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In Vitro Metabolism of T-2 Toxin in Rats†

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T-2 toxin was rapidly converted in the 9,000 × *g* supernatant fraction of rat liver homogenate into HT-2 toxin, T-2 tetraol, and two unknown metabolites designated as TMR-1 and TMR-2. TMR-1 was characterized as 4-deacetylneosolaniol (15-acetoxy-3α,4β,8α-trihydroxy-12,13-epoxytrichothec-9-ene) by spectroscopic analyses. Since the same metabolites were also obtained from HT-2 toxin used as substrate, it was concluded that T-2 toxin was hydrolyzed preferentially at the C-4 position to give HT-2 toxin, which was then metabolized to T-2 tetraol via 4-deacetylneosolaniol. In addition to HT-2 toxin, 4-deacetylneosolaniol, and T-2 tetraol, a trace amount of neosolaniol was transformed from T-2 toxin by rat intestinal strips. In vitro metabolic pathways for T-2 toxin in rats are proposed.

T-2 toxin, 4β,15-diacetoxy-8α-(3-methylbutyloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene (III), is a toxic metabolite produced by *Fusarium* spp. (1). This toxin is one of the most important trichothecene mycotoxins occurring naturally in agricultural products (5, 9, 10, 14) and is possibly involved in serious field cases of human toxicoses (7, 15, 17) and farm animals (C. M. Hibbs, C. D. Osweiler, W. B. Buck, and G. P. Macfee, Proc. 17th Annu. Meet. Am. Assoc. Vet. Diag., p. 305, 1974; 6, 13, 16).

Up to now, a few papers have reported on the in vivo metabolism of T-2 toxin in animals. Orally administered T-2 toxin is eliminated into the excreta of rats as HT-2 toxin, neosolaniol, and several unknown metabolites (8). In addition to these metabolites, T-2 tetraol and 4-deacetylneosolaniol were found in the excreta of broiler chickens administered tritium-labeled T-2 toxin, but the major metabolites were not identified (20). On the other hand, HT-2 toxin was reported as a sole metabolite in bovine and human liver homogenates (3) and in liver microsomes of various animals (11, 12). Nevertheless, the in vitro metabolic pathway of T-2 toxin is still unknown.

The investigation reported here was initiated in an effort to provide more precise information on the in vitro metabolism of T-2 toxin both in the liver homogenate and in the gastrointestinal tract of rats.

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MATERIALS AND METHODS

Chemicals. T-2 toxin and its derivatives were prepared as previously described (2). The purity of T-2 toxin and HT-2 toxin used as substrates was >95% by gas-liquid chromatography (GLC). ^{3}H-labeled T-2 toxin was synthesized by oxidation of T-2 toxin in the C-3 position with dimethylsulfide-*N*-chlorosuccimide, followed by reduction with sodium [^{3}H]borohydride as described by Wallace et al. (18). The specific activity was 59.0 mCi/mmol and the radiochemical purity, as determined by thin-layer chromatography (TLC) followed by liquid scintillation counting, was >99%. 15-Deacetylneosolaniol (VII) was synthesized by partial hydrolysis of neosolaniol (V) with 1 N ammonium hydroxide in methanol. 3-Acetyl T-2 tetraol (IX) was prepared from T-2 tetraol (X) by partial acetylation with acetic anhydride in pyridine.

Chromatography. GLC was performed on a Hewlett-Packard model 5710A gas chromatograph equipped with a hydrogen flame ionization detector. A stainless-steel column (1 m by 3 mm ID) packed with 3% OV-17 on Gas-Chrom Q of 100 to 120 mesh was used throughout the analyses. The operating conditions were: column temperature programed for 150 to 290°C at 8°C/min; injection temperature, 250°C; detector temperature, 300°C; flow rate of nitrogen, hydrogen, and air, 30, 30, and 240 ml/min, respectively. The quantification of metabolites was based on calculation of peak heights by external method. Neosolaniol was used as an external standard for quantification of unknown metabolites.

TLC was carried out on precoated Silica Gel 60 plates (150-μm gel thickness, 20 by 20 cm and 5 by 20 cm; E. Merck AG) for analysis and purification of metabolites. Two TLC solvent systems were used: solvent A consisted of chloroform-methanol (9:1, vol/vol); solvent B consisted of chloroform-acetone (3:2, vol/vol). Compounds were made visible under long-wave (354 nm) ultraviolet light by charring with 20% H_2SO_4 in methanol. Thin-layer radiochromatography was performed on high-performance silica gel TLC

plates (200-μm gel thickness, 10 by 10 cm; Whatman Inc., Clifton, N.J.). After development, the silica gel layer was divided into 3-mm-wide zones and each zone was scraped directly into a scintillation vial. One milliliter of absolute ethanol was added to the vial followed by 10 ml of Aquasol 2 (New England Nuclear Corp., Boston, Mass.). Radioactivity was counted on a Beckman LS-8000 liquid scintillation spectrometer.

Spectroscopy. Gas chromatography-mass spectroscopy (GC-MS) was carried out in an LKB-9000 mass spectrometer at 20 and 70 eV. Proton and ^{13}C nuclear magnetic resonance (NMR) spectra were measured in $ChCl_3$ solution with Me_4Si as an internal standard on a Varian XLFT-100 spectrometer (100 MHz).

Derivatization of metabolites. For preparation of trimethylsilyl (TMS) ether and trifluoroacetate (TFA) derivatives, metabolites were reacted with Tri-Sil-BT and *N*-methylbis(trifluoroacetamide) (Pierce Chemical Co., Rockford, Ill.) and then analyzed directly by GC-MS.

About 25 μg of the metabolite was hydrolyzed by reacting with 25 μl of 1 N KOH in 80% ethanol for 30 min at room temperature. After evaporation of solvent, the reaction products were analyzed by TLC and by GLC as TMS ethers.

For acetylation of the metabolite in question, about 25 μg of the material was reacted with 20 μl of acetic anhydride in pyridine (25 μl) for 90 min at 70°C. The reaction products were analyzed directly by TLC and GLC.

Preparation of tissue fractions. (i) Liver homogenates. Male Wistar rats (6 weeks old, 250 to 300 g) were fasted overnight and sacrificed by decapitation. Livers, 10 to 12 g from two rats, were combined and rinsed with cold 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5 with 0.7% [wt/vol] NaCl) and homogenized in 4 volumes of this buffer with Super Dispax Tissumizer (Tekmar Co., Cincinnati, Ohio) for 1 min at 0°C. The homogenate was centrifuged at 9,000 × *g* for 20 min at 3°C, and the supernatant was filtered through glass wool to give the filtrate, which is referred to as the liver S-9 fraction.

(ii) Gastrointestinal strips. After decapitation, rat stomachs and small intestines were collected and emptied of their contents by washing with cold isotonic saline. Each tissue was cut into 3-mm-wide strips. Stomach strips (1 to 2 g) were suspended in 4 ml of 0.9% NaCl-HCl (pH 2.2) or 50 mM Tris-HCl buffer (pH 7.5 with 0.9% [wt/vol] NaCl). Intestinal strips (1 to 2 g) were suspended in 4 ml of 50 mM Tris-HCl buffer (pH 7.5 with 0.9% [wt/vol] NaCl).

