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IMMUNODETECTION OF T-2 METABOLITES IN RAT URINES AFTER DERMAL, ORAL, OR INTRAMUSCULAR EXPOSURE TO T-2 TOXIN

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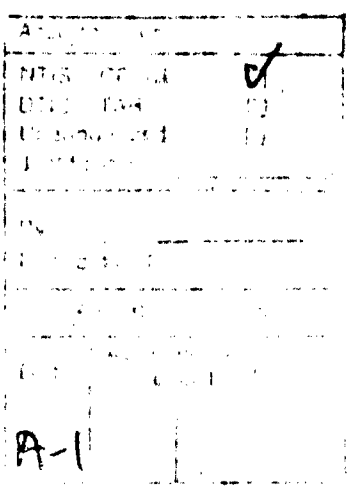

Immunodetection of T-2 Metabolites in Rat Urines after Dermal, Oral, or Intramuscular Exposure to T-2 Toxin. Hewetson, J.F., Wannemacher, R.W. Jr., and Hawley, R.J. (1988). *Fundam. Appl. Toxicol.* --,-- - --. T-2 toxin metabolites were detected in rat urines up to 2 weeks after the animals' exposure to sublethal dose of T-2 toxin. Urines were assayed for HT-2 or T-2 tetraol by radioimmunoassay (RIA) with polyclonal antibody. Sensitivity of the RIA was 5 ng/ml for HT-2 and 50 ng/ml for T-2 tetraol. Some urines were assayed in parallel with an enzyme-linked immunosorbent assay (ELISA) developed for T-2 with a monoclonal antibody that cross reacts with HT-2. The sensitivity of the ELISA for detection of HT-2 in rat urines was 250 ng/ml. Urines were diluted 1:2 or 1:5 to eliminate high backgrounds, which appeared as false positives in the T-2 tetraol RIA and ELISA assays. T-2 or its metabolites could be detected up to 96 hr after an im injection of T-2 toxin. In rats exposed orally or dermally, metabolites were detected up to 10 or 16 days, respectively."T-2 equivalents" detected by the ELISA correlated with the HT-2- or HT-2-like compounds detected by RIA.

The presence of T-2 toxin in contaminated grain (Chung, *et al.* 1974; Ellison and Kotsonis 1973; Hsu, *et al.* 1972; Jemmali, *et al.* 1978; Mirocha, *et al.* 1979; Romer, *et al.* 1978) and its reported use as a biological warfare weapon in Southeast Asia and Afghanistan (Mirocha, *et al.* 1983; Watson, *et al.* 1984) has generated many studies on the detection of T-2 by mass spectrometry (Mirocha, *et al.* 1983), high pressure liquid chromatography (Stack and Eppley 1980) thin layer chromatography (Pace 1986; Takeda 1979), bioassay (Sukroongreung 1984; Sukroongreung, *et al.* 1984a; Chung, *et al.* 1974) and immunoassay techniques (Fan, *et al.* 1984; Fontelo, *et al.* 1983; Chu 1986; Pestka, *et al.* 1981; Peters, *et al.* 1982). Studies carried out with tritiated toxin show that T-2 is rapidly metabolized to HT-2 and T-2 tetraol and the parent compound is not present in urines of exposed guinea pigs (Pace, *et al.* 1985; Pace, *et al.* 1985a) and monkeys (Mereish, *et al.* 1987). Radio-labeled toxins are appropriate for laboratory studies, but not useful for evaluation of natural exposure. Several conventional immunoassay techniques based on tritiated ligands (Fontelo, *et al.* 1983; Lee and Chu 1981, 1981a) and enzymes conjugated to ligands or antibodies (Fan, et al. 1984; Gendloff, *et al.* 1984; Pestka, *et al.* 1981; Peters, *et al.* 1982) have formed the basis of most of the efforts for rapid detection both in environmental samples and biological fluids. Cross reactivities occur and are exploited to broaden the number of substances that a given assay can detect. Therefore, each assay must be standardized for a particular substance and all known cross reactivities investigated before it can be stated with certainty what is present in an unknown sample. The previous studies have used spiked samples of urine or blood to demonstrate the feasibility of such procedures and often have had problems with background and non-specific reactions (Fan, *et al.* 1984; Lee and Chu 1981). In order to demonstrate the practical feasibility of confirming an environmental exposure to T-2 toxin, we evaluated two assays for the detection of T-2 metabolites in rat urines. These studies were compatible with an evaluation of accidental or intentional environmental exposure. Three routes of exposure were used and the kinetics of excretion evaluated.

