Metabolism and Clearance of T-2 Mycotoxin in Perfused Rat Livers[1,2]

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Isolated perfused rat livers were used to study the metabolism and clearance of T-2 mycotoxin, a non-protein Fusarium metabolite known to cause illness or death on contact or by ingestion. To evaluate the in vitro hepatic metabolism, clearnace and rate of biliary excretion of T-2 toxin, [^{3}H]T-2 toxin was delivered under constant perfusate flow (8 ml/min) in a single-pass experiment. Steady-state conditions were achieved within 10 min as indicated by a constant exit rate of radiolabel in the effluent. At steady-state, 70 ± 4% of the total (cont.)

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delivered radiolabel was extracted by the liver, 38 ± 4% remained in the perfusate. Liver actively metabolizes trichothecenes, therefore, the extraction ratio for total radiolabel does not reflect the actual extraction ratio for T-2 toxin. At steady-state, 93% of the delivered [^{3}H]T-2 was extracted and metabolized by the liver, while 4.6 ± 0.3% remained unmetabolized in the effluent perfusate. The excretion rate of metabolites and conjugates into bile was constant after a 10-min perfusion. Radioactivity measured in bile accounted for 55% of the total radiolabel delivered during the perfusion experiment (1 hr). T-2 toxin was metabolized and eliminated as 3'hydroxy HT-2, 3'hydroxy T-2 triol, 4-deacetylneosolaniol, T-2 tetraol, and glucuronide conjugates of HT-2, 3'hydroxy HT-2, and T-2 tetraol. Approximately 7% of the administered radiolabel remained in the liver and was identified as 4-deacetylneosolaniol (18%), T-2 tetraol (41%), and conjugated metabolites (41%). Total recovery of administered radiolabel associated with T-2 and its metabolites equalled 97.6% (bile, 52.5%; perfusate, 38.0%; and liver, 7.1%). Approximately 3% of the biliary radiolabel was not identified. These studies describe the use of a perfused organ system to determine the rate of formation of T-2 metabolites and their elimination into bile.

Metabolism and clearance of T-2 mycotoxin in perfused rat livers. PACE, J.G. (1984). Fundam. Appl. Toxicol. , - . Isolated perfused rat livers were used to study the metabolism and clearance of T-2 mycotoxin, a non-protein Fusarium metabolite known to cause illness or death on contact or by ingestion. To evaluate the in vitro hepatic metabolism, clearance and rate of biliary excretion of T-2 toxin, [^{3}H]T-2 toxin was delivered under constant perfusate flow (8 ml/min) in a single-pass experiment. Steady-state conditions were achieved within 10 min as indicated by a constant exit rate of radiolabel in the effluent. At steady-state, 70 ± 4% of the total delivered radiolabel was extracted by the liver, 38 ± 4% remained in the perfusate. Liver actively metabolizes trichothecenes, therefore, the extraction ratio for total radiolabel does not reflect the actual extraction ratio for T-2 toxin. At steady-state, 93% of the delivered [^{3}H] T-2 was extracted and metabolized by the liver, while 4.6 ± 0.3% remained unmetabolized in the effluent perfusate. The excretion rate of metabolites and conjugates into bile was constant after a 10-min perfusion. Radioactivity measured in bile accounted for 55% of the total radiolabel delivered during the perfusion experiment (1 hr). T-2 toxin was metabolized and eliminated as 3'hydroxy HT-2, 3'hydroxy T-2 triol, 4-deacetylneosolaniol, T-2 tetraol, and glucuronide conjugates of HT-2, 3'hydroxy HT-2, and T-2 tetraol. Approximately 7% of the administered radiolabel remained in the liver and was identified as 4-deacetylneosolaniol (18%), T-2 tetraol (41%), and conjugated metabolites (41%). Total recovery of administered radiolabel associated with T-2 and its metabolites equalled 97.6% (bile, 52.5%; perfusate, 38.0%; and liver, 7.1%). Approximately 3% of the biliary radiolabel was not identified. These studies describe the use of a perfused organ system to determine the rate of formation of T-2 metabolites and their elimination into bile.

T-2 toxin, 4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12,13 epoxytrichothec-9-ene, is produced by the Fusarium species (Bamburg et al., 1968a, b). T-2 and other trichothecene mycotoxins have been associated with moldy-food toxicoses in man and farm animals (Joffe, 1971; Hsu et al., 1972; Ueno et al., 1972a, b; Ueno, 1977). In vivo studies (Yoshizawa et al., 1982; Matsumoto et al., 1978; Pace et al., 1985a) suggest that liver is the main site for T-2 toxin metabolism, although metabolism may also occur in other tissues (Pace et al., 1985a). For this reason the liver provides a highly useful model for examining the metabolism of T-2 toxin.

Recently there has been an increased interest in the metabolism of trichothecene mycotoxins. Emphasis in this area of research is given to identifying new metabolites (Yoshizawa et al., 1982, 1985; Roush et al., 1985a, b) and detection of metabolites in biological fluids for diagnostic purposes (Mirocha et al., 1983; Pace et al., 1985a; Lee et al., 1984).