Incubation and extraction. (i) S-9 fraction. The S-9 fraction (20 ml) was incubated at 37°C for 60 min in a 50-ml Erlenmeyer flask containing 6 mg of T-2 toxin or HT-2 toxin dissolved in 100 μl of ethanol. For radioactive studies ^3H-labeled T-2 toxin (1.8×10^7 dpm in 10 μl of ethanol) was incubated with the S-9 fraction. After incubation, the mixtures were heated for 3 min at 85°C to denature the protein and centrifuged for 5 min at 5,000 rpm. The precipitate was resuspended in 5 ml of water and recentrifuged. The supernatant solutions were combined and applied onto Amberlite XAD-2 resin columns (5 g, 1 by 14 cm, 20 to 50 mesh; Mallinckrodt Inc., Paris, Ky.). The column was washed successively with 100 ml of water and 100 ml of methanol-water (9:1, vol/vol). After concentration of the methanol eluate, the residue was redissolved in 2 ml of acetone and equal portions of this were analyzed by TLC and GLC. The overall recovery of T-2 toxin added to the buffer was estimated to be about 88% by GLC tracing, and about 7% of the added toxin was hydrolyzed nonenzymatically into HT-2 toxin under experimental conditions.

(ii) Gastrointestinal incubation. Three milligrams of T-2 toxin dissolved in 50 μl of ethanol was incubated with 4 ml of the tissue suspension for 60 or 180 min at 37°C in a 20-ml glass vial. After incubation, the mixture was homogenized with 20 ml of acetone and filtered, and the filtrate was concentrated. The residue was applied onto a column (1 cm ID) containing Florisil (5 g, 60 to 100 mesh; Fisher Scientific Co., Fair Lawn, N.J.) and eluted with 50 ml of chloroform-methanol (9:1, vol/vol). The eluate was concentrated and redissolved in 2 ml of acetone. T-2 toxin added to the buffer (pH 7.5) was very stable during extraction and was recovered in yield of 71.6%, calculated by GLC analysis.

Metabolite isolation. Extracts from the liver S-9 incubations were combined, redissolved in 2 ml of water, and introduced onto a Sep-Pak C_{18} cartridge (Waters Associates Inc., Milford, Mass.) as described previously (21). The cartridge was eluted sequentially with 2 ml of the following: water and 20, 50, 70, and 100% methanol in water. The 20% methanol eluate was concentrated and applied onto a silica gel TLC plate followed by development in TLC solvent system A. The band corresponding to the metabolite in question was scraped and eluted with acetone. To isolate the metabolite, the eluate was applied onto a second TLC plate and developed twice in TLC solvent system B.

RESULTS

Metabolism of T-2 and HT-2 toxins by liver homogenates. After 60 min of incubation in liver S-9 fractions, T-2 toxin was completely metabolized to yield four products, HT-2 toxin (IV), T-2 tetraol (X), and two unknown metabolites named TMR-1 and TMR-2, in yields of 49.3, 4.3, 18.7, and 1.6% of the substrate, respectively (Table 1). The tritium-labeled T-2 toxin incubated under the same conditions gave a similar metabolite profile on TLC, and a trace amount of radioactivity remained at the origin of the TLC plate (Fig. 1). Metabolites HT-2 toxin and T-2 tetraol were identical with standard compounds by TLC and GC-MS. Unknown metabolites TMR-1 and TMR-2 had retention times of 9.24 and 8.91 min (as TMS ethers), respectively, on GLC and R_f values of 0.19, 0.18 (solvent system A), 0.10, and 0.06 (solvent system B), respectively, on TLC (Table 2).

These unknown metabolites, in addition to T-2 tetraol, were also found in the incubation mixture of HT-2 toxin in yields of 11.5, 0.9, and 6.6%

TABLE 1. *In vitro metabolism of T-2 toxin and HT-2 toxin by the S-9 fraction of rat liver homogenates*[a]

Substrate	Product (% of added substrate)					
	T-2 (III)	HT-2 (IV)	TMR-1 (VI)	TMR-2	Tetraol (X)	Total
T-2 toxin[b]		49.3 ± 4.3	18.7 ± 3.1	1.6 ± 1.5	4.3 ± 2.2	73.9 ± 5.7
HT-2 toxin[c]		55.3 ± 1.8	11.5 ± 4.9	0.9 ± 0.4	6.6 ± 0.5	74.3 ± 5.8
Control[c]	88.0 ± 2.7	7.7 ± 5.1				95.6 ± 7.8

[a] T-2 toxin and HT-2 toxin (6 mg each) were incubated in 20 ml of the S-9 fraction of liver homogenates. In the control, T-2 toxin was incubated in 20 ml of 50 mM Tris-hydrochloride buffer under the same condition. Metabolites were extracted with an XAD-2 column and quantitated as TMS ethers by GLC on 3% OV-17.

