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METABOLISM OF THREE TRICHOTHECENE MYCOTOXINS, T-2 TOXIN, DIACETOXYSCIRPENOL AND DEOXYNIVALENOL, BY BOVINE RUMEN MICROORGANISMS

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SUMMARY

The three trichothecene mycotoxins T-2 toxin, diacetoxyscirpenol (DAS) and deoxynivalenol (DON) were incubated in vitro for 12, 24 and 48 h with rumen microorganisms obtained from a fistulated dairy cow. Gas chromatographic and gas chromatographic–mass spectrometric analyses of extracts indicated all three toxins were biotransformed to a variety of deepoxy and deacylated products. DON was partially converted to a product identified as deepoxy DON. DAS was rapidly converted to four products including 15-monoacetoxyscirpenol (MAS), scirpentriol and two new compounds identified as 15-acetoxy-3α,4β-dihydroxytrichothec-9,12-diene (deepoxy MAS) and 3α,4β,15-trihydroxytrichothec-9,12-diene (deepoxy scirpentriol). T-2 toxin was also completely biotransformed to the products HT-2, T-2 triol and two new metabolites identified as 15-acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (deepoxy HT-2) and 3α,4β,15-trihydroxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (deepoxy T-2 triol).

INTRODUCTION

The trichothecene mycotoxins are a group of biologically active fungal metabolites characterized by a tetracyclic 12,13-epoxytrichothecene skeleton [1]. Diacetoxyscirpenol (DAS, anguidine), T-2 toxin and deoxynivalenol (DON, vomitoxin) are three of the more important members of this group of mycotoxins which have been detected in agriculture commodities. These mycotoxins have been implicated in a variety of diseases in man and animals with clinical signs

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including hemorrhaging, diarrhea, skin lesions, emesis, feed refusal, weight loss, leukopenia, radiomimetic injury to tissues and death [1–6].

Several authors have recently suggested that ruminants are less sensitive to the effects of trichothecenes [7,8]. This may be due at least in part to differences in metabolism of trichothecene mycotoxins by endogenous microorganisms present in the alimentary tracts of ruminants and non-ruminants. In the present study we have utilized an in vitro rumen system to compare the metabolism of three trichothecene mycotoxins by rumen microorganisms.

EXPERIMENTAL

Inoculum

Fresh rumen fluid was obtained from a rumen fistulated dairy cow maintained on a timothy hay diet. Inoculum was prepared by filtering the rumen contents through cheese cloth and blending the filtrate for 1 min in a waring blender under a carbon dioxide atmosphere.

Culture conditions

To 125-ml Erlenmeyer flasks were added 1.25 mg of the trichothecenes T-2 toxin, DON or DAS dissolved in 0.5 ml of 10% aqueous ethanol, 12.5 ml of Mineral Solution A (0.6% potassium dihydrogen phosphate, 0.6% ammonium sulfate, 1.2% sodium chloride, 0.25% magnesium sulfate heptahydrate and 0.16% calcium chloride dihydrate), 12.5 ml of 12% dipotassium hydrogen phosphate and 0.125 ml of 2.5% (w/v) L-cysteine hydrochloride. The mixture was then saturated with oxygen-free carbon dioxide for 10 min. Inoculum (25 ml) was then added to each flask, the pH adjusted to 6.8 with sodium bicarbonate and the contents were bubbled with carbon dioxide for an additional 5 min. The flasks were stoppered and then incubated on a water bath shaker at 38°C. Incubations with each of the three toxins were run in triplicate. Controls were prepared by adding inoculum to flasks without toxins. Toxin recovery determinations were performed by extraction of triplicate flasks to which heat-killed inoculum was added.

Bacterial enumeration

At 0 and 48 h, 1-ml aliquots were removed from selected flasks for bacterial enumeration. The enumeration medium and the techniques used for the enumeration were performed as outlined by Hungate [9] and modified by Bryant [10]. Inoculation of roll tubes for enumeration was as described by Leedle and Hespell [11].

Sample analysis

At 0, 12, 24 and 48 h, 10-ml aliquots of the incubation mixtures were removed under a carbon dioxide atmosphere, diluted with 5 ml water and centrifuged 10 min at 2000 *g*. A 10-ml aliquot of the supernatant was placed on a 1×12 cm column of Amberlite XAD-4. The column was rinsed with 100 ml water, the trichothecenes eluted with 100 ml acetone and the eluate concentrated on a rotary

evaporator. The residue was transferred to a Florisil column (2.5 g, 60–100 mesh, Fisher Scientific, Chicago, IL, U.S.A.) with 3×2 ml of dichloromethane–methanol (9:1). The toxins were eluted with an additional 50 ml of the same solvent, the eluate was concentrated and the residue redissolved in 1 ml of ethanol.