Many studies use tissue homogenates and isolated enzyme preparations for the production and identification of toxin metabolites. Figure 1 represents the current proposed scheme for the metabolism of T-2 toxin. Ellison and Kotsonis (1974) first reported that the S-9 fraction of liver homogenates metabolized T-2 to HT-2 toxin. A non-specific microsomal esterase was found to be responsible for the deacetylation of T-2 toxin (Ohta et al., 1978). Yoshizawa et al. (1980) further defined the in vitro metabolic pathway to include 4-deacetylneosolaniol (4-DANS), 15-deacetylneosolaniol, neosolaniol, and T-2 tetraol. In addition to these hydrolzyed products, Yoshizawa et al. (1982) identified two hydroxylated derivatives, 3'hydroxy T-2 (3'OH T-2) and 3'hydroxy HT-2 (3'OH HT-2), in hepatic homogenates containing the NADPH-generating system. Roush et al., (1985a) recently described the in vitro production of a glucuronide conjugate of T-2 or HT-2 toxin. All the above

metabolites, in addition to deepoxydation metabolites of 3'OH HT-2 and T-2 tetraol (Yoshizawa et al., 1985), have since been identified in vivo (Pace et al., 1985a; Matsumoto et al., 1978; Chi et al., 1978).

Although in vitro systems provide significant qualitative information regarding the synthesis of T-2 metabolites, isolated perfused organs are generally better models of in vivo metabolism (Meijer et al., 1981). Use of the isolated perfused liver is advantageous because the liver retains the intracellular and extracellular integrity of in vivo tissue, blood and bile flow can be regulated and perfusate composition can be controlled. While perfused liver is an attractive system for studying metabolism of trichothecene toxins, few such studies appear in the literature (Pace and Watts, 1983). The results of the present investigation indicate that perfused rat liver is a valid model for studying metabolism and clearance of T-2 mycotoxin.

METHODS

Reagents. T-2 toxin was purchased from Myco-Labs[3]. Purity was determined to be 99% by thin-layer chromatography (TLC) and gas chromatography-mass spectrometry (GC/MS). Standards for TLC (T-2, HT-2, T-2 triol, and T-2 tetraol) were purchased from Calbiochem[4]. 3'OH T-2 and 3'OH HT-2 toxin were kindly provided by F.S. Chu[5]. T-2 toxin was labeled with tritium in the C-3 position by New England Nuclear[6] by the method of Wallace et al. (1977). The purity (93% radiochemical purity, specific activity 8.9 Ci/mmol of a-T-2) was determined by high-pressure liquid chromatography (HPLC) and TLC.

Animals. Male, Fisher-Dunning rats[7], weighing 175-200g, were housed in wire-bottom cages and allowed food[8] and water ad libitum. Rats were maintained in a light- and temperature-controlled room (12-hr light-dark cycle, 23°C).

Surgical Procedure. Rats were anesthetized (ip.) with 60 mg/kg sodium pentobarbital. The abdominal hair was removed with a clipper. The abdomen was scrubbed with Prepodyne Swabs[9] and opened by means of a long, U-shaped incision. Stomach and intestines were reflected to the left to expose the portal vein and the inferior vena cava. The liver was freed from attachments to esophagus and stomach but was not removed (in situ). The esophagus and vagus nerve were divided between two encompassing ties and the right renal vein was tied off. The bile duct was cannulated with PE 20 tubing[10] followed by cannulation of the inferior vena cava. The anesthetized rat was transferred to the perfusion chamber where a cannula attached to the perfusion system was secured in the portal vein. To ensure unidirectional flow of the perfusate, the chest was opened, and the thoracic vena cava cross-clamped below the right atrium. The anesthetized rat died within one min after the chest was opened. The outflow from the liver was collected via the cannula inserted through the upper portion of the inferior vena cava.

Perfusion of Isolated Livers. Isolated liver perfusions were performed by modifying the apparatus of Miller (1961) as previously described by Zenser et al. (1974). The perfusion medium consisted of 30% washed sheep erythrocytes, 3% bovine serum albumin, and 5 units of heparin/ml in Krebs-Ringer bicarbonate solution, buffered to pH 7.4. The atmosphere in the closed perfused system consisted of 95% O_2 and 5% CO_2. Perfusion of the liver was maintained at 8 ml/min.

Cytosolic and mitochondrial $[NADH]/[NAD]^+$ ratios (Veech et al., 1970; Brunengraber et al., 1975) were determined as an estimate of the metabolic integrity of the liver. The [lactate]/[pyruvate] ratio values were 6 to 10 and the [3-hydroxybutyrate]/[acetoacetate] ratios were 0.28 to 0.82. These ratios remained constant throughout the perfusion study.

At the end of a 30-min equilibration period, 264 µl of a 10 mg/ml solution of radiolabeled T-2 toxin (specific activity 8.9 Ci/mmol) was injected into 580 ml of perfusion medium. The effluent perfusate leaving the liver was not returned to the reservoir (single-pass or noncirculating). Two determinations of the input medium were taken before and after the perfusion period to be used as the steady-state input concentration. The output medium was sampled at 5-min intervals.