[b] Mean of four replications ± standard deviation.

[c] Mean of two replications ± standard deviation.

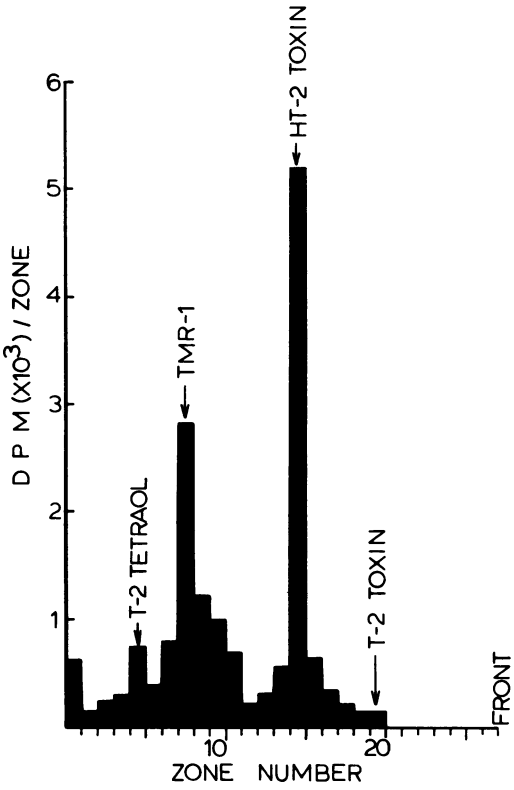


FIG. 1. *Thin-layer radiochromatogram of the incubation products of T-2 toxin in the S-9 fraction of rat liver homogenates.*

of the substrate for TMR-1, TMR-2, and T-2 tetraol, respectively (Table 1). This suggests that two unknown compounds are converted from T-2 toxin via HT-2 toxin. The production of TMR-2, however, was much more variable as compared with the other metabolites detected.

Metabolism of T-2 toxin by gastrointestinal strips. T-2 toxin incubated with stomach strips at pH 2.2 was metabolized to HT-2 toxin after 60 min in yield of 7.6%, and no other metabolites were found by GLC and TLC (Table 3), indicating that T-2 toxin was considerably stable at the physiological pH of rat stomach. At pH 7.5, however, stomach strips converted T-2 toxin into HT-2 toxin, TMR-1, and neosolaniol after 180 min of incubation in yields of 18.0, 3.5, and 4.4%, respectively (Table 3). All metabolites were confirmed by GC-MS as TMS ethers.

T-2 toxin incubated with intestinal strips for 60 min at pH 7.5 was efficiently metabolized to yield two major metabolites, HT-2 toxin and TMR-1, in yields of 45.5 and 12.9%, respectively. Only 5% of the substrate remained intact (Table 3). In addition to the above two metabolites, a trace amount of neosolaniol was detected by TLC and GLC and confirmed by GC-MS.

