Table IV. Recovery of Radioactivity in Monkey Livers following Administration of [^{14}C]HCB

Step	Sample	Relative radioactivity No. 1079	No. 1036	No. 1111
Soxhlet extraction	Original liver sample	100	100	100
	Benzene extract	83.2	75.4	95.8
	CH_3OH extract	7.5	4.6	3.8
	H_2O extract	0.2	0.6	0
	Dried liver residue	13.0	18.3	4.4
	(Total recovery)	103.9	98.9	103.9
Florisil column chromatography	Benzene extract	100	100	100
	Pentane-CH_2Cl_2 eluate	96.3	89.4	90.5
	First CH_3OH eluate	0.8	6.2	11.6
	Second CH_3OH eluate	0	0	0.7

methanolic Soxhlet extract indicated that the radiochemical was [^{14}C]HCB. It is interesting to note that the dried liver residue contains a fairly large portion of the radioactivity. The significance of this observation is unknown at the present time.

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Excretion and Metabolism of 3,4,5-Tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide in the Rat

Rex E. Hornish* and John L. Nappier

The title compound, a potential preemergence herbicide, labeled in the C-3(5) position with carbon-14, was administered orally to rats to determine the excretion pattern and the metabolism scheme. The herbicide was rapidly absorbed and metabolized, and the metabolites were readily excreted; 90% of the dose was excreted within 72 h. No significant residue levels were found in any tissue. The metabolism was characterized by two successive side chain N-demethylations, followed by hydrolysis to 3,4,5-tribromo-α-methyl-1*H*-pyrazole-1-acetic acid. This tribromo acid was the major metabolite, but was not the end product of metabolism. A reductive debromination of the tribromo acid occurred selectively at the C-5 position to produce the second most abundant metabolite. Debromination at either C-3 or C-4 was not detected.

3,4,5-Tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide (I) has shown exceptional preemergence herbicidal activity against sedges, grasses, and broadleafs in various crops including peanuts and sugar beets. The by-products of these crops, peanut hay and sugar beet tops, are often incorporated into animal fodder. Thus, the herbicide and its residues become potential feed ingredients in the diet of food-producing animals. We were, therefore, interested in establishing the mammalian excretion patterns and

The Upjohn Co., Kalamazoo, Michigan 49001.

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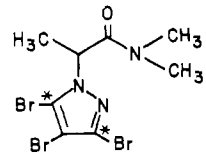


I

Figure 1. 3,4,5-Tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide.

metabolism schemes for this compound. In this our initial study, the herbicide, labeled with C-14 in the C-3(5) position, was orally administered to Sprague-Dawley rats at a level of 6–10 mg/kg.

There are two structural features of this herbicide to be noted (Figure 1). First is the *N*,*N*-dimethylamide side chain, a functional group frequently found in pesticide chemistry. Second is the pyrazole ring, here substituted with three bromine atoms, which as a class of compounds have been only recently applied to weed control (Difenzoquat, for example). The metabolism of I in peanuts and sugar beets has been studied (Kornis and Friedman, 1972). A side chain degradation pattern involving successive N-demethylations was found. In addition, debromination of the pyrazole ring was indicated; small amounts of the 3,5-dibromo analogue of I and 3,4-dibromopyrazole were found. We were thus interested in the in vivo metabolism of I in the rat with regard to side chain degradation and pyrazole ring transformations.

EXPERIMENTAL SECTION

Radiolabeled I. The compound was synthesized (Mallinckrodt Chemical Co.) by condensing [^{14}C]malondialdehyde (^{14}C in the 1 and 3 positions) with hydrazine, followed by bromination and N-alkylation. The label was thus located in the 3(5) position of the pyrazole ring. The compound was purified by preparative TLC on silica gel before use. The sp act. was 4.57 mCi/mmol.

Reference Standards. Unlabeled standards, prepared by known synthetic manipulations (Kornis et al., 1972), were generously provided by G. Kornis of the Upjohn Company.

Animal Treatment. A. *For Excretion Pattern (Single Dose).* Two Sprague-Dawley rats, one male (248 g) and one female (170 g), were housed in conventional metabolism cages (Acme Metal Products, Chicago) which allowed for the collection and separation of urine and feces. They were orally dosed by intubation with 1.50 and 1.70 mg, respectively, of ^{14}C-labeled I in 1:9 ethanol–water. The dose rate was 6.03 and 10.0 mg/kg, respectively. The urine and feces were collected at 24-h intervals for 10 days posttreatment. The rats were then sacrificed for ^{14}C tissue residue determinations.

B. *For Metabolite Analysis (Multiple Dose).* One male Sprague-Dawley rat (235 g), housed as above, was orally dosed with ^{14}C-labeled I at a level of 1.70 mg/day for 10 successive days. The excreta were collected at 24-h intervals, at which time the rat was dosed and transferred to a clean cage. The animal was sacrificed 24 h after the tenth dose for tissue assay (^{14}C content only). The radioactivity in the daily excreta was measured, then the excreta pooled for metabolite isolation and identification.

Radioactive Assay. A Packard Tri-Carb Liquid Scintillation Counter, Model 3375, was used. Aliquots of the urine and cage wash were counted in Diotol Scintillation Cocktail (Burdick and Jackson, Muskegon, Mich.). Fecal samples were homogenized with a three–four-fold excess of water in a Waring Blendor fitted with a mini container, Model 5019. Aliquots were then taken for

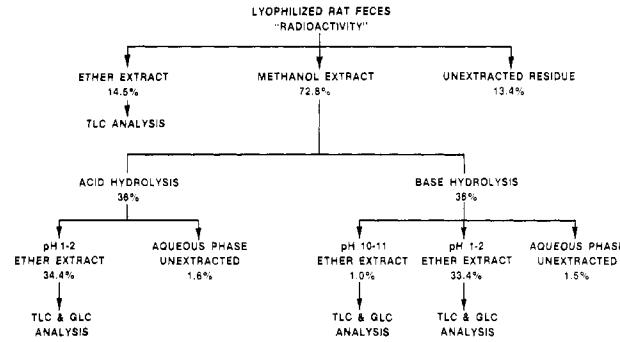


Figure 2. Flow chart of the extraction of the rat feces radioactivity.

combustion (Beckman Biological Material Oxidizer) and scintillation counting in CