal muscle and rumen mucosa were taken at necropsy for histopathologic examination. Tissues were fixed in formalin, embedded in paraffin, sectioned at 5 µ, stained with hematoxylin and eosin and examined by light microscopy.

G. Clinical Pathology

Packed cell volume (PCV) and total protein determinations were made on each blood sample. Determinations were made on serum for sorbitol dehydrogenase (SDH), total and direct bilirubin, alkaline phosphatase, aspartate aminotransferase (AST) and blood urea nitrogen (BUN) according to the directions provided by the manufacturers. Serum was analyzed for glycocholic acid by radioimmunoassay which quantitated cholic acid and its glycine and taurine conjugates in serum. In this procedure, an unknown

3Sigma Diagnostics, St. Louis, Missouri.
4Gilford Diagnostics, Cleveland, Ohio.
5Cooperbiomedical, Freehold, New Jersey.
6Farmos Diagnostica, Oulunsalo, Finland.
amount of glycocholic acid to be assayed was added to a standard amount of radioactively labeled derivative of glycocholic acid. These labeled and unlabeled antigens competed for the limited number of binding sites of a specific antibody, and the amount of radioactive antigen in the antigen-antibody complex was reversely proportional to the amount of unlabeled antigen in the mixture. The free antigen was separated from the antigen-antibody complex, and the radioactivity of either fraction was counted. Actual concentrations were calculated with the aid of a standard curve based on known amounts of unlabeled antigen analyzed along with the unknowns.

H. Assay of Tissues and Fluids for Aflatoxin B₁ and M₁

Blood, urine and bile were analyzed as follows: Twenty-five ml of blood, 50 ml of bile or 50 ml of urine were poured onto a 35 g hydrophilic matrix column and eluted 3 times with 50 ml 4:1 v/v methylene chloride:acetone into a 250 ml evaporating flask. Extract was dried under vacuum, rediluted with methylene chloride, transferred quantitatively to dram vials and evaporated to dryness under flowing nitrogen. Liver and kidney tissue were analyzed by the meat method of Stubblefield and Shotwell (1981). Recoveries of aflatoxin B₁ and M₁ added to liver and kidney tissue were approximately 90 percent with a detection limit < 0.1 ng/g of tissue. Rumen contents and feces were extracted by the procedure of Richard and Lyon (1985) as follows: Fifty g of rumen contents or feces, 25 g of diatomaceous earth, 25 ml of distilled water and 250 ml of methy-
lene chloride were added, mixed in that order in a 500 ml Erlenmeyer flask and shaken for 30 minutes on a rotary shaker. The sample was then filtered through a #1 Whatman filter paper into an evaporating flask. Remaining solid material was washed with 100 ml of methylene chloride and filtered. Extract was dried under vacuum, rediluted with 50 ml of 30:20 v/v acetonitrile:water and poured onto a 35 g hydrophilic matrix column. The flask was rinsed 2 times with 5 ml of methylene chloride and added to the column. The column was eluted 3 times with 50 ml 4:1 methylene chloride:acetone. Eluate was collected in a 250 ml evaporating flask, dried under vacuum and the extract transferred quantitatively to dram vials and evaporated to dryness under flowing nitrogen. With this procedure a 92% average recovery was obtained within a detection limit of approximately 0.5 ng from samples spiked at 28.8 ng/g. Two-dimensional thin layer chromatography, fluorometric densitometry, and a 2.5 ng/µl B1-M1 standard were used to quantify aflatoxin B1 and M1. Samples for quantitative analysis were initially rediluted in 100 µl of 99:1 v:v benzene:acetone, and portions with or without dilutions were plated on 10 x 10 cm silica gel coated thin layer chromatography plates (Fig. 3). Ten to 20 µl of the aflatoxin B1-M1 standard was plated in each of 2 channels at right angles to each other. Plates were developed first in 84:10:6 v:v:v chloroform:acetone:isopropanol,

7 CT-2050 Chemtubes, CT-001 Hydromatix, Analyticchem International, Inc., Harbor City, Calif.
8 Merck silica gel 60 glass TLC plates, MCB Manufacturing, Cincinnati, Ohio.
9 Shimadzu CS-920, Shimadzu Corp., Kyoto, Japan.
10 Northern Regional Research Center, Peoria, Illinois.
air-dried, turned 90 degrees and developed in 93:6:1 v:v:v ether:methanol:water. Plates were examined qualitatively for aflatoxin fluorescence under a 375 nm ultraviolet light before quantitation by fluorometric densitometry. Trifluoroacetic acid was used to confirm the identity of aflatoxin B₁ and M₁ in sample extracts from each steer (Stubblefield and Shotwell, 1981).