METHODS

Toxin. T-2 toxin was obtained from Myco Labs, Inc., Chesterfield, Missouri.

Polyclonal antiserum. Antibodies to HT-2 were raised by conjugating the molecule to bovine serum albumin and immunizing rabbits or goats (Fan, *et al.* 1987; Chu 1986). Antibodies to T-2 tetraol were produced in rabbits by first conjugating T-2 tetraol to sheep gamma globulin. The resulting antibodies were titrated against the homologous labeled ligand for optimal reactivity in an RIA. HT-2 antisera were supplied by Dr. Chu, University of Wisconsin (Fan, *et al.* 1987) and T-2 tetraol antisera were produced and supplied by Bio-Metric Systems, Inc., Eden Prairie, Minnesota.[1]

Monoclonal antibody. A monoclonal antibody to T-2 (15H6) was originally developed by Dr. Ken Hunter at the Uniformed Services University of the Health Sciences, Bethesda, Maryland. (Hunter, *et al.* 1985). Quantities of this monoclone as IgG-purified ascites fluid were produced by Charles River Biotechnical Service, Inc. Wilmington, Massachusetts. The antibody was titrated in the RIA against homologous ligand for optimal reactivity.

Radioimmunoassay. A standard RIA was used as previously described (Fontelo, *et al.* 1983) with slight modifications. 100 µl of sample, 50 µl of ^{3}H-ligand (20,000 dpm), and 50 µl of diluted antibody were combined and incubated at room temperature for 1 hr. Ammonium sulfate (250 µl, 76%) was added, the tubes incubated for 1 hr, and centrifuged at 3000 rpm in an International Model PR6 centrifuge. Supernatants were counted for radioactivity and the amount of toxin present in the samples determined from standard curves run in phosphate buffered saline (PBS). Normal urines were included in all assays as a control for nonspecific binding.

1 Dr. Chu and Bio-Metric Systems are under contract with the U. S. Army.

Enzyme-Linked Immunosorbent assay. An ELISA test kit selected as the best prototype for detection of T-2 in environmental and biological samples, consisted of a standard competitive ELISA with T-2 adsorbed onto the surface of plastic plates. Unknown sample plus monoclonal T-2 antibody conjugated to horseradish peroxidase were added to the wells and incubated. After 2 hr, wells were washed and 2,2'Azinobis(3- ethylbenz-thiazoline sulfonic acid) (ABTS) substrate added. The absence of color indicated a positive reaction, that is, presence of toxin. The results were read as a + or - by comparison to controls included in each assay.

Animals. Fisher F344 rats (250-400g) from Charles River, Wilmington, Massachusetts,were used for all studies. Rats were housed in plastic metabolic cages (Nalgene, Inc. Rochester, N.Y.) with free access to food and water. Urine and feces were collected daily and stored at 4°C.

Experimental design. Three groups of rats were exposed to T-2 toxin either im, orally, or dermally. Group 1 received 0.5 mg/kg, im in PBS by injection in the thigh muscle. Group 2 was fasted 24 hr before receiving an oral dose of 2 mg/kg T-2 in propylene glycol by inoculation with a 23-gauge, 3-in. intubation needle attached to a 1 ml syringe. Group 3 animals were shaved with electric clippers and 24 hr later, 0.1 ml of the toxin (0.5 or 1.0 mg/kg) in dimethylsulfoxide was applied to a 4 x 4 cm2 area on the shaved skin. A jacketed barrier was applied over the application site, as described previously (Wannemacher, *et al.* 1985). Controls of carrier only were included in all groups.

RESULTS

Titration of Antisera

Both polyclonal sera and 15H6 monoclonal anti-sera had good reactivity against the homologous ligand in the RIA (data not shown). Based on 50% binding, dilution of the antisera selected for further studies was 1:25, 1:20, and 1:35 for T-2, HT-2 and T-2 tetraol, respectively. Cross reactivities with related mycotoxins were determined to show the specificity of each antiserum. Anti-T-2 showed complete cross reactivity with HT-2 as also shown by Hunter (Hunter, *et al.* 1985), and no cross reactivity with T-2 tetraol. Anti HT-2 was 30% cross reactive with T-2 and unreactive with T-2 tetraol. Anti-T-2 tetraol did not cross react with T-2, but was 30% cross reactive with HT-2.