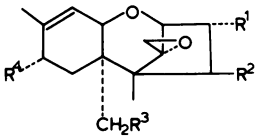
Identification of the new metabolite. The unknown metabolite TMR-1 isolated from the incubation mixture of the liver S-9 fraction gave T-2 tetraol after alkaline hydrolysis, and the acetylation of the metabolite with acetic anhydride-pyridine yielded diacetylneosolaniol (II), indicating that the metabolite retained a tetrahydroxytrichothecene nucleus. GC-MS of the TMS ether of TMR-1 gave the following major fragmentations: 556, 466, 335, 275, 247, 193, and 157 (Fig. 2). The molecular ion (*m/e* 556) was derived from a TMS ether of monoacetylated T-2 tetraol. The absence of an isovaleryl ion at *m/e* 85 and the diagnostic ion at *m/e* 466, which corresponds to a 12,13-epoxytrichothec-7,9-diene formed by elimination of the TMS-OH fragment (*m/e* 90) at the C-8 position, indicated the presence of a hydroxyl group at the C-8 position of the compound. The NMR gave conclusive evidence of an acetoxyl group located at the C-15 position. The ^{13}C NMR data of TMR-1 (VI) are shown in Table 4 in comparison with T-2 tetraol and its acetylated derivatives. The new metabolite gave one acetate carbonyl signal at δ170.78. The signal at δ65.0 was assigned to C-15 by comparing it with chemical shifts of the C-15 both of T-2 tetraol (δ61.0) and of neosolaniol (δ65.0). The signals at 64.8, 66.6, 78.8, 80.1, and 81.2 were assigned to C-12, C-8, C-2, C-3, and C-4, respectively. These data indicated one acetyl group of TMR-1 attached to the C-15 position. In addition, as shown in Tables 2 and 4, TMR-1 possessed different chromatographic

TABLE 2. *Chemical structures and resolution of T-2 toxin and its derivatives by TLC and GLC*

Compound	R^1	R^2	R^3	R^4	TLC R_f value[a]		GLC t_R (min)[b]	
					A	B	TMS	TFA
I Acetyl T-2 toxin	OAc	OAc	OAc	X[c]	0.71	0.66	14.70	14.70
II Diacetylneosolaniol	OAc	OAc	OAc	OAc	0.70	0.58	13.65	13.65
III T-2 toxin	OH	OAc	OAc	X	0.63	0.48	12.73	11.04
IV HT-2 toxin	OH	OH	OAc	X	0.36	0.19	11.87	7.80
V Neosolaniol	OH	OAc	OAc	OH	0.42	0.32	10.16	6.97
VI 4-Deacetylneosolaniol (TMR-1)	OH	OH	OAc	OH	0.19	0.10	9.24	3.99
VII 15-Deacetylneosolaniol	OH	OAc	OH	OH	0.34	0.24	8.01	5.15
VIII Deacetyl HT-2 toxin	OH	OH	OH	X	0.23	0.09	10.38	5.73
IX 3-Acetyl T-2 tetraol	OAc	OH	OH	OH	0.28	0.18	9.00	5.31
X T-2 tetraol	OH	OH	OH	OH	0.10	0.04	7.35	2.52

[a] On silica gel TLC plates developed in solvent systems A and B.
[b] Retention times of TMS ether and TFA ester derivatives.
[c] X = $OCOCH_2CH(CH_3)_2$.

TABLE 3. *In vitro metabolism of T-2 toxin by strips of rat gastrointestinal tracts*[a]



Tissue	pH of buffer	Product (% of added substrate)				Total
		T-2 (III)	HT-2 (IV)	TMR-1 (VI)	Neosolaniol (V)	
Stomach[b]	2.2	63.5 ± 3.9	7.6 ± 0.5			71.1 ± 4.0
Stomach[c]	7.5	50.8 ± 5.8	18.0 ± 5.0	3.5 ± 0.8	4.4 ± 1.4	76.6 ± 5.4
Intestine[b]	7.5	5.0 ± 1.5	45.5 ± 1.9	12.9 ± 5.3	Trace	63.4 ± 6.0
Control[d]	7.5	71.6 ± 0.7				71.6 ± 0.7

[a] Three milligrams of T-2 toxin was added to 4 ml of the buffer solution and incubated at 37°C with tissue strips.
[b] Strips from half of the tissue were incubated for 60 min; mean of four replications ± standard deviation.
[c] Strips from whole tissue were incubated for 180 min; mean of four replications ± standard deviation.
[d] T-2 toxin was incubated without tissue strips under the same conditions for 180 min. Products were extracted with acetone, chromatographed on a Florisil column, and quantitated as TMS ethers by GLC on a 3% OV-17 column; mean of two replications ± standard deviation.