I. Volatile Fatty Acid Analysis

Samples of rumen contents for VFA analysis were rapidly thawed and centrifuged at 5,000 RPM for 10 minutes. Analyses were performed on 0.5 ml of supernatant according to the method of Salanitro and Muirhead (1975). Samples were analyzed on a Hewlett Packard 5830 A gas chromatograph. The column was a 2 M x 0.32 cm steel column packed with Chromasorb W(80-100 mesh HP, DMCS, AW) coated with 10% Dexsil 300 GC. Flame ionization detector temperature was 270° C. Initial column bath was 75° C followed by 5° C/minute temperature increase up to 265° C.

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11Analabs, North Haven, Connecticut.
IV. RESULTS

Clinical signs appeared 24 to 32 hours after aflatoxin administration and consisted of cessation or decrease in frequency of rumen contractions, total and/or partial anorexia, dry rumen contents, and decreased fecal output. Clinical signs were more prolonged in steers dosed with greater concentrations of aflatoxin. Changes in appetite ranged from partial anorexia for 1 day in the lesser dosed steer to partial or complete anorexia for 3-4 days in steers given the greater doses. Total anorexia never persisted for more than 1 day. The steer given 0.2 mg of aflatoxin/kg BW was not depressed, but depression was observed for 1 day in steer given 0.4 mg of aflatoxin/kg BW and for 3 days in the steers given 0.6 and 0.8 mg of aflatoxin/kg BW. Blood was present in feces from the steer given 0.8 mg of aflatoxin/kg BW at 24-48 hours post dosing. Consistency of rumen contents was very dry for 2, 3, and 5 days in the steers given 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW respectively, but remained normal in the steer given 0.2 mg of aflatoxin/kg BW. Fecal output was markedly reduced for approximately 2 days in all animals.

Icteric serum and excessive urination occurred for 1-3 days commencing 24 hours after aflatoxin administration in all steers. Rectal temperatures increased by 0.8° C to 3° C above baseline in all steers from 24-48 hours after dosing.

Alterations in rumen motility were detected by radiotelemetry at all doses of aflatoxin administered (Figs. 4-7). Amplitude (strength) and frequency of rumen contractions appeared to be affected beginning 24 hours after aflatoxin administration. Only a decrease in amplitude of rumen
contractions was observed in the steer given 0.2 mg of aflatoxin/kg BW, but steers given greater doses had a decrease in amplitude and/or frequency of rumen contractions (Figs. 8-9). Normal rumen motility resumed within 3 days after alterations appeared. The transmitter from the steer given 0.2 mg of aflatoxin/kg BW was present in the reticulum at necropsy. In other steers, transmitters were present in the rumina at necropsy.

Aflatoxin $B_1$ and $M_1$ reached maximum concentrations in rumen contents within 4-8 hours, and in some steers appeared to increase again later (Figs. 10-11). Aflatoxin $M_1$ was detected in rumen contents at all concentrations of aflatoxin given within 2 hours. The steers given 0.6 and 0.8 mg of aflatoxin/kg BW contained detectable concentrations of aflatoxin $B_1$ and $M_1$ in rumen contents at necropsy on day 7. Aflatoxin in rumen contents was calculated on a dry weight basis because it was observed that aflatoxin in rumen contents was associated primarily with particulate matter and because rumen contents are extremely variable in percent water.

Aflatoxin $B_1$ and $M_1$ were detected in blood of all steers within 30 minutes from the time of aflatoxin administration (Figs. 12-13). Steers dosed with 0.2, 0.6, and 0.8 mg of aflatoxin/kg BW achieved concentrations of aflatoxin $B_1$ in blood greater than aflatoxin $M_1$. Aflatoxin $M_1$ reached maximum concentrations in blood earlier than aflatoxin $B_1$. Concentrations of aflatoxin $B_1$ and $M_1$ increased in blood from 4-8 hours after administration and appeared to rise again later. Aflatoxin $B_1$ and $M_1$ were detected for a longer time in blood from steers given the larger doses of aflatoxin.