False Positive Reactions and Toxin Detection Limits

Previous studies showed that individual normal urines exhibited false positive reactions in the T-2 tetraol RIA (Hewetson, unpublished). The false positive reaction was eliminated by diluting the urine 1:2. Therefore, the detection limit of T-2 tetraol in urine was defined at a 1:2 dilution as the bound/free (B/F) value that was greater than 2 standard errors from the B/F value of a group of 20 control urines diluted 1:2. The same definition applied to HT-2 detection, except that undiluted urines were assayed with no false positive reactions. Detection limits for each of the toxins was 5 ng/ml for HT-2 and 50 ng/ml for T-2 tetraol.

Standardization of ELISA Kit

Since the monoclonal antibody against T-2 cross reacts with T-2 (Hunter, *et al.* 1985, Hewetson, unpub'ished), we included HT-2 in our evaluation. The ELISA kit was capable of detecting of both T-2 and HT-2 in buffer at

concentrations of 25 ng/ml and 50 ng/ml, respectively (Table 1). The ELISA kit also produced sporadic false-positive reactions with control urine. A 1:5 dilution of the urine was necessary to eliminate this problem, making the ELISA detection limit of HT-2 in urines about 250 ng/ml.

T-2 Metabolite Detection in Urine by RIA

A series of studies were designed to evaluate the patterns of HT-2 and T-2 tetraol excretion in urines of exposed rats. Table 2 presents the results obtained after their im exposure. T-2 tetraol and trace amounts of HT-2 were detected up to day 3 in rat 1. Since this rat did not produce urine on day 1, total toxin excreted was similar to that excreted by rats 2 and 3.

T-2 tetraol and HT-2 excretion after oral exposure occurred up to 9 days after exposure (Fig. 1 and 2). Dermal exposure to T-2 showed an excretion pattern that extended beyond that observed for the other two routes of exposure (Fig. 3 and 4). T-2 toxin metabolites, HT-2 and T-2 tetraol, were still detected up to 16 days after exposure.

T-2 Metabolite Detection in Urine by ELISA

Selected urine samples from the oral-exposure series were tested in the ELISA kit. All urines were not evaluated by this method because of a limitation on the number of tests available. Table 3 presents a comparison between the two methods for detection of HT-2 (RIA) and "T-2 equivalents" (ELISA). Urine samples containing HT-2 >250 ng/ml as measured by RIA were positive by the ELISA. Lower amounts of HT-2 could not be detected by the ELISA.

DISCUSSION

These studies describe the excretion patterns of T-2 metabolites in urine after three routes of exposure. We also compared detection results obtained with an RIA specific for HT-2 to results from a commercially available kit for detecting "T-2 equivalents." Previous studies show that the principal metabolites of T-2 are HT-2 and T-2 tetraol (Pace, *et al.* 1985; Pace, *et al.* 1985a; Corley, *et al.* 1985; Yoshizawa, *et al.* 1980). Therefore an assay for these urinary metabolites should provide evidence of T-2 exposure. Pace, et al (1985a) reported that in guinea pigs exposed im to ^{3}H T-2, radioactivity in urine peaked at 24 hr and rapidly decreased over the next 4 days. Matsumoto, *et al.* (1978) reported rapid excretion of radioactivity after oral administration of ^{3}H T-2 Our results also showed rapid excretion of T-2 metabolites after im exposure as detected by the RIA. After oral administration of T-2, the majority of metabolites were excreted by day 4 (Fig. 3 and 4), but there was a low, detectable amount of HT-2 up to day 8 in all rats. Low levels of T-2 tetraol were detected up to day 10 in two rats studied. Matsumoto, *et al.* (1978) reported most of the radioactivity was excreted by 72 hr in two rats orally dosed with T-2. Most of the HT-2 and T-2 tetraol excreted from rats dosed dermally with T-2 was complete by day 10. However, low levels of both metabolites were detected up to day 16 after exposure. This was most likely due to the slow absorption of T-2 from the skin.