Determination of metabolites. Perfusate and bile were analyzed for radioactivity in a liquid scintillation counter[11]. Aliquots (10-20 µl) of perfusate and bile were analyzed by TLC to separate and identify toxin metabolites. Precoated silica gel TLC plates[12] (20 x 20 cm, 0.25-mm thick) were developed by two sequential solvent systems of chloroform:ethyl acetate:ethanol in ratios of (1) 50:25:25 and (2) 80:10:10 (Pace et al., 1985a). Mycotoxin standards were visualized by on a chromogenic reaction between 4-(p-nitrobenzyl) pyridine and the 12,13-epoxy group (Takitani et al., 1979), and samples were scanned for radioactivity with a Bioscan BID100 radioisotopic scanner[13]. The radioactive zones were then scraped, extracted with boiling ethyl acetate, and filtered through a glass-wool plug. The filtrates were evaporated under a stream of nitrogen. The residues were derivatized with 1 drop each of methylene chloride and trifluoroacetic anhydride, sealed, and allowed to stand at room temperature 1.5-2 hr before nitrogen removal of

the liquids. The residues were dissolved in a minimum of acetone and analyzed immediately by GC/MS using a Hewlett Packard 5985 B[14] equipped with a 25 m x 0.2 mm ID fused silica capillary column (cross-linked OV-1, .11 mm thick) interfaced directly to the source. Source temperature was 200°C, and GC conditions were 160°C, 1 min, 20° C/min to 250°C. Electron-impact spectra were run using 70 eV.

A 0.2-g liver sample was extracted three times with 0.3 ml of methanol (100%). Recovery of total radioactivity was 97 ± 2% (n=6). The extract was dried under nitrogen and reconstituted in 0.2 ml of methanol (Pace et al., 1985b). A 50-µl aliquot was spotted on TLC plates, developed as described above, and scanned for radioactivity.

The polar fraction, remaining at the origin of the TLC plate, was extracted with methanol, dried under nitrogen, dissolved in 3 ml of 0.1 M acetate buffer (pH 3.8), and heated at 90°C for 10 min. After cooling, either 3.1 mg of β-glucuronidase (1500 units/mg)[15] or sulfatase (10 units/mg, pH 5.0)[15] or 0.1 M acetate buffer was added to duplicate sample tubes and incubated at 38°C for 18 hr. A specific inhibitor of β-glucuronidase, saccharic acid 1,4-lactone (10 mM)[15], was added to a separate sample tube as an additional control. The reaction volume was dried under nitrogen and reconstituted in 100 µl of methanol. A 50-µl sample was spotted on TLC plates and processed as described above.

Theoretical and Statistical Analyses. The steady-state extraction ratio (E) is the fraction of toxin eliminated during passage through the liver and was calculated as follows:

$$E = (C_{in} - C_{out})/C_{in} \quad (1)$$

where C_{in} is the steady-state toxin concentration in perfusate entering the liver; C_{out} is the steady-state toxin concentration leaving the liver. The steady-state hepatic clearance (CL), the volume of perfusate that is cleared of toxin per unit time, was calculated as follows:

$$CL = E * Q \quad (2)$$

where Q is the hepatic perfusate flow (Pang and Gillette, 1978). Results are expressed as mean ± SD for data obtained from three separate experiments.

RESULTS

To study the relative contributions of metabolism and biliary excretion to total elimination of T-2 toxin by liver, a single-pass experiment was performed. Steady-state conditions were reached within 10 min for T-2 toxin and glucuronide-conjugates (Figure 2). The steady-state extraction ratio

(Equation 1) of total toxin equivalents (total radioactivity) was 0.70 ± 0.04. The steady-state extraction ratio for T-2 toxin was 0.93 ± 0.04. A twofold change in the input concentration had no effect on extraction ratios (data not shown). The steady-state hepatic clearance (Equation 2) was 7.47 ± 0.32 ml/min.

The excretion rates of glucuronide conjugates and metabolites into bile were constant after 20 min of perfusion (Figure 2). Radioactivity measured in bile over a 1-hr perfusion study accounted for 54.6% of the total radiolabel perfused through the liver. The rate of biliary excretion was 1% of total per min. There was no significant difference in the metabolite distribution profile after the 20-min time point (Figure 3). Figure 4 shows the metabolites detected in bile after 1 hr. The polar-conjugate was the predominant metabolite (88%). T-2 tetraol (3%), 3'OH HT-2 (4%), 3'OH T-2 triol (1.5%), HT-2 (1%), 4-deacetylneosolaniol (4-DANS) (1%), T-2 triol (0.5%), and 3'OH T-2 (0.2%) accounted for 11% of the total biliary radioactivity, while unconjugated T-2 accounted for less than 0.3%. Several minor unidentified metabolites, denoted as toxin metabolite perfusion 1 and 2 (TMP-1, -2), were also present in bile. Upon hydrolysis of the polar-conjugate (peak remaining at TLC plate origin) with β-glucuronidase from limpets, greater than 92% of the radiolabel was associated with metabolites shown in Figure 5. The major metabolite was HT-2 (80%). Lesser amounts of 3'OH HT-2 (11%) and T-2 tetraol (1%) were also conjugated as glucuronides. The polar-conjugates were poor substrates for bovine β-glucuronidase and sulfatase (Figure 5). This is in agreement with the findings of Roush et al. (1985b). Approximately 7% of the

total administered radiolabel remained in liver and was identified as 4-deacetylneosolaniol (18%), T-2 tetraol (41%), and conjugated metabolites (41%). Table 1 shows the distribution and recovery of total delivered radiolabel in the three compartments (bile, perfusate, and liver). Greater than 97% of the radiolabel was recovered.

DISCUSSION

This study demonstrates that the perfused liver system is a valid model for investigating metabolism and hepatic clearance of T-2 mycotoxin. The metabolic integrity of the perfused liver, assessed by the levels of oxidation-reduction ratios, remained physiologically intact throughout this study. Since steady-state conditions for toxin uptake and biliary excretion were reached within 10 min, it appears that T-2 toxin was not significantly bound to liver tissue. The extraction ratio for T-2 and its metabolites was 0.70, while that for T-2 alone was 0.93. Thus, liver rapidly and efficiently extracted T-2 toxin. In a highly extracted toxin, clearance and half-life are both flow-dependent (Wilkenson and Shand, 1976). Therefore, because the extraction ratio for T-2 toxin approached 1, it is expected that alterations in blood flow to the liver will produce proportional changes in clearance (Studies in progress).