Urine contained greater concentrations of aflatoxin $M_1$ than $B_1$ (Figs. 14-15). Generally, aflatoxin $M_1$ reached maximum concentrations in
urine within 24 hours and increased again later. Different patterns of elimination of aflatoxin $B_1$ were observed, but in the steer given 0.8 mg of aflatoxin/kg BW aflatoxin $B_1$ concentrations peaked in urine at 8 and 72 hours after dosing. Aflatoxin $M_1$ was detected in all urine samples, but aflatoxin $B_1$ was not detected for the last 3 days in urine from the steer given 0.2 mg of aflatoxin/kg BW.

Feces from steers given 0.2 to 0.6 mg of aflatoxin/kg BW contained higher concentrations of aflatoxin $M_1$ than $B_1$ (Figs. 16-17). Generally, aflatoxin $B_1$ and $M_1$ attained maximum concentrations in feces within 24-32 hours after dosing.

Aflatoxin $M_1$ was detected in kidney tissue collected at necropsy from all steers and in greater concentrations than aflatoxin $B_1$ (Table 1). Aflatoxin $B_1$ concentrations were greater than aflatoxin $M_1$ when detected in liver tissue. Aflatoxin $B_1$ and $M_1$ were not detected in bile collected at necropsy.

Table 1. Aflatoxin residues (ng/g) in liver and kidney tissue

<table>
<thead>
<tr>
<th>Dose (mg of aflatoxin/kg BW)</th>
<th>Toxin</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>$B_1$</td>
<td>ND$^a$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>$M_1$</td>
<td>ND</td>
<td>.029</td>
</tr>
<tr>
<td>0.4</td>
<td>$B_1$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>$M_1$</td>
<td>ND</td>
<td>.035</td>
</tr>
<tr>
<td>0.6</td>
<td>$B_1$</td>
<td>.089</td>
<td>.06</td>
</tr>
<tr>
<td></td>
<td>$M_1$</td>
<td>.061</td>
<td>.26</td>
</tr>
<tr>
<td>0.8</td>
<td>$B_1$</td>
<td>.025</td>
<td>.047</td>
</tr>
<tr>
<td></td>
<td>$M_1$</td>
<td>ND</td>
<td>.14</td>
</tr>
</tbody>
</table>

$^a$ND = not detected.
Serum SDH and AST enzymes increased above normal published values at all levels of aflatoxin given (Duncan and Prasse, 1978) (Figs. 18-19). Little change was noted before 8 hours, but enzymes rapidly reached maximum concentrations at 24-48 hours and decreased quickly thereafter. An aflatoxin dose related enzyme response was observed; steers receiving greater amounts of aflatoxin on a BW basis had greater serum enzyme concentrations. Steers given the greater aflatoxin doses also had greater increases in serum glycocholic acid that generally reached maximum concentrations after several days (Fig. 20). No significant change was observed in PCV, total serum protein, bilirubin, BUN or alkaline phosphatase.

No pattern changes were observed in total VFA, ratios of acetic to butyric acid, or the individual volatile fatty acids (acetic, propionic or butyric acid) in rumen contents after aflatoxin administration. Marked fluctuations in individual and total VFA did occur from 8-48 hours after aflatoxin administration when compared with other times.

Necropsy of steers revealed a tan colored liver in the steer given 0.8 mg of aflatoxin/kg BW.

Lesions were observed in liver tissue from all steers at histopathologic examination. Severity of liver lesions was approximately related to the concentration of aflatoxin given (Table 2). Marked centrilocular hemorrhage with infiltration of lymphocytes and plasma cells occurred in liver tissue from steers given 0.6 and 0.8 mg of aflatoxin/kg BW (Fig. 21). Occasional foci of lymphocytes and plasma cells were scattered throughout the liver parenchyma and around bile ducts. The liver of the steer given 0.4 mg of aflatoxin/kg BW contained the same distribution of lymphocytes and plasma cells, but to a lesser degree. Swollen hepatocytes with diffuse
cytoplasmic vacuolization appeared in liver tissue at all concentrations of aflatoxin given. Hepatocyte swelling and nuclear pleomorphism were more marked in liver tissue from steers given greater concentrations of aflatoxin. Vacuoles stained more positive with PAS for glycogen in liver tissue from steers given the lesser concentrations of aflatoxin. Vacuoles didn't stain positive for fat with Oil Red O. Dilated periportal veins, retention of bile pigment indicative of cholestasis, and accumulation of hemosiderin laden macrophages around centrilobular veins were evident in liver tissue from steers dosed with 0.4-0.8 mg of aflatoxin/kg BW. There was no fibrosis or proliferation of bile ducts observed.