As HT-2 showed complete cross reactivity with the anti-T-2 monoclonal antibody (Hunter, *et al.* 1985), we evaluated exposure to T-2 by testing urines with an ELISA kit designed for T-2 testing. These results compared favorably with results obtained with the RIA specific for HT-2.

False-positive reactions were not a problem with HT-2 detection in the RIA, even in undiluted urine. Sensitivity was similar to that of HT-2 in buffer, or about 5 ng/ml. T-2 tetraol detection by RIA and HT-2 detection by ELISA presented significant interference. These false-positive reactions were eliminated by diluting the urines to a point where no false-positives

occurred in a battery of control urines. Although this simple solution does not provide an answer to the identification of the interfering material, it offers a practical alternative. By diluting samples 1:5 for the ELISA, practical detection limits for HT-2 in undiluted urine were raised to about 250 ng/ml. The RIA can detect HT-2 at lower levels than the ELISA (Table 3), but the 250 ng/ml level for the ELISA was more than adequate for routine detection in urines up to several days after exposure to a sublethal dose of T-2.

These studies support Mirocha's observation (Mirocha, *et al.* 1983) in human samples from Southeast Asia that HT-2 could be detected 2 weeks after exposure. Although we document a useful assay for detection of sublethal exposure to T-2 by an oral or dermal route, incorporation of either anti-HT-2 or anti-T-2 tetraol antibodies into the kit should provide greater sensitivity for detection of T-2 metabolites.

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TABLE 1

T-2 AND HT-2 TITRATION OF STANDARD TOXIN SAMPLES IN ELISA KIT

ng/ml	T-2	HT-2
10,000	+	+
1,000	+	+
100	+	+
50	+	+
25	+	-
12	+/-	-
6	-	-
PBS	-	-

TABLE 2

HT-2 AND T-2 TETRAOL RECOVERED FROM RAT URINE (μg/kg)

IM EXPOSURE TO T-2

RAT	24 HR		48 HR		72-96 HR		120 HR	
	Ta	Hb	T	H	T	H	T	H
1	[c]	-	3.841	0.911	1.614	(TR)[d]	0.484	(TR)
2	4.587	1.974	1.367	(TR)	(TR)	(TR)	-	(TR)
3	12.506	0.992	1.965	1.910	1.846	(TR)	-	-

[a] T T-2 tetraol

[b] H HT-2

[c] - Not detected

[d] TR Trace

TABLE 3

HT DETECTION IN RAT URINES

ORAL EXPOSURE

DAY	ELISA[a]	RIA (ng/ml)
1	+	2116
2	+	5533
3	+	1250
4	-	196
5,6	-	133
7,8	+	270
9	-	-
10	-	-