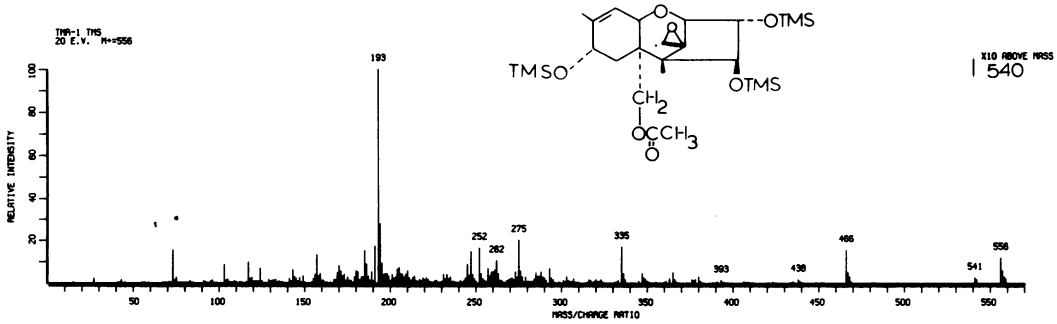


FIG. 2. *Mass spectrum of the TMS ether of the new metabolite, TMR-1, in rat liver homogenates. The spectrum was obtained on a combination GC-MS at 70 eV with a gas chromatographic column and conditions as described in the text.*

and spectroscopic properties from monoacetylated derivatives such as 3-acetyl T-2 tetraol (IX) and 15-deacetylneosolaniol (VII). This evidence is consistent with structure VI of TMR-1, 4-deacetylneosolaniol (15-acetoxy-3α,4β,8α-trihydroxy-12,13-epoxytrichothec-9-ene).

DISCUSSION

T-2 toxin was readily metabolized in rat liver homogenates and intestinal tracts. The former

TABLE 4. *^{13}C chemical shifts of the trichothecenes*

Position	Chemical shift (ppm from Me_4Si)				
	Neosolaniol (V)	3-Acetyltetraol (IX)	15-Deacetylneosolaniol (VII)	4-Deacetylneosolaniol (VI)	T-2 tetraol[a] (X)
C-2	78.3	77.1	78.1	78.8	78.5
C-3	78.9	82.5	78.9	80.1	79.1
C-4	84.6	78.7	84.7	81.2	80.0
C-5	48.7	49.1	49.4	49.1	48.0
C-6	43.5	44.6	45.1	43.0	45.1
C-7	30.5	28.4	28.3	30.3	28.5
C-8	66.7	66.2	66.5	66.6	64.8
C-9	139.9	139.6	139.8	139.6	138.1
C-10	121.3	121.3	121.8	121.0	121.6
C-11	67.8	68.6	68.8	67.6	67.6
C-12	65.0	64.3	64.6	64.8	64.3
C-13	47.3	46.7	47.2	46.9	45.1
C-14	6.7	6.8	6.9	6.8	6.3
C-15	65.0	62.0	62.3	65.0	61.0
C-16	20.5	20.5	20.7	20.5	19.9

[a] From Ellison and Kotsonis (4).

preparation was highly active, as 6 mg of T-2 toxin was completely converted into other metabolites within 60 min by 20 ml of the S-9 fraction, which represented a whole liver (4 to 5 g) of an adult rat. Therefore, this suggests that the lethal dose of T-2 toxin administered to rats (oral 50% lethal dose in adult rats is around 5 mg/kg) seems to be converted rapidly into other metabolites in the liver.