Table 2. Relative degree of selected histopathologic liver lesions

<table>
<thead>
<tr>
<th>Aflatoxin Dose (mg/kg BW)</th>
<th>Hemorrhage</th>
<th>Lymphocytes/Plasma Cells</th>
<th>PAS Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>0.4</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>0.6</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>0.8</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

- = not present; + lesion present and graded on scale from 1-4.

The kidney tissue in the steer dosed with 0.2 mg of aflatoxin/kg BW contained eosinophilic casts, and there was mild vacuolization of epithelial cells in proximal convoluted tubules (Fig. 22).
V. DISCUSSION AND CONCLUSIONS

An apparent decrease in strength and frequency of rumen contractions was observed in steers given from 0.2-0.8 mg of aflatoxin/kg BW. Alterations appeared to be dose related; the lowest dosed steer given 0.2 mg of aflatoxin/kg BW had changes only in strength of rumen contractions while steers given greater doses had decreases in frequency and strength of rumen contractions. Future studies with more animals at each dose would be necessary to provide a basis for statistical analysis of the changes observed in this study. In the present study, aflatoxin doses that resulted in recordings of altered rumen motility; 0.2-0.8 mg of aflatoxin/kg BW, were similar to those given in previous studies, 0.35 and 0.5 mg of aflatoxin/kg BW, that resulted in clinically observed alterations or suspected alterations in rumen motility (Stubblefield et al., 1983; Trucksess et al., 1983). In the present study, rumen motility was altered in the steer given 0.2 mg of aflatoxin/kg BW. This suggested that an evaluation of rumen motility in steers given doses of aflatoxin less than this may be warranted. A dose of 0.2 mg of aflatoxin/kg BW corresponds to approximately 6 mg of aflatoxin/kg of feed in a total ration. Steers fed a diet containing 0.450 mg of aflatoxin/kg for a longer period of time, 15 weeks, had delayed elimination of aflatoxin from the rumen, but had normal weight gain (Richard et al., 1983). Alterations in rumen motility could occur that may delay aflatoxin elimination, but not affect weight gain.

Elimination of aflatoxin from rumen contents reflected changes in rumen motility as recorded by radiotelemetry. Assuming good mixing of aflatoxin in rumen contents by 8 hours post dosing and normal rumen
motility thereafter (halving of rumen contents every 20-25 hours) (Church, 1975) elimination of aflatoxin from rumens in the steers given 0.4-0.8 mg of aflatoxin/kg BW was delayed (Fig. 10). These steers had a marked decrease in frequency and strength of rumen contractions as recorded by radiotelemetry. Aflatoxin was cleared faster than projected from the rumen of the steer given 0.2 mg of aflatoxin/kg BW. Radiotelemetry indicated this steer had decreased strength of rumen contractions, but no change in frequency of contractions. Examination of rumen contents and feces for aflatoxin would have diagnostic potential. First, unlike blood or urine, rumen contents and feces are usually readily available in large quantities at necropsy, whereas other samples such as blood or urine may not be.

Aflatoxin B₁ and M₁ in the present study were detected for a longer time after dosing in feces than in rumen contents. Second, as a result of decreased rumen motility and delayed elimination of aflatoxin from the rumen, rumen contents and feces may contain detectable concentrations of aflatoxin after other tissues and fluids do not. Aflatoxin could be detected in rumen contents, but not in other tissues or fluids collected at necropsy from steers withdrawn from an aflatoxin contaminated ration 18 days earlier (Richard et al., 1983).