[a] Urine diluted 1:5

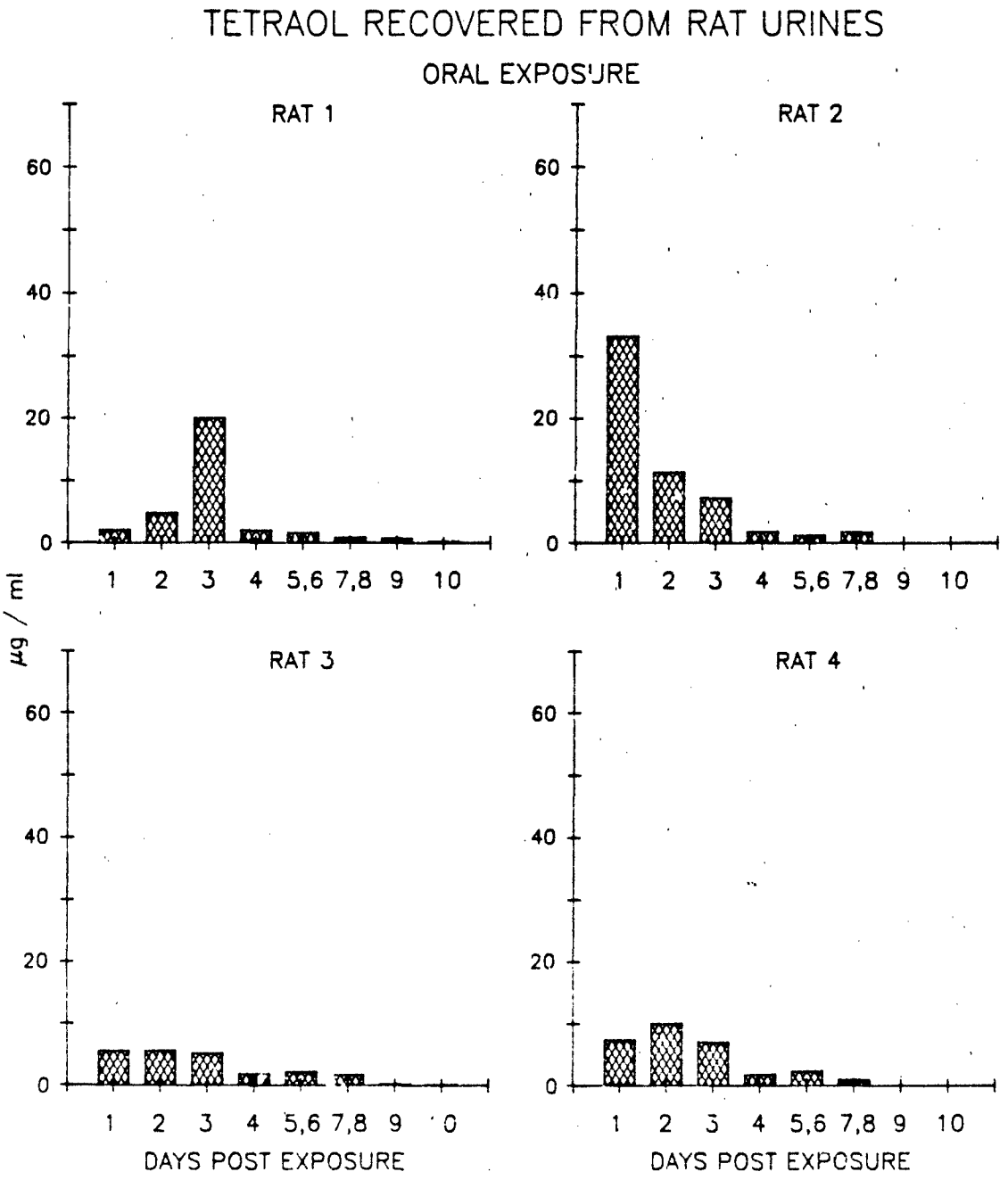
LEGENDS

FIG 1 AND 2

Recovery of HT-2 and T-2 tetraol from individual rat urines after oral exposure to 2 mg/kg T-2 in propylene glycol. Recovery is expressed as µg toxin recovered per kg body weight.