Gas chromatography (GC)

A 0.2-ml aliquot of each extract was concentrated and redissolved in 1 ml of toluene–acetonitrile (95:5). Heptafluorobutyrylimidazole (HFBI, 0.05 ml, Pierce, Rockford, IL, U.S.A.) was added and the mixture incubated at 60°C for 1 h. After cooling, the excess HFBI was removed by mixing with 1 ml of 5% aqueous sodium bicarbonate. A 0.10-ml aliquot of the organic phase containing the resulting heptafluorobutyryl ester derivatives was removed and diluted to 5.0 ml with hexane. Aliquots of 2 μl were injected into a Hewlett-Packard 5840A gas chromatograph equipped with an electron-capture detector and a 1.8 m×2 mm I.D. glass column packed with 3% OV-17 on 100–120 mesh Supelcoport. Other GC conditions were as follows: injector, 275°C; detector, 325°C; argon–methane (95:5) carrier gas flow-rate, 30 ml/min.

Gas chromatography–mass spectrometry (GC–MS)

Mass spectra of the metabolites were obtained on a Hewlett-Packard 5985 GC–MS system and a VG 7070E GC–MS system using electron impact (EI) (70 eV) or methane positive chemical ionization (CI) of the corresponding trifluoroacyl (TFA) or trimethylsilyl (TMS) derivatives. The TFA ester derivatives were prepared as previously described [12]. Formation of the corresponding TMS ethers was accomplished by adding 50 μl of TMS reagent [bis(trimethylsilyl)trifluoroacetamide (BSTFA)–trimethylsilylimidazole (TMSI)–trimethylchlorosilane (TMCS) (11:3:2)] to small vials containing aliquots of extracts concentrated to dryness, and heating for 10 min at 60°C. The mixture was then diluted with an equal volume of ethyl acetate prior to GC–MS analysis.

RESULTS

The concentration of anaerobic bacteria averaged $4.9 \cdot 10^7$ per ml immediately after innoculation. At 48 h the concentration of bacteria had declined to $2.8 \cdot 10^6$ per ml.

GC analysis of the incubation mixtures demonstrated a steady decline in toxin substrate concentration over time for all three trichothecenes, with a corresponding appearance of one or more new products. Average recoveries of the three parent toxins from control flasks containing heat-killed inoculum ranged from 69% to 92%. Although trace amounts of HT-2 (<3%) and 15-monoacetoxyscirpenol (<2% of added substrate) were detected in 48-h killed inoculum control incubations, no other metabolites were observed.

DON was partially biotransformed to a single metabolite with a GC retention time of 2.25 min (Table I). The mass spectrum of this metabolite (TMS deriv-

TABLE I

BIOTRANSFORMATION OF DON BY ANAEROBIC BOVINE RUMEN MICROORGANISMS

Compound	Retention time* (min)	Recovery** (%)		
		Incubation time		
		12 h	24 h	36 h
DON	3.45	88.0	62.5	41.2
DOM-1	2.25	7.4	14.2	18.7
Ratio DON/DOM-1		11.9	4.4	2.2

*GC retention times of the heptafluorobutyryl ester derivatives on OV-17 at a column temperature of 170°C.

**Each value is the average molar percent recovery of three replications. The mean recovery of DON added to control rumen samples containing heat-killed inoculum was 69 ± 4%.

ative) displayed a molecular ion (EI) at *m/z* 496 with major fragment ions at *m/z* 481, 406, 391, 361 and 309. These fragment ions were 16 mass units less (loss of one oxygen atom) than the corresponding fragments for DON and the spectrum was identical to authentic deepoxy DON (DOM-1) obtained from the urine of rats administered DON [13]. Although DOM-1 was detected at the earliest sampling and increased over time, the parent compound DON was the major compound present at all time periods examined.

T-2 toxin was metabolized more rapidly and to a greater extent than DON. Only 3.6% of the parent T-2 was detected at 12-h incubation and none was detected at later time periods (Table II). The hydrolysis products HT-2 and T-2 triol were

TABLE II

BIOTRANSFORMATION OF T-2 TOXIN BY ANAEROBIC BOVINE RUMEN MICROORGANISMS

Compound	Retention time* (min)	Recovery** (%)		
		Incubation time		
		12 h	24 h	48 h
T-2 toxin	21.82	3.6	0	0
HT-2	6.14	60.4	44.8	30.9
DE HT-2	3.92	4.9	14.3	26.5
Triol	2.97	1.5	4.3	9.5
DE Triol	2.01	0	1.0	5.9
Ratio epoxy/deepoxy metabolites		13.4	3.2	1.2

*GC retention times of the heptafluorobutyryl ester derivatives on OV-17 at a column temperature of 220°C.