In the present study with steers, the periods of altered rumen motility detected by radiotelemetry coincided in time with clinical observations of anorexia and maximum severity of liver damage detected by clinical pathology. Generally, evidence of altered rumen motility, anorexia, and maximum severity of liver damage commenced 24 hours after aflatoxin administration and were present for 48 hours. The steer given the least concen-
tration of aflatoxin and that had the least indications of liver damage, based on clinical pathologic and histopathologic results, never became totally anorexic and maintained a relatively consistent frequency of rumen contractions over the course of the experiment. Steers given greater concentrations of aflatoxin and with more severe clinical pathologic and histopathologic evidence of liver damage became totally anorexic and had a marked decrease in number and frequency of rumen contractions. A fair return of appetite preceded evidence by radiotelemetry of good rumen motility. Liver damage was the most likely cause of anorexia and decreased feed consumption. Consumption of feed is known to stimulate rumen motility (Church, 1975).

Increased or sustained concentrations of aflatoxin B<sub>1</sub> and M<sub>1</sub> were detected in rumen contents 32-72 hours after aflatoxin administration. Return of stronger rumen motility may have been responsible for better mixing of feed containing aflatoxin, which had settled in the ventral rumen as a result of poor motility, with the more recently consumed noncontaminated feed in the dorsal sac of the rumen (Church, 1975). The rapid rise of aflatoxin B<sub>1</sub> and M<sub>1</sub> concentrations observed in blood at 48 hours most likely represented the time the aflatoxin contaminated feed was passing through the small intestine with increased absorption. A return of rumen motility during this time could have enhanced the absorption. The biphasic increase in aflatoxin B<sub>1</sub> and M<sub>1</sub> that we observed in blood has been observed in blood and milk in a previous study with aflatoxin in adult cows (Trucksess et al., 1983).

Aflatoxin M<sub>1</sub> was detected in rumen contents from all steers within 2 hours after aflatoxin administration. Even though most studies have not
demonstrated a significant breakdown of aflatoxin $B_1$ to $M_1$ in the rumen, the speed with which aflatoxin $M_1$ was present in the rumen and feces, and the concentrations at which it was detected would suggest the possibility of rumen metabolism of aflatoxin $B_1$ to $M_1$. The likelihood of mixed function oxidase activity occurring in the rumen has not been explored. Notably, concentrations of aflatoxin $M_1$ in rumen contents were less than, but closely paralleled those of aflatoxin $B_1$ over time in rumen contents, and the pattern of aflatoxin $M_1$ in blood was similar to those of aflatoxin $B_1$ and $M_1$ in rumen contents. Aflatoxin $M_1$, known to be metabolized from aflatoxin $B_1$ in the liver, could be partitioned into the rumen and feces from blood and indirectly into the rumen from blood by saliva, thus explaining aflatoxin $M_1$ in rumen contents and feces.

The rapid presence of aflatoxin $B_1$ and $M_1$ in blood within 30 minutes in all steers supports previous work that has suggested aflatoxin can be absorbed directly across the rumen mucosa (Polan et al., 1974; Trucksess et al., 1983). This may account for the early peaks of aflatoxin $B_1$ and $M_1$ in blood.

Urinary excretion of aflatoxin $B_1$ and $M_1$ paralleled or slightly lagged in time behind the concentrations of aflatoxin $B_1$ and $M_1$ in blood. In the present study, after a one time aflatoxin dose, concentrations of aflatoxin $B_1$ and $M_1$ were measured in urine during an extended period of time. This has not been reported before. Aflatoxin $M_1$ was detected in all urine samples throughout the study and was present when aflatoxin could not be detected in other tissues and fluids. Aflatoxin $M_1$ in urine was the most sensitive indicator of aflatoxin exposure in this study and
supports previous work that indicated urine to be of significant diagnostic value in determining aflatoxin exposure in the bovine (Richard et al., 1983).

Greater concentrations of aflatoxin $M_1$ than $B_1$ were present in feces from steers given 0.2-0.6 mg of aflatoxin/kg BW. Previous acute studies with aflatoxin in adult cows found greater concentrations of aflatoxin $B_1$ than $M_1$ in feces (Stubblefield et al., 1983).