FIG 3 AND 4

Recovery of HT-2 and T-2 tetraol from rat urines after dermal exposure to T-2. Two groups of 3 rats received 1 mg/kg or 0.5 mg/kg, respectively. Recovery is expressed as µg toxin recovered per kg body weight.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RAT 3
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40
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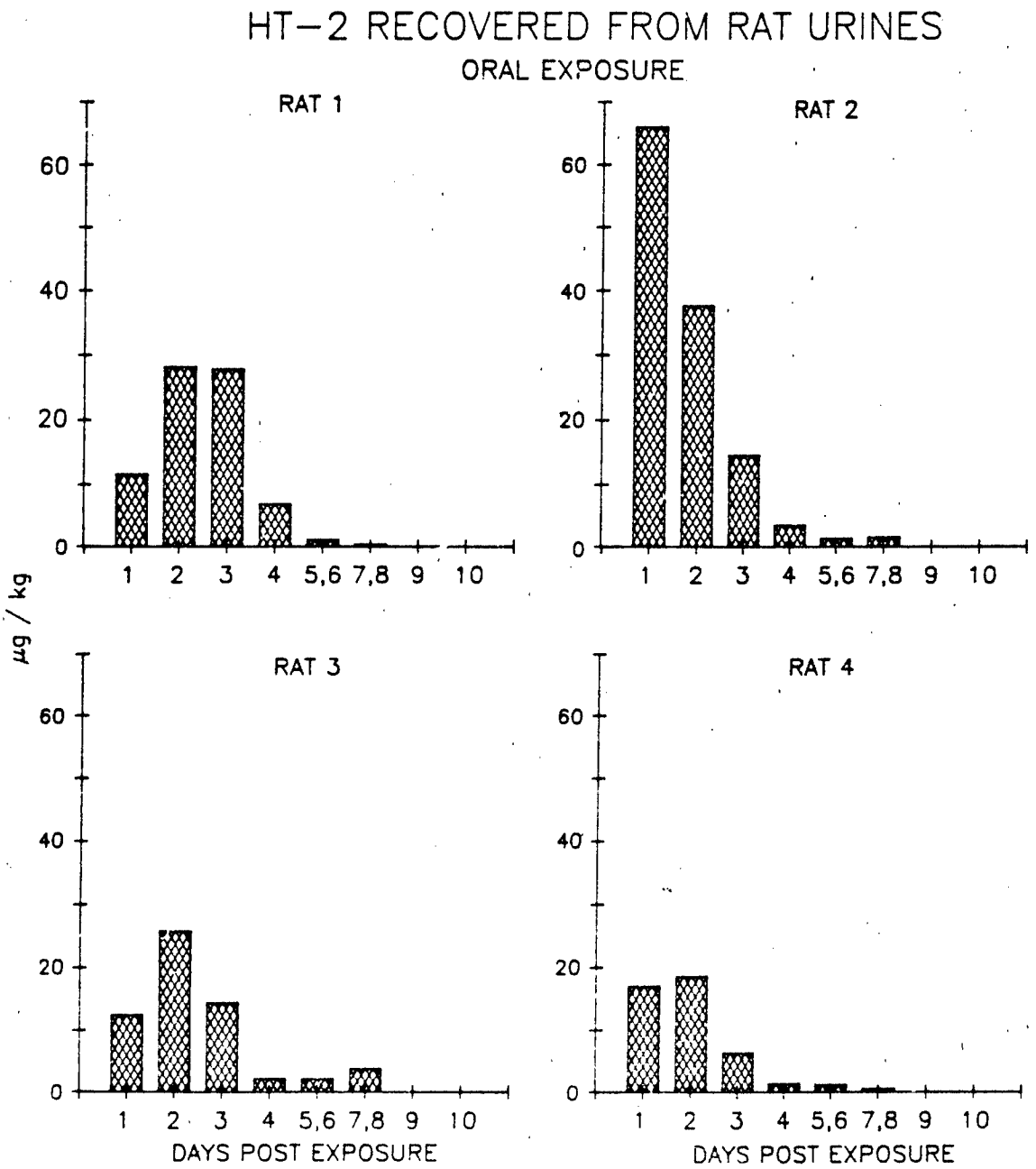