**Each value is the average molar percent recovery of three replications. The mean recovery of T-2 added to control flasks containing heat-killed inoculum was 86 ± 16%.

TABLE III

BIOTRANSFORMATION OF DAS BY ANAEROBIC BOVINE RUMEN MICROORGANISMS

Compound	Retention time* (min)	Recovery** (%)		
		Incubation time		
		12 h	24 h	48 h
DAS	14.20	0	0	0
MAS	3.92	47.9	47.6	24.1
DE MAS	2.14	5.3	12.2	21.4
Striol	1.48	13.2	25.0	25.7
DE Striol	0.93	0.8	2.1	15.1
Ratio epoxy/deepoxy metabolites		11.9	4.4	2.2

*GC retention times of the heptafluorobutyryl derivatives on OV-17 at a column temperature of 190°C.

**Each value is the average molar percent recovery of three replications. The mean recovery of DAS added to control flasks containing heat-killed inoculum was 92 ± 8%.

detected as metabolites, in addition to two new compounds having GC retention times of 3.92 and 2.01 min, respectively. The CI mass spectra of the TFA derivatives of the two unknowns displayed M+1 peaks at *m/z* 601 (M+1 of HT-2−oxygen) with fragment ions at *m/z* 541, 499, 487 and 439 for the unknown eluting at 3.92 min and an M+1 peak at *m/z* 655 (M+1 of T-2 triol−oxygen) with fragment ions at *m/z* 553, 439 and 325 for the unknown eluting at 2.01 min. The mass spectra obtained were consistent with the loss of oxygen from the epoxide group of HT-2 and T-2 triol to give deepoxy HT-2 (DE HT-2) and deepoxy T-2 triol (DE Triol), respectively. Alkaline hydrolysis [12] of both new metabolites yielded the same product, deepoxy T-2 tetraol (TMS ether molecular ion at *m/z* 470 by EI and M+1 of *m/z* 471 by methane CI, i.e. TMS T-2 tetraol−one oxygen) rather than T-2 tetraol. However, neither deepoxy T-2 tetraol nor T-2 tetraol were detected as products directly. The structures of the two new metabolites were therefore proposed as 15-acetoxy-3α,4β-dihydroxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (DE HT-2) and 3α,4β,15-triacetoxy-8α-(3-methylbutyryloxy)trichothec-9,12-diene (DE Triol).

DAS was extensively biotransformed, no parent DAS was observed at any of the three incubation time periods. Four products were detected including scirpentriol, MAS and two new products with GC retention times of 0.93 and 2.14 min (Table III). CI mass spectra of the earliest-eluting product yielded an M+1 peak at *m/z* 555 with fragment ions at *m/z* 441, 387 and 327. All these fragments were 16 mass units less, compared with the corresponding fragments in scirpentriol. The CI mass spectrum of the second unknown displayed an M+1 ion at *m/z* 501 (M+1 of MAS−oxygen). Alkaline hydrolysis [12] of this compound yielded a new product identical to the metabolite eluting at 0.93 min. In addition,

both new products did not react with 4-(*p*-nitrobenzyl)pyridine [14] on thin-layer chromatography plates suggesting the absence of the 12,13-epoxide group. Based upon the above evidence the two new DAS metobolites were identified as 15-acetoxy-3α,4β-trihydroxytrichothec-9,12-diene (deepoxy MAS; DE MAS) and 3α,4β,15-trihydroxytrichothec-9,12-diene (deepoxy scirpentriol; DE Striol).

DISCUSSION

The ability of bovine rumen microorganisms to reduce the 12,13-epoxide group with the loss of oxygen to yield a carbon–carbon double bond was demonstrated for all three trichothecenes. King et al. [7] and Cote et al. [15] have previously reported the reduction of DON to the deepoxy product, DOM-1, by rumen microorganisms. The present data demonstrates that the deepoxidation of DON by bovine rumen microorganisms occurs slowly over time and incubation periods longer than 48 h are required for complete biotransformation of DON to DOM-1.