Biochemical pathways of T-2 metabolism in perfused liver were the same as those existing in vivo (Pace et al., 1985a; Yoshizawa et al., 1980), confirming that liver is the major organ for metabolism of T-2 toxin. Unconjugated T-2 and glucuronide conjugates of T-2 metabolites were the major metabolites detected in hepatic venous perfusate. Over half of the total toxin

equivalents extracted by liver appeared as biliary excretion products. In vivo studies (Pace et al., 1985a; Chi et al., 1978; Matsumoto et al., 1978) suggest that biliary excretion is the major route of toxin elimination and emphasize the importance of enterohepatic circulation to the overall toxicity of T-2 toxin. Essentially all biliary radioactivity was associated with metabolites and the glucuronide conjugate of HT-2. Negligible amounts of the parent compound were present in bile. This suggests that liver rapidly and efficiently converts T-2 to HT-2 which is then further metabolized and conjugated as glucuronides. Similar conclusions were drawn in in vivo studies of guinea pigs exposed to T-2 toxin (Pace et al., 1985a), and in in vitro glucuronide synthesis studies of Roush et al. (1985a).

Hydrolysis products of glucuronide conjugates were confirmed by GC/MS to be HT-2, 3'OH HT-2, and T-2 tetraol. Roush et al. (1985a) have identified T-2 and HT-2 glucuronide conjugates in vitro and Pace et al. (1985b) have identified them in vivo. Minor metabolites, both conjugated and unconjugated, were also present in the liver perfusion study. Approximately 3% of the radioactivity remaining at the TLC plate origin was not hydrolyzed by β-glucuronidase or sulfatase (Figure 5). We are currently investigating the identities of this polar metabolite and the two minor unknowns shown in Figure 4 (TMP-1, -2).

The in vitro liver perfusion system can provide a means of producing metabolites for structural analysis. Identification of minor metabolites has not been practical in in vitro microsomal systems, and in vivo studies require the use of large animal models, such as swine or cow, to produce urinary metabolites in large enough quantities for positive identification (Robison et al., 1979; Yoshizawa et al., 1981). In the isolated perfused liver system, there was a dose-dependent appearance of metabolites in bile that peaked

between 10 and 30 min and remained high for the duration of the perfusion (data not shown). Therefore, perfusing high concentrations of toxin over an extended time will potentially allow accumulation and concentration of minor biliary metabolites that cannot be readily obtained in other systems.

The major conclusions drawn from these in vitro liver perfusion studies are 1) liver rapidly and efficiently converted T-2 toxin to more-polar metabolites and glucuronide conjugates of these metabolites; 2) bile was the major route of elimination of T-2 metabolites and glucuronide conjugates; and 3) the perfusion model has potential as a tool for isolation of minor metabolites for structural analysis.

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FOOTNOTES

1. Portions of this work have been presented in abstract form: Fed. Proc. 42, 1809, (1983).

2. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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9. West Chemical Products, Inc., New York, NY 11101.

10. Clay-Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ 07054.

11. Mark III Scintillation Counter, Searle Analytic, Inc., Des Plaines, IL 60018.

12. Precoated silica gel thin layer plates, EM, Science, Cincinnati, OH 45212.

13. Bioscan, Inc., Washington, DC 20007.

14. Hewlett Packard, 11000 Wolfe, Rd., Cupertino, CA 95014.

15. Sigma Chemical Co., St. Louis, MO 63178.

TABLE 1

Percent Recovery and Distribution of Radiolabel in Bile, Perfusate, and Liver

Compartment	Metabolites					
	Conjugates	tetraol	4-DANS	3'OH HT-2	T-2	Total
Bile	48.4	1.6	<0.1	2.2	<0.3	52.5[a]
Perfusate	33.4	ND	ND	ND	4.6	38.0
Liver	2.9	2.9	1.3	ND	ND	7.1
TOTAL	84.7	4.5	1.4	2.2	4.7	97.6

4-DANS, 4-deacetylneosolaniol.

ND - metabolite not detected

[a] - The percent radiolabel associate with 3'OH T-2 triol, T-2 triol, HT-2, and 3'OH HT-2 was <0.1%. These percentages are included in the biliary total recovery.

