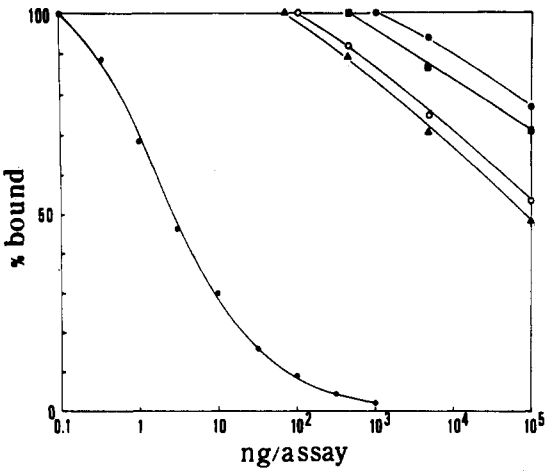

Figure 3. Effect of structural analogues of ochratoxin A on the binding of [^{14}C]ochratoxin A with IgG: ochratoxin A (●); ochratoxin B (▲); bovine serum albumin (○); β-L-phenylalanine (■); ochratoxin α (*). The extent of binding of [^{14}C]ochratoxin A with IgG in the absence of unlabeled toxin was considered as 100% of binding.

Table III. Comparison between RIA and HPLC Analysis of Porcine Serum Samples[b]

	RIA, ng/mL	HPLC, ng/mL
neg sample	<0.4[a]	<5[a]
pos sample 1	350.4 ± 6.4	363.1 ± 9.3
pos sample 2	199.6 ± 3.7	209.4 ● 5.5
pos sample 3	274.7 ± 5.3	282.7 ± 7.4

[a] Detection limit. [b] Data are means of three to five analyses.

(0.4 ng/mL) and as the entire procedure can be done in 1 day, the method can be useful for broad surveys of biological samples of slaughter animals.

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Registry No. Ochratoxin A, 303-47-9.

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In Vitro Metabolism of T-2 Toxin by Rat Liver Microsomes

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Rat liver microsomes biotransform T-2 toxin in vitro to a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3′-OH T-2, and 3′-OH HT-2, in addition to two unidentified compounds. Metabolism resulting from cleavage of the ester linkages of the parent T-2 toxin was more extensive than hydroxylation of the 3′-carbon side chain by mixed-function oxidases. Treatment of rats with phenobarbital resulted in enhanced oxidative hydroxylation of T-2 toxin at the 3′-carbon position, and addition of paraoxon to the microsomal preparation inhibited the hydrolysis of the C3′-oxidized product.

INTRODUCTION

T-2 toxin (4β,15-diacetoxy-8α-[(3-methylbutyryl)oxy]-3α-hydroxy-12,13-epoxytrichothec-9-ene) is a trichothecene mycotoxin produced by species of *Fusarium* (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). Although detected only sporadically in nature compared with other trichothecene mycotoxins such as deoxynivalenol, T-2 has been implicated in several serious cases of human and animal toxicoses (Hsu et al., 1972; Joffe, 1971; Bamburg et al., 1971).

The distribution and metabolism of tritium-labeled T-2 toxin was investigated after oral administration in chickens (Yoshizawa et al., 1980a), mice (Matsumoto et al., 1978), and a lactating cow (Yoshizawa et al., 1981) and after intravascular administration in swine (Corley et al., 1985). In all species, T-2 was rapidly biotransformed to a variety of metabolites. Minor metabolites in the cow and chicken were initially identified as simple hydrolysis products including HT-2, 4-deacetylneosolaniol, and neosolaniol; however, the major metabolites remained unidentified.

Yoshizawa et al. (1982) characterized two of the main metabolites present in bovine urine, initially labeled TC1 and TC3, as 3′-OH T-2 and 3′-OH HT-2, respectively. Recently a third metabolite, TC6, was tentatively identified as 3′-OH-7-OH HT-2 (Pawlosky and Mirocha, 1984).

The compound HT-2 was reported as the only in vitro metabolite in human and bovine liver homogenates (Ellison and Kotsonis, 1974) and laboratory animals (Ohta et al., 1977). In addition to HT-2, 4-deacetylneosolaniol,

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T-2 tetraol, and neosolaniol were detected in rat liver homogenates and stomach strips incubated with T-2 toxin (Yoshizawa et al., 1980b). The hydroxylated products 3′-OH T-2 and 3′-OH HT-2 were subsequently identified for the first time in vitro, using monkey and mice liver homogenates or microsomal fractions supplemented with a NADPH-generating system, cofactors necessary for mixed-function oxidase activity.