In the present study, aflatoxin $B_1$ occurred in blood at greater concentrations than aflatoxin $M_1$ except from the steer given 0.4 mg of aflatoxin/kg BW. Previous work in the adult bovine revealed greater concentrations of aflatoxin $M_1$ than $B_1$ in blood, plasma and red blood cells (Trucksess et al., 1983). Steers averaging 183 kg and fed a ration of feed containing 450 ng of aflatoxin/g of feed for 17.5 weeks had greater concentrations of aflatoxin $M_1$ than $B_1$ in blood samples collected weekly (Richard et al., 1983). The relatively higher concentrations of aflatoxin $B_1$ and $M_1$ in the present study may be a result of differences in metabolism due to sex and breed.

In general, the relative concentrations detected of aflatoxin $B_1$ in samples in this study were as follows: rumen contents $>$, feces $>$, urine $>$, blood. The relative concentrations detected of aflatoxin $M_1$ in samples was urine $>$, feces $>$, rumen contents $>$, blood.

Rumen contents in steers given 0.4-0.8 mg of aflatoxin/kg of BW became firm and less fluid within 24-36 hours after aflatoxin administration. The duration of time that the consistency of rumen contents was altered increased with greater doses of aflatoxin. A decrease in water
consumption may have been the cause, but water consumption was not measured. Systemic dehydration was not present in these animals based on PCVs and total protein concentrations recorded during these times. Decreased rumen motility may have been a factor and resulted in poor mixing of the ventral fluid layer of the rumen with drier ingesta in the dorsal layer of the rumen (Church, 1975).

Primary bile acids, such as glycocholic acid, are steroids formed from metabolism of sterols, predominantly liver cholesterol, in mitochondrial, endoplasmic reticulum and lysosomal compartments in liver tissue. Bile acids are excreted into bile in the intestine where they are partly metabolized into secondary bile acids. Bile acids are reabsorbed from the intestinal lumen into the circulation from which they are excreted once again in conjugated forms by the biliary system. Liver parenchymal damage and biliary damage causing cholestasis reduce clearance of bile acids from the serum and biliary excretion of bile acids with an elevation of serum bile acid concentration. Liver parenchymal damage and cholestasis have been common features in experimental and field cases of bovine aflatoxicosis and were observed in this study. The dose dependent response of serum glycocholic acid in this study appeared to be a more sensitive indicator of biliary impairment or cholestasis after aflatoxin administration than alkaline phosphatase or bilirubin. This finding supports previous results with glycocholic acid, alkaline phosphatase and aflatoxin in the bovine (Richard et al., 1983). Previous studies have found increased concentrations of serum cholesterol in calves given aflatoxin (Pier et al., 1976). Perhaps decreased biliary excretion of glyco-
cholic acid results in reduced metabolism of cholesterol to glycocholic acid and an increase in serum cholesterol concentrations.

Histopathologic lesions around centrilobular veins in liver tissue consisted of marked hemorrhage and accumulation of lymphocytes and plasma cells. These lesions were different from those previously reported in the bovine that have not included lymphocytes and plasma cells around centrilobular veins. Minimal centrilobular hemorrhage or massive lobular hemorrhage have been described. In a case of acute aflatoxicosis in which calves were fed aflatoxin contaminated peanut hay for 11 days, minimal hemorrhage was observed around centrilobular veins (McKenzie et al., 1981). The finding of lymphocytes with their known pleuropotentiality may be a precursor in the fibrosis that is often observed in cases of chronic aflatoxicosis in the bovine. Reported liver lesions in cases of aflatoxicosis in the bovine include bile duct hyperplasia, fibrosis with possible pseudo-lobulation of the liver, fatty vacuolization of hepatocytes, necrosis of hepatocytes and edema of centrilobular veins. Fatty change in liver tissue has been commonly reported in chronic bovine aflatoxicosis but not in acute toxicosis. Extensive loss of cellular glycogen has been observed in dairy calves fed 0.03, 0.04, and 0.08 mg of aflatoxin B1/kg BW but not at lesser concentrations down to 0.008 mg of aflatoxin/kg BW (Lynch et al., 1970). In the present study, liver tissue from steers given greater concentrations of aflatoxin stained less positive for glycogen. The lesions in the present study may have been different because studies with aflatoxin in the bovine have usually been performed after chronic aflatoxin exposure and/or a longer time after exposure to aflatoxin. The lesions
observed in this study may be more representative of those occurring in acute to subacute aflatoxicosis in the bovine.
VI. SUMMARY