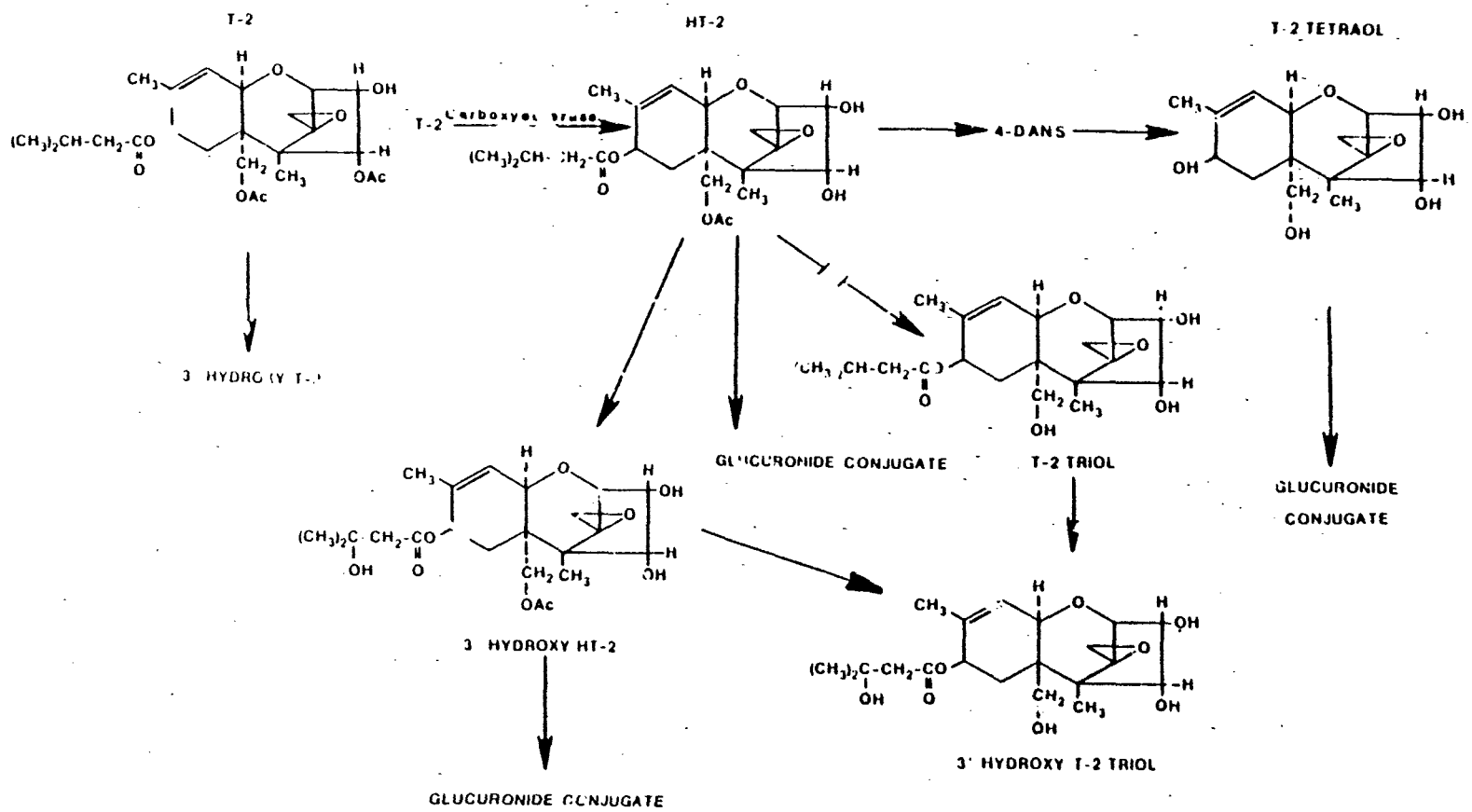
Figure 1 Proposed pathways for metabolism of T-2 toxin. 4-DANS, 4-deacetylneosolaniol.

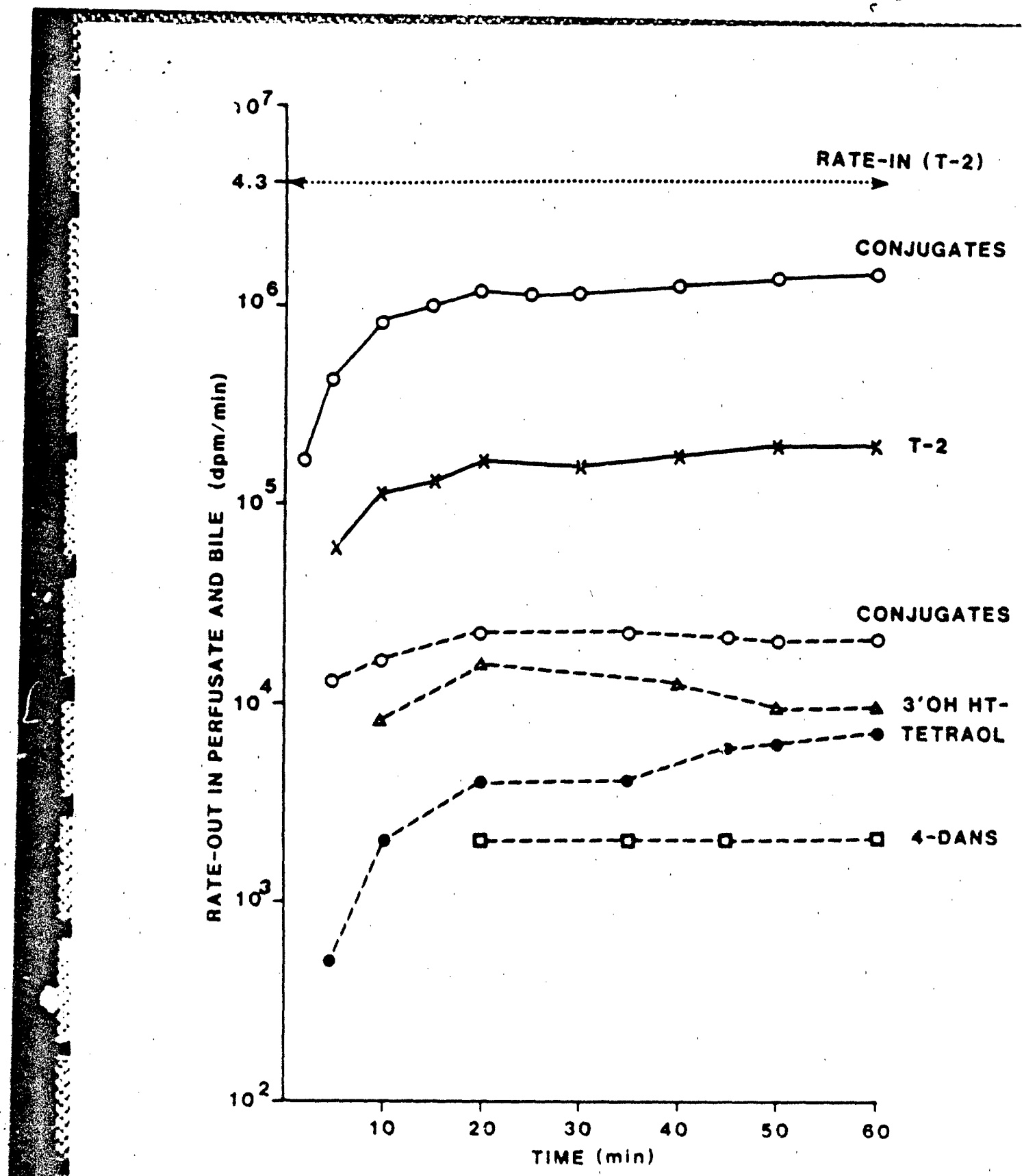
Figure 2 The disposition of [^{3}H]T-2 during its single passage through the isolated perfused rat liver. Perfusate was delivered at a constant flow of 8 ml/min. The input concentration was maintained at 5.4 x 10^5 dpm/ml. The Rate-in is equal to the product of the perfusate flow and the input concentration. Rate-out in bile (---- -) and perfusate (———) is equal to the product of flow and the output concentration.