The high metabolizing activity of animal livers was previously reported by Ellison and Kotsonis (3) and Ohta et al. (11, 12), but they detected HT-2 toxin as a sole metabolite of T-2 toxin in in vitro metabolic studies. The experiments reported here show that in the liver S-9 fraction, T-2 was biotransformed to HT-2 toxin and more polar metabolites such as 4-deacetylneosolaniol and T-2 tetraol. The latter two metabolites were also converted from HT-2 toxin by the liver S-9 fraction. No other metabolites, including neosolaniol (8-deacyl T-2 toxin), 15-deacetyl T-2 toxin, 15-deacetyl HT-2 toxin (T-2 triol), and 15-deacetylneosolaniol, were detected in the incubation mixture of T-2 toxin or HT-2 toxin. From these facts we conclude that in the rat liver in vitro system, T-2 toxin is deacetylated preferentially at the C-4 position to give HT-2 toxin, which is converted stepwise into T-2 tetraol via 4-deacetylneosolaniol. It is noteworthy that the enzymatic elimination of the acetyl group in HT-2 toxin occurred preferentially at the C-8 position of the substrate, and that this pathway is distinctly different from that of chemical hydrolysis with base, in which HT-2 toxin is converted to T-2 tetraol via 15-deacetyl HT-2 toxin (20).

Ohta et al. (12) reported that T-2 toxin was hydrolyzed selectively to HT-2 toxin by the microsomal carboxyesterase, which showed no more transformation of the product HT-2 toxin. The method used by these authors, however, involved partitioning an aqueous reaction mixture with organic solvents such as ethyl acetate and chloroform (3, 11, 12). The XAD-2 column used in this study extracts polar metabolites such as T-2 tetraol and 4-deacetylneosolaniol more efficiently than the method described by Ohta et al. (11, 12). We believe that the microsomal hydrolyzing enzyme(s) is also involved in the biotransformation of HT-2 toxin to T-2 tetraol via 4-deacetylneosolaniol.

In addition to HT-2 toxin and 4-deacetylneosolaniol, a trace amount of neosolaniol was found in the intestinal incubation, but this system did not show any metabolizing activity of the above metabolites into TMR-2 and T-2 tetraol as did the liver incubation. In the case of rat stomach strips, only 7% of T-2 toxin was converted to HT-2 toxin at pH 2.2, whereas at pH 7.5 the metabolic profile of T-2 toxin was similar to that for the intestinal strips. These observations suggest that the metabolic pathway as well as the metabolizing activity for T-2 toxin are different between tissues of animals and that the substrate specificity of enzyme(s) involved in this metabolism is probably affected by the pH of the incubation system.

From the above evidence, the pathway shown in Fig. 3 is proposed for the in vitro metabolism

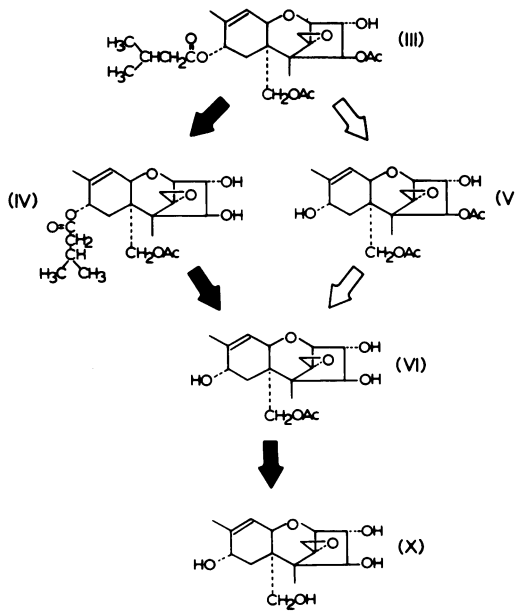


FIG. 3. *Proposed pathways for the in vitro metabolism of T-2 toxin in rats. Solid arrows indicate major pathways in liver and intestines, and open arrows indicate minor pathways in intestines.*

of T-2 toxin in rats. We conclude that T-2 toxin is also metabolized in vivo partially by the above pathway, not only in rats, but also in other animals. Actually, we detected T-2 metabolites, including HT-2 toxin, 4-deacetylneosolaniol, and T-2 tetraol, by GC-MS from the excreta of broiler chickens administered orally with T-2 toxin (20), confirming the above suggestion. The identification of TMR-2 will be the subject of a separate paper.

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