DAYS POST EXPOSURE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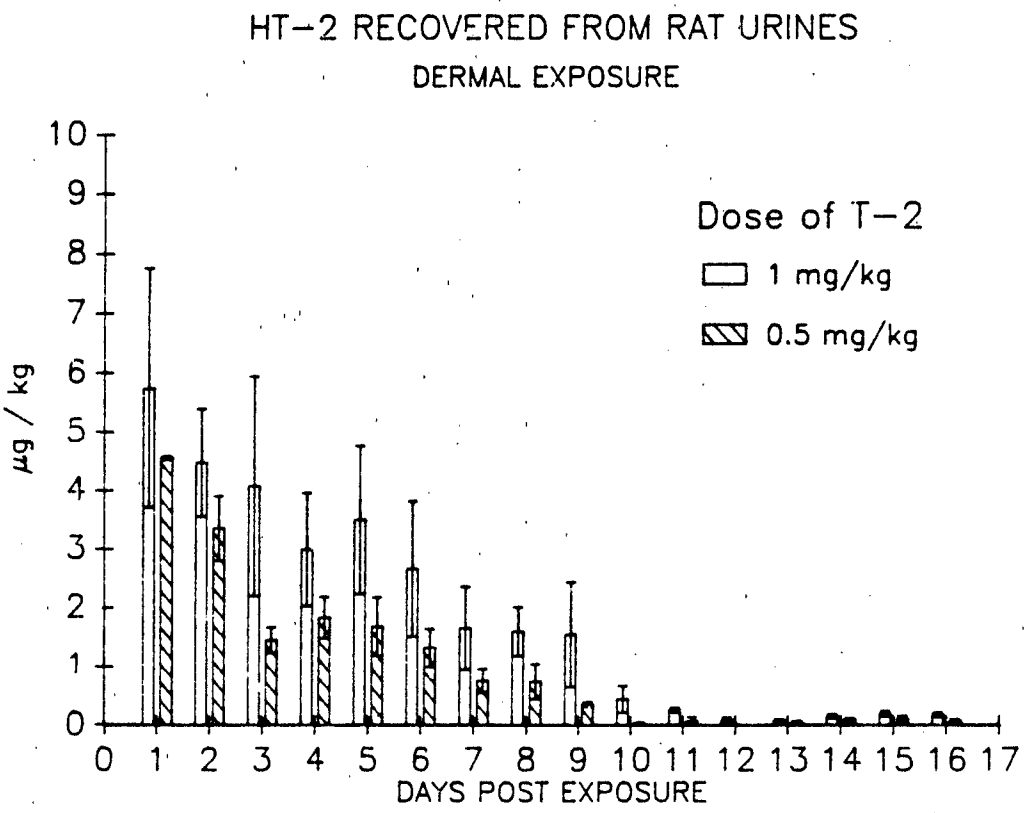
HT−2 RECOVERED FROM RAT URINES
DERMAL EXPOSURE
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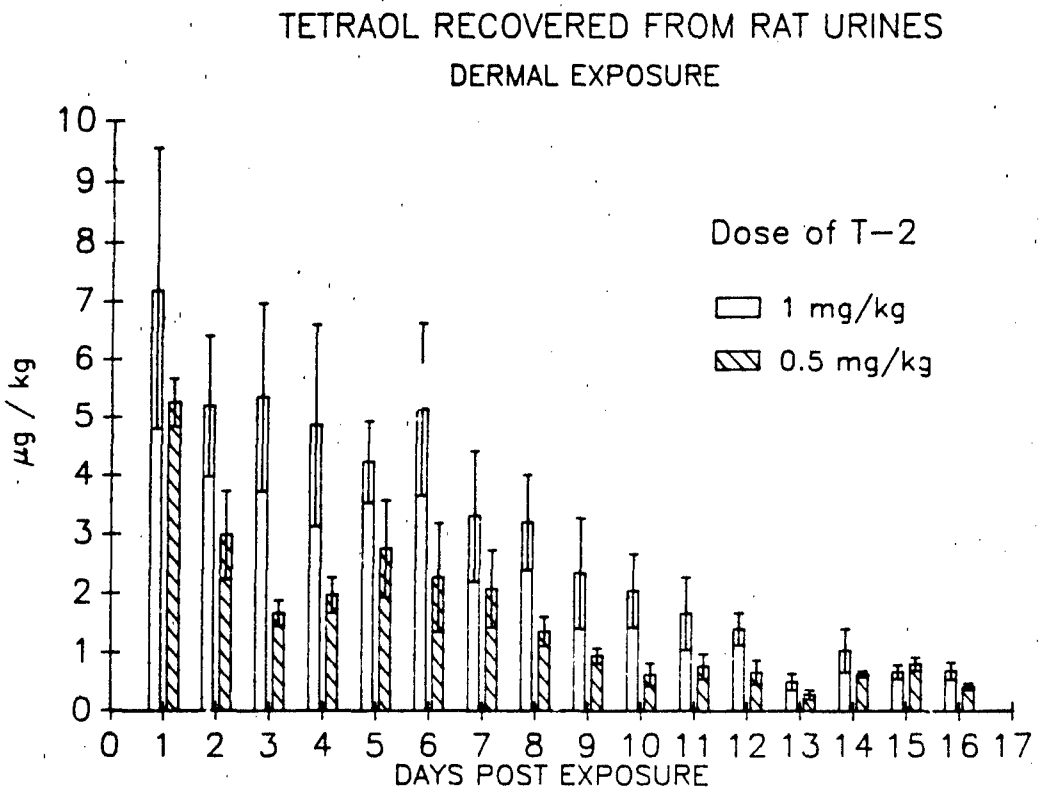
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DERMAL EXPOSURE
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DAYS POST EXPOSURE