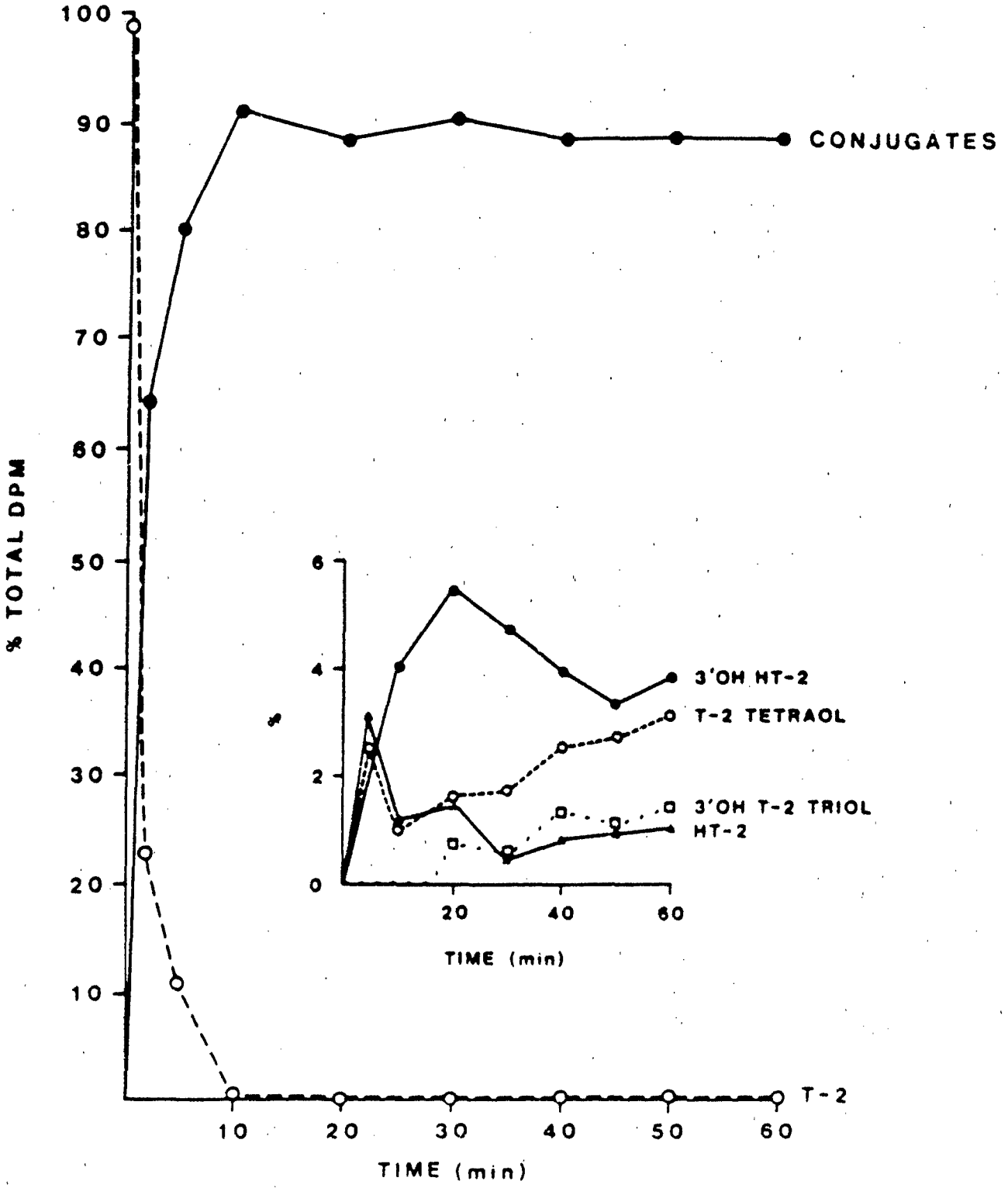
Figure 3 Distribution of radiolabel in bile (% total dpm measured in bile) during the single-pass perfusion. Radioactivity measured in bile accounted for 54.6% of the radiolabel delivered during the 1-hr perfusion experiments. Inset: Expanded scale.

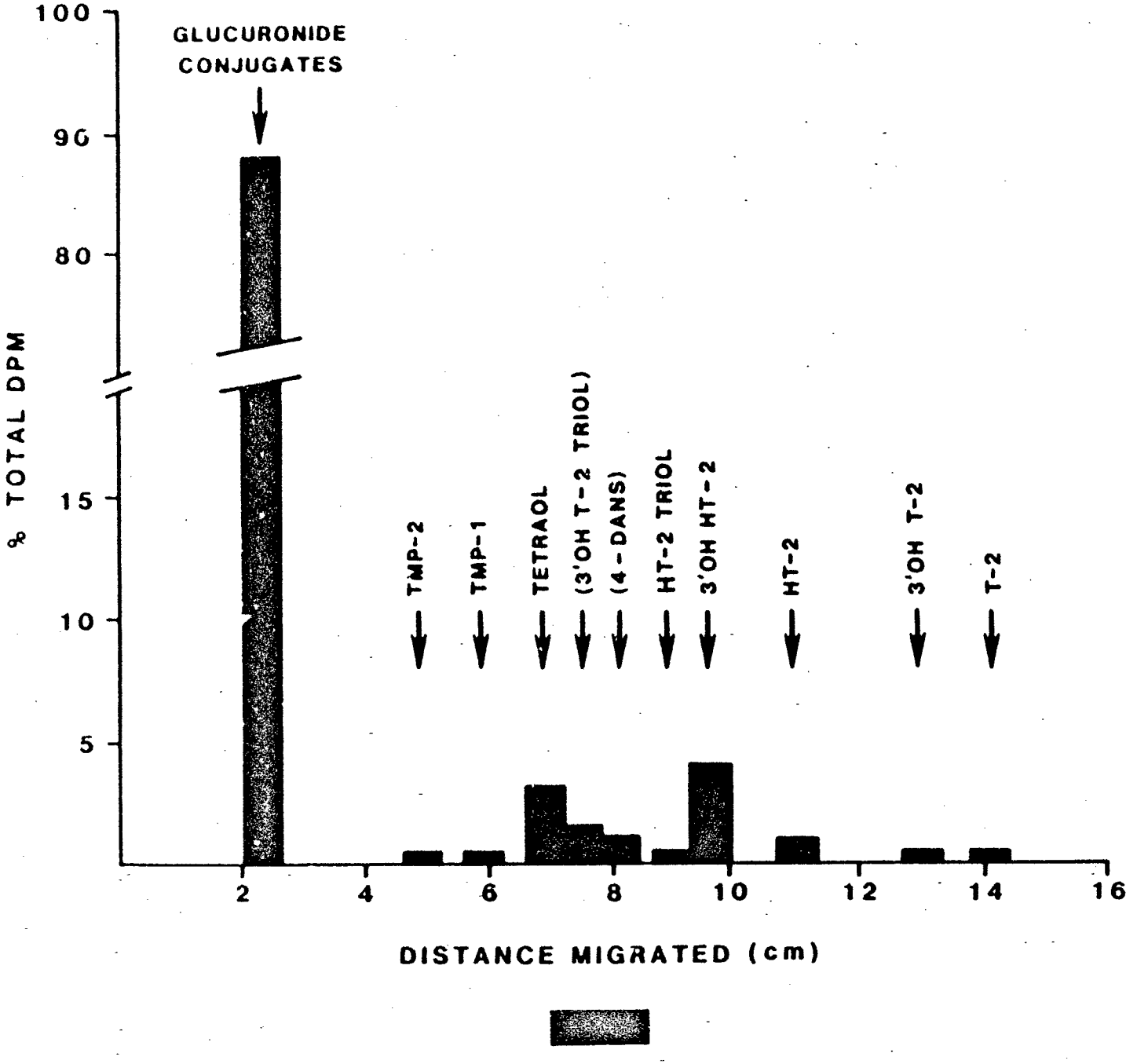
Figure 4 Thin-layer radiochromatogram of biliary metabolites (1 hr). Perfusate was delivered at a constant flow of 8 ml/min. Input concentration of [^{3}H]T-2 was maintained at 5.4 x 10^5 dpm/ml. Aliquots (10-20 µl) of bile were analyzed by TLC by two sequential solvent systems of chloroform:ethyl-acetate:ethanol (50:25:25) (80:10:10).