The effects of aflatoxin on bovine rumen motility were investigated with the aid of radiotelemetry. Aflatoxin markedly altered amplitude and/or frequency of rumen contractions in steers given 0.2 to 0.8 mg of aflatoxin/kg BW. Decreased elimination of aflatoxin from rumens was observed in steers given 0.4 to 0.8 mg of aflatoxin/kg BW. Effects of aflatoxin on rumen motility appeared to be dose dependent. Changes in rumen motility coincided in time with severity of liver damage and feed intake. Aflatoxin M₁ was detected in relatively high concentrations in rumen contents 2 hours after aflatoxin administration. This suggested intraruminal metabolism of aflatoxin B₁ to M₁. Rumen contents and feces were concluded to be of possible diagnostic value in detecting cases of bovine aflatoxicosis, while aflatoxin M₁ in urine was the most sensitive indicator of aflatoxin exposure in this study.
VII. APPENDIX
Figure 1. Silicone rumen fistula
Figure 2. Rumen transmitter
Figure 3. A developed two dimensional thin layer chromatography plate. Aflatoxin B₁ (B) and aflatoxin M₁ (M) standards are on side channels. Aflatoxin B₁ (B) and aflatoxin M₁ (M) have been chromatographed out of a sample and are present in the center of the plate.
Figure 4. Rumen motility from the steer given 0.8 mg of aflatoxin/kg BW as recorded by radiotelemetry.
Figure 5. Rumen motility from the steer given 0.6 mg of aflatoxin/kg BW as recorded by radiotelemetry.
STEER +37 0.6 mg/kg AFLATOXIN

PREDOSE

0h
+8h
+24h
+48h
+56h
+72h
+80h
+104h
+120h

--- 10 MINUTES ---
Figure 6. Rumen motility from the steer given 0.4 mg of aflatoxin/kg BW as recorded by radiotelemetry.
Figure 7. Rumen motility from the steer given 0.2 mg of aflatoxin/kg BW as recorded by radiotelemetry.
STEER +3 0.2 mg/kg AFLATOXIN

PREDOSE

0h
+8h
+24h
+32h
+48h
+58h
+72h
+88h

10 mm Hg

10 MINUTES
Figure 8. Frequency of rumen contractions in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW. The zero time is a predose, or control time in all steers.
Figure 9. Amplitude (strength) of rumen contractions in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW. The zero time is a predose, or control time in all steers.
AFLATOXIN
0.2 mg/kg BW

AFLATOXIN
0.4 mg/kg BW

AFLATOXIN
0.6 mg/kg BW

AFLATOXIN
0.8 mg/kg BW

AMPLITUDE (mm)

0 20 40 60 80 100
TIME (h)
Figure 10. Aflatoxin B₁ (ng/g) in rumen contents (dry weight basis) in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 11. Aflatoxin M₁ (ng/g) in rumen contents (dry weight basis) in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 12. Aflatoxin B1 (ng/ml) in blood in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 13. Aflatoxin M₁ (ng/ml) in blood in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW.
AFLATOXIN M1 (ng/ml)

TIME (h)

0 20 40 60 80 100 120 140

0 10 20 30 40 50

0.2 mg/kg BW
0.4 mg/kg BW
0.6 mg/kg BW
0.8 mg/kg BW
1.0 mg/kg BW

20 40 60 80 100 120 140
Figure 14. Aflatoxin B₁ (ng/ml) in urine in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 15. Aflatoxin M₁ (ng/ml) in urine in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW.
Figure 16. Aflatoxin B₁ (ng/g) in feces (dry weight basis) in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 17. Aflatoxin M₁ (ng/g) in feces (dry weight basis) in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 18. Sorbitol dehydrogenase (IU/L) in serum in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 19. Aspartate aminotransferase (IU/L) in serum in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 20. Glycocholic acid in serum in steers given 0.2, 0.4, 0.6, and 0.8 mg of aflatoxin/kg BW
Figure 21. Marked hemorrhage, lymphocytes and plasma cells around a centrilobular vein in liver tissue from the steer given 0.6 mg of aflatoxin/kg BW
Figure 22. Eosinophilic hyaline casts in renal tubules of the steer given 0.2 mg of aflatoxin/kg BW
VIII. REFERENCES


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