T-2 toxin and DAS were reduced in a similar manner to DON by bovine rumen microorganisms to yield deepoxy products. In contrast to DON, direct deepoxidation of T-2 or DAS to yield deepoxy T-2 (DE T-2) and deepoxy DAS (DE DAS) was not observed but rather their deacylated products DE HT-2, DE Triol, DE MAS and DE Striol, respectively. Although culture conditions in this study may not have been optimal, as suggested by the concentration of anaerobic bacteria observed (10^7 cells per ml) compared to literature values of 10^9 cells per ml for rumen contents [11,16], deepoxy metabolites were produced in significant amounts. Reduction of the epoxide groups in the trichothecene toxins were presumed to be the result of bacterial biotransformations; however, since the population of protozoa was not determined in this study, additional work is needed to confirm which type of microorganisms and which species are responsible for this deepoxidation reaction.

Kiessling et al. [17] incubated both T-2 toxin and DAS with rumen microorganisms but detected only deacylation products after incubation times of up to 3 h. In the present study, deepoxy biotransformation products were found only in small amounts after 12 h incubation, but increased steadily over time for the following 36 h. Similarly to DON, deepoxidation of DAS and T-2 metabolites by bovine rumen microorganisms appears to require incubation times greater than 12 h.

Although deepoxy metabolites of T-2 toxin (DE HT-2, DE Triol) and DAS (DE MAS and DE Striol) were significant products after 48 h incubation, the simple hydrolysis products HT-2 and scirpentriol were the predominant metabolites present. This observation, in addition to the failure to detect DE T-2 and DE DAS directly, suggests deacylation of the C-4 acetyl group by microbial esterases occurred prior to the deepoxidation reactions. Neither T-2 tetraol nor deepoxy tetraol were detected as biotransformation products of T-2, demonstrating the isovaleryl ester group at the C-8 position of T-2 is resistant to microbial hydrolysis. The pathway shown in Fig. 1, which includes both enzymic reduction and ester hydrolysis of the parent compounds, is proposed for the in vitro metab-

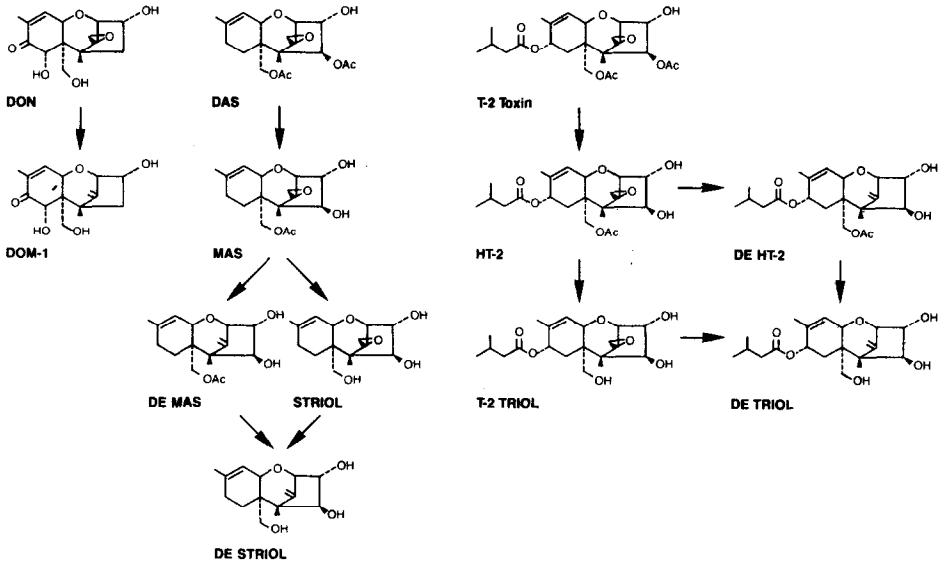

Fig. 1. Proposed pathway for the anaerobic biotransformation of T-2, DAS and DON by bovine rumen microorganisms.

olism of the trichothecene mycotoxins DON, DAS and T-2 toxin by anaerobic bovine rumen microorganisms.

Reduction of the 12,13-epoxide to yield a carbon–carbon double bond has been reported with several trichothecenes by fungi, animals and microorganisms. Verrucarin K, the deepoxy equivalent of verrucarin A, was observed as a metabolite of *Myrothecium verrucaria* by Breitstein and Tamm [18]. DOM-1 has been found in the urine and feces of rats orally administered DON [13], and recently in the urine, feces and milk of dairy cattle given DON-contaminated feed [19,20]. Deepoxy metabolites of T-2 toxin including deepoxy T-2 tetraol, deepoxy-4-deacetylneosolaniol and deepoxy-3′-hydroxy HT-2 have been identified in excreta of rats administered T-2 tetraol and 3′-hydroxy HT-2 [21]. Recently, we have identified DE MAS and deepoxy scirpentriol as major metabolites in the urine and feces of rats orally administered DAS [22].

The identification of deepoxy metabolites in the urine and feces of animals administered trichothecenes suggests gastrointestinal microflora are capable of performing reductive deepoxidation reactions similar to the bovine rumen microorganisms. The toxicological significance of this reaction, however, is still unclear. Several authors have demonstrated that alteration of the 12,13-epoxide group resulted in elimination of toxicity [6,23,24] although the reaction products examined were significantly altered in structure either by ring rearrangements or reductive cleavage of the epoxide to give a tertiary alcohol. Further work is needed to determine whether reduction of the epoxide to yield a carbon–carbon double bond also eliminates toxicity.

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REFERENCES

1 J.R. Bamburg and F.M. Strong, in S. Kadis, A. Ciegler and S.J. Ajl (Editors), Microbial Toxins, Academic Press, New York, 1971, p. 207.
2 L.M. Cote, J.D. Reynolds, R.F.Vesonder, W.B. Buck, S.P. Swanson, R.T. Coffee and D.C. Brown, J. Am. Vet. Med. Assoc., 184 (1984) 189.
3 C.J. Mirocha, S.P. Pathre, B. Schauerhamer and C.M. Christensen, Appl. Environ. Microbiol., 32 (1976) 553.
4 C.J. Mirocha, S.V. Pathre and C.M. Christensen, in T.D. Wyllie and L.G. Morehouse (Editors), Mycotoxic Fungi, Mycotoxins and Mycotoxicoses, Marcel Dekker, New York, 1977, p. 365.
5 N. Sato and Y. Ueno, in J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (Editors), Mycotoxins in Human and Animal Health, Pathotox Publishers, Park Forest South, 1976, p. 295.
6 E.B. Smalley, and F.M. Strong, in I.F.H. Purchase (Editor), Mycotoxins, Elsevier, Amsterdam, 1973, p. 199.
7 R.D. King, R.E. McQueen, D. Levesque and R. Greenhalgh, J. Agric. Food Chem., 32 (1984) 1181.
8 T. Romer, Feedstuffs, 11 (1983) 30.
9 R.E. Hungate, Bacteriol. Rev., 14 (1950) 1.
10 M.P. Bryant, Am. J. Clin. Nutr., 25 (1972) 1324.
11 J.A.Z. Leedle and R.B. Hespell, Appl. Environ. Microbiol., 39 (1980) 709.
12 H.D. Rood, Jr., S.P. Swanson and W.B. Buck, J. Chromatogr., 378 (1986) 375.
13 T. Yoshizawa, H. Takeda and T. Ohi, Agric. Biol. Chem., 47 (1983) 2133.
14 S. Takitani, T. Asabe, T. Kato, M. Suzuki and Y. Ueno, J. Chromatogr., 172 (1979) 335.
15 L.M. Cote, J. Nicoletti, S.P. Swanson and W.B. Buck, J. Agric. Food Chem., 34 (1986) 458.
16 R.E. Hungate, The Rumen and its Microbes, Academic Press, New York, 1966, p. 31.
17 K.H. Kiessling, H. Peterson, K. Sandholm and M. Olson, Appl. Environ. Microbiol., 47 (1984) 1070.
18 W. Breitstein and C. Tamm, Helv. Chim. Acta, 60 (1977) 1522.
19 T. Yoshizawa, L.M. Cote, S.P. Swanson and W.B. Buck, Agric. Biol. Chem., 50 (1986) 227.
20 L.M. Cote, A.M. Dahlem, S.P. Swanson, T. Yoshizawa and W.B. Buck, J. Dairy Sci., 69 (1986) 2416.
21 T. Yoshizawa, T. Sakamoto and K. Kuwamura, Appl. Environ. Microbiol., 50 (1985) 676.
22 T. Sakamoto, S.P. Swanson, T. Yoshizawa and W.B. Buck, J. Agric. Food Chem., 34 (1986) 698.
23 J.F. Grove and P.H. Mortimer, Biochem. Pharmacol., 18 (1969) 1473.
24 M.D. Grove, H.R. Burmeister, S.L. Taylor, D. Wesleder and R.D. Plattner, J. Agric. Food Chem.,. 32 (1984) 541.