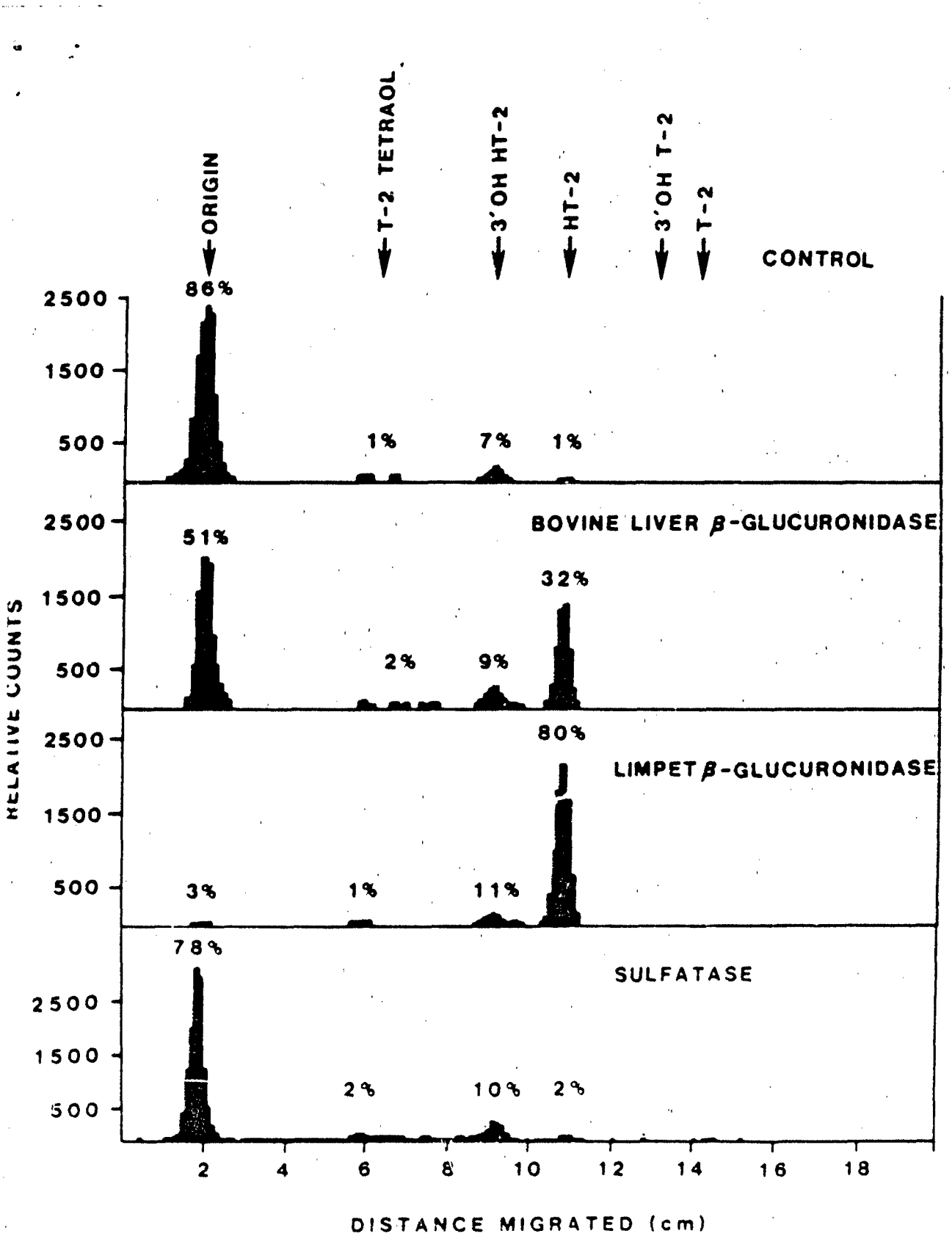
Figure 5 Thin-layer radiochromatogram of the polar fraction remaining at the origin in Figure 4. The polar fraction was hydrolyzed with β-glucuronidase (bovine liver and limpet) or sulfatase at 38°C for 18 hr, pH 3.8 and 5, respectively. The reaction volume was dried under nitrogen, reconstituted in 100 µl methanol, spotted (50 µl) on TLC plates, developed as described in text, and scanned for radioactivity. Relative counts are Bioscan counts and equal 0.3% of the actual counts present on the TLC plate.


T-2
HT-2
T-2 TETRAOL
4-DANS
3 HYDROXY T-2
GLUCURONIDE CONJUGATE
T-2 TRIOL
GLUCURONIDE
CONJUGATE
3 HYDROXY HT-2
GLUCURONIDE CONJUGATE
3' HYDROXY T-2 TRIOL


RATE-IN (T-2)
4.3
CONJUGATES
T-2
CONJUGATES
3'OH HT-
TETRAOL
4-DANS
RATE-OUT IN PERFUSATE AND BILE (dpm/min)
TIME (min)
10
20
30
40
50
60


100
90
80
70
60
50
40
30
20
10
% TOTAL DPM
CONJUGATES
T-2
10
20
30
40
50
60
TIME (min)
6
4
2
0
%
3'OH HT-2
T-2 TETRAOL
3'OH T-2 TRIOL
HT-2
20
40
60
TIME (min)


100
90
80
15
10
5
% TOTAL DPM
GLUCURONIDE
CONJUGATES
TMP-2
TMP-1
TETRAOL
(3'OH T-2 TRIOL)
(4-DANS)
HT-2 TRIOL
3'OH HT-2
HT-2
3'OH T-2
T-2
2
4
6
8
10
12
14
16
DISTANCE MIGRATED (cm)


ORIGIN
T-2 TETRAOL
3'OH HT-2
HT-2
3'OH T-2
T-2
CONTROL
86%
1%
7%
1%
BOVINE LIVER β-GLUCURONIDASE
51%
2%
9%
32%
LIMPET β-GLUCURONIDASE
3%
1%
11%
80%
SULFATASE
78%
2%
10%
2%
2500
1500
500
RELATIVE COUNTS
2
4
6
8
10
12
14
16
18
DISTANCE MIGRATED (cm)