The present study is concerned with the metabolism of T-2 by rat liver microsome fractions sampled at specific time intervals (5, 10, 15, 30, 60 min) and the role of microsomal esterase and oxidase systems in the biotransformation of T-2 toxin in vitro.

EXPERIMENTAL SECTION

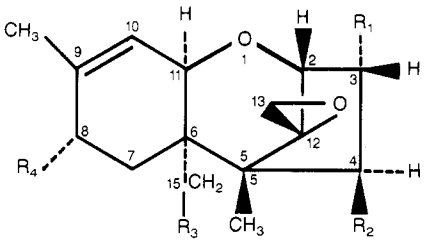
Microsomal Preparation. Male Sprague–Dawley rats weighing 400–500 g were obtained from Harlan Co., St. Louis, MO. Liver microsome mixed-function oxidase activity was induced by treating rats for 3 consecutive days with intraperitoneal injections of 75 mg/kg of sodium phenobarbital (PB) dissolved in physiologic buffered saline. Following exsanguination, individual livers were perfused with cold homogenizing medium (0.25 M sucrose contining 0.05 mM EDTA) through visible blood vessels and then homogenized with 3 volumes of the same medium. The homogenate was centrifuged at 10000*g* for 10 min at 0° C and the supernatant filtered through glass wool. The filtrate was centrifuged at 100000*g* for 60 min at 4 °C and the supernatant decanted. The microsomal pellet was resuspended in the homogenizing medium to give 1.0 g of liver fresh weight/mL, flash frozen in liquid nitrogen, and stored at −70 °C. All microsome preparations were utilized within 2 weeks of preparation. For all assays, 1.0 mL of microsomal preparation was resuspended in 9.0 mL of Tris buffer (0.05 M, pH 7.4) containing 0.15 M KCl. The final microsomal suspension averaged 22 mg of protein/mL by the Hartree (1972) modification of the Lowry et al. (1951) procedure.

Microsomal Assay. The reaction mixture consisted of 1.0 mL of microsomal suspension from PB-induced or -noninduced control rats and 3.0 mL of the Tris–KCl buffer containing the following cofactors: 2.0 μmol of $NADP^+$, 10.0 μmol of glucose 6-phosphate, 30 μmol of $MgCl_2$, and 1.33 units of glucose 6-phosphate dehydrogenase. To each vial was added 2.15 μmol of T-2 toxin (99+% pure) dissolved in 0.05 mL of ethanol, and the vials were incubated at 37 °C in a water bath shaker. Additional microsomes from PB-induced rats were also incubated with T-2 toxin in the presence of 50 nmol of paraoxon for 60 min. Controls consisted of a microsomal blank from each animal containing 1.0 mL of microsomal suspension plus 3.0 mL of buffered cofactor solution (incubated for 60 min), in addition to samples without microsomes (reagents only) with 2.15 μmol of T-2 toxin added. At the completion of each incubation period (5, 10, 15, 30, 60 min), enzyme activity was stopped by adding 0.5 mL of 1 N HCl. Each incubation mixture was then applied to a 500-mg C18 cartridge (J. T. Baker; preconditioned with methanol and water), and the toxins were eluted with 60% aqueous methanol (2 × 0.9 mL) followed by 100% methanol (2 × 0.9 mL) on an Analytichem Vac Elute system (Habor City, CA). The solvent was evaporated and the residue redissolved in ethanol. An aliquot was removed from each fraction for gas chromatographic analysis.

Derivatization and Gas Chromatographic Conditions. For GLC-ECD analysis sample extracts were dissolved in 1.0 mL of toluene–acetonitrile (95:5) and derivatized with 0.05 mL of trifluoroacetic anhydride at 60 °C for 60 min. Samples were cooled to room temperature and vortexed with 1.0 mL of a 5% aqueous sodium bicarbonate solution. An aliquot was removed from the organic layer and diluted with isooctane, and 1 μL was injected (splitless) into the gas chromatograph. Gas chromatography was performed on a Hewlett-Packard 5790 gas chromatograph equipped with a ^{63}Ni electron capture detector and a 12-m length × 0.2-mm i.d. (0.2 μm film coating) J & W DB1701 fused silica capillary column. A multiramp column temperature program was used as follows: 90 °C (hold 1 min) to 170 °C at 30 °C/min and then from 170 °C (hold 1 min) to 245 °C at 5.5 °C/min (hold 2 min). Other operating parameters were as follows: injector, 275 °C; detector, 340 °C; hydrogen carrier gas flow rate, 45 cm/s.

For capillary GLC-fid, sample extracts were dissolved in 0.04 mL of ethyl acetate–isooctane (1:1) containing 0.25 mg/mL of triacontane ($C_{30}H_{62}$) as an internal standard and derivatized with 0.01 mL of (trimethylsilyl)imidazole–trimethylchlorosilane (5:1) at 60 °C for 10 min. A split injection mode was used with a split ratio of 30:1. A 30-m length × 0.2-mm i.d. (0.2-μm film coating) J & W DB1701 fused silica capillary column was used. The column temperature was programmed from 250 °C (hold 2 min) to 285 °C at 10 °C/min (hold 5.5 min). Other operating parameters were as follows: injector, 285 °C; detector, 300 °C; hydrogen carrier gas flow rate, 45 cm/s. Identification of metabolites was achieved by comparing retention times to those of known GC standards, as both the TFA and Me_3Si derivatives. Metabolites were quantitated by fid as the Me_3Si derivatives. Recovery of T-2 toxin averaged 95% (n = 5, SE = 4%) when added to microsomes inactivated with 0.5 mL of 1 N HCl prior to incubating for 60 min.

Table I. Chemical Structures and Retention Times of T-2 Toxin and Its Metabolites by Capillary Gas Chromatography



compd	R_1	R_2	R_3	R_4	GLC ret time,[a] min TFA	GLC ret time,[a] min Me_3Si
T-2 toxin	OH	OAc	OAc	X1[b]	16.73	7.35
3′-OH T-2	OH	OAc	OAc	X2	16.82, 17.87[c]	9.88
3′-OH HT-2	OH	OH	OAc	X2	13.77, 14.80[c]	9.10
HT-2 toxin (HT-2)	OH	OH	OAc	X1	13.68	6.86
T-2 triol (Triol)	OH	OH	OH	X1	11.76	5.73
neosolaniol (NEO)	OH	OAc	OAc	OH	11.59	5.25
4-deacetylneosolaniol (4-DN)	OH	OH	OAc	OH	8.79	4.82
T-2 tetraol (TOL)	OH	OH	OH	OH	7.39	3.74

[a] Retention times. See text for conditions. [b] b_{X_1} = $OCOCH_2C(H)(CH_3)_2$; X_2 = $OCOCH_2C(OH)(CH_3)_2$. [c] The 3′-hydroxy metabolites form two isomers when derivatized with TFAA (Pawlosky et al., 1984).

RESULTS AND DISCUSSION

The structures of T-2 metabolites and their gas chromatographic retention times as both TFA and Me_3Si derivatives are given in Table I. The metabolic profiles from PB-treated and control rat liver microsomes are compared in Table II. The major metabolite at all time periods and with both treatment groups was HT-2, demonstrating that enzymatic ester hydrolysis at the C4 position occurs more

Table II. In Vitro Metabolism of T-2 Toxin by Microsomal Liver Fractions of Untreated Rats and Rats Treated with Phenobarbital

	nmol of product[a]									
	5 min		10 min		15 min		30 min		60 min	
compd	PB[b]	C[c]	PB	C	PB	C	PB	C	PB	C
T-2	1630	1620	1440	1480	1300	1330	830	1020	324	620
	(133)	(186)	(110)	(166)	(42)	(138)	(122)	(65)	(67)	(84)
3′-OH T-2	26	9	38	11	54	13	87	22	124	35
	(9)	(2)	(11)	(4)	(11)	(4)	(24)	(7)	(67)	(6)
3′-OH HT-2	0.3	ND[d]	0.6	ND	1.1	ND	6.5	1.5	20.7	4.6
	(0.3)		(0.6)		(1.0)		(2.0)	(0.6)	(4.4)	1.0)
HT-2	235	173	412	301	555	433	970	740	1420	1160
	(26)	(22)	(34)	(37)	(23)	(60)	(25)	(105)	(73)	(126)
Triol	0.6	0.2	0.7	0.4	2.2	1.2	1.2	1.6	4.9	4.4
	(0.6)	(0.2)	(0.7)	(0.4)	(1.2)	(0.4)	(0.9)	(0.3)	(1.6)	(1.6)
Neo	8.1	6.0	10.0	5.9	12.0	6.3	18.0	7.2	27.0	8.8
	(3.3)	(1.7)	(3.5)	(1.9)	(3.1)	(1.9)	(4.9)	(2.1)	(4.3)	(1.5)

[a] To each vial was added 2150 nmol of T-2 toxin. Values are means of five replications, standard error in parentheses. [b] Animal treated with 75 mg/kg of sodium phenobarbital intraperitoneally for 3 consecutive days prior to microsomal preparation. [c] Noninduced control rats. [d] ND = none detected.

readily than hydroxylation at C3′ or hydrolysis at the C15 or C8 carbons. Five metabolites including HT-2, 3′-OH T-2, 3′-OH HT-2, NEO, and T-2 triol were detected within 5 min when incubated with microsomes from PB-treated rats. In contrast, 3′-OH HT-2 was not detected until 30 min in the noninduced rats. The metabolite 4-DN was detected in trace quantities (less than 0.3% of the added T-2) by TFA/ECD in all samples from PB-induced rats and in the 60-min sample from the noninduced rats but was not detected by the less sensitive Me_3Si/fid method.

Although analysis by TFA/ECD provided greater sensitivity, quantitation was accomplished by Me_3Si/fid for the following reasons: (1) The 3′-hydroxyl metabolites yield a single peak as the Me_3Si derivative, rather than the split peak formed during TFA derivatization. The detection of a single peak allows for more consistent quantitation and facilitated separation from other compounds. (2) Flame ionization detection provided better run-to-run reproducibility and quantitation due to the similar response factors of each metabolite and less base-line interference as compared to EC detection. (3) The Me_3Si derivatives provided more complete chromatographic separation and are more stable than the corresponding TFA derivatives. Actual analysis time was also considerably shortened since gas chromatograph run times of under 10 min were possible when using a split fid mode, as compared to 25-min run times when splitless injection capillary ECD was used.

Treatment of rats with phenobarbital induced both esterase and mixed-function oxidase activity as indicated by the increase in HT-2 and the two 3′-hydroxylated metabolites at all time periods compared with noninduced controls. The induction of mixed-function oxidase activity by PB, however, was much more significant. The ratio of metabolite produced in PB-induced compared to noninduced microsomes, averaged over all time intervals, was only 1.3 for HT-2 compared with a ratio of 3.7 for 3′-OH T-2 and 4.8 for 3′-OH HT-2.

At 60 min, 15% and 30% of the added T-2 remained unmetabolized in PB-treated and noninduced microsomal preparations, respectively. All metabolites were still increasing in concentration at this time period.

The ratio of substrate to microsomal protein is critical in determining both extent and the pathway of metabolism. When the amount of T-2 toxin added to the PB-induced microsomal incubation mixtures was reduced from 2150 nmol (975 nmol/mg of protein) to 86 nmol (39 nmol/mg of protein), only a trace amount of substrate remained unmetabolized by 60 min. In addition, the percentage of 3′-OH T-2 formed at the lower substrate to protein ratio increased threefold with no corresponding change in the HT-2 produced (data not shown).

Complete metabolism of T-2 toxin was reported in PB-treated mice liver microsomes and a 60-min incubation time with a substrate to microsomal protein ratio (nmol/mg of protein) of 154 (Yoshizawa et al., 1984). However, 3′-OH HT-2 was the major hydroxylated metabolite detected with mice microsomes compared with 3′-OH T-2 in the present study, suggesting differences in biotransformation of T-2 toxin between these rodent species.

The addition of 50 nmol of paraoxon to PB-induced microsomes increased by 6 times the amount of 3′-OH T-2 formed as compared to untreated control microsomes during a 60-min incubation period. When incubated with paraoxon, PB-induced microsomes produced 1.5–2.0 times more 3′-OH T-2 than did nontreated PB microsomes. Paraoxon is a potent esterase (carboxyesterase, aliesterase) inhibitor (Stitzel et al., 1972). Therefore, it was expected that addition of paraoxon to the microsomal preparation would increase the percentage of the C3′-hydroxylated product 3′OH T-2. Esterase inhibition not only increased the availability of added substrate (T-2) for oxidation but also decreased the rate of hydrolysis of the resulting oxidized product.

Two new compounds (presumed to be T-2 metabolites) were detected in the microsomal suspensions in addition to the six confirmed metabolites. These compounds were designated RLM-2 and RLM-3 and had GC retention times of 17.11 and 18.41 min as the TFA derivatives and 8.69 and 10.56 min as the Me_3Si derivatives, respectively. They were not found in either the T-2 plus reagents or microsomal blank incubations. Both compounds increased steadily over time and with phenobarbital induction. They also showed a 50% increase after the addition of paraoxon to the phenobarbital-induced microsomes. These compounds were relatively minor metabolites and did not exceed 3% of the administered T-2 even in the paraoxon plus PB microsomal system. Production of larger quantities of these compounds for structural identification is in progress.

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Registry No. T-2, 21259-20-1; HT-2, 26934-87-2; T-2 triol, 2270-41-9; 3′-OH T-2, 84474-35-1; 3′-OH HT-2, 78368-54-4; neo-

solaniol, 36519-25-2; deacetylneosolaniol, 74833-39-9; mixed-function oxidase, 9040-60-2.

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Disposition of T-2 Toxin, a Trichothecene Mycotoxin, in Intravascularly Dosed Swine

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Metabolite profiles in plasma and tissues of two swine were determined following intravascular administration of tritium-labeled T-2 toxin. The plasma elimination phase half-life was 90 min for total tritium residues. At 4-h postdosing (time of death), the greatest amount of radioactivity was located in the gastrointestinal tract (15.5 and 24.1% of the dose for the two swine). The remaining tissues accounted for 5.2 and 4.7% of the dose for the two swine. A total of 21 metabolites was identified by reversed-phase HPLC radiochromatography. Approximately 55% of the extractable radioactivity in the tissues and gastrointestinal tract of both swine corresponded to T-2 toxin, HT-2, deepoxy HT-2, T-2 triol, deepoxy T-2 triol, 3′-OH T-2, 3′-OH HT-2, T-2 tetraol, and deepoxy T-2 tetraol. The major metabolite in tissues, PM-XV, did not correspond to any standard and represented an additional 27% of the extractable radioactivity.

INTRODUCTION

T-2 toxin, 4β,15-diacetoxy-3α-hydroxy-8α-[(3-methylbutyryl)oxy]-12,13-epoxytrichothec-9-ene, is a toxic fungal metabolite produced by several species of *Fusaria* (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). T-2 toxin has been found in naturally contaminated corn, barley, and mixed feeds in the U.S. and Canada at concentrations as high as 25 ppm (Vesonder, 1983). When present in the diets of livestock and poultry, T-2 toxin has been associated with feed refusal, perioral and pharyngeal irritation, intestinal irritation, and possibly hemorrhage, diarrhea, infertility, and lowered immunity (Hsu et al., 1972; Palyusik and Koplik-Kovacs, 1975; Speers et al., 1977; Weaver at al., 1977; Weaver et al., 1978a,b; Rafai and Tuboly, 1972; Hoerr et al., 1982).

Studies on the fate of T-2 toxin in laboratory animals, poultry, and livestock have demonstrated that the parent compound is rapidly cleared from body fluids and tissues. Tritium-labeled T-2 toxin and its metabolites rapidly distributed to tissues of orally dosed mice with maximum levels reached within 30 min, declining thereafter to nondetectable levels by 72 h (Matsumoto et al., 1978). Metabolites were eliminated in a feces to urine ratio of 3:1 over a 72-h time period. Matsumoto et al. (1978) identified T-2 and HT-2 in rat feces at 2.7 and 7.5% of the administered dose, respectively, in addition to two unknown metabolites at 25.8 and 9.1% of the administered dose. No parent T-2 toxin was detected in the urine of rats; however, neosolaniol, HT-2, and three unknowns totaling less than 8% of the administered dose were identified. The chemical structures of T-2 toxin and various metabolites are given in Table I. Rats eliminated metabolites in a feces to urine ratio of 5:1 over a 24-h period. Ueno (1977) reported similar results, finding HT-2 and neosolaniol in rat excreta.

T-2 and its metabolites were eliminated primarily through the bile into the gastrointestinal tract and excreta of orally dosed chickens (Chi et al., 1978). Maximum levels were reached by 4 h in blood, plasma, abdominal fat, liver, heart, kidneys, and carcass and by 12 h in muscle, skin, bile, and gall bladder. Yoshizawa et al. (1980) identified neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol in the excreta of broiler chickens. However, several unknown compounds labeled TB-1 through TB-8 were found to be quantitatively more significant. TB-1 and TB-2 were later identified as 3′-OH T-2; TB-3 (major metabolite) was identified as 3′-OH HT-2, TB-4 as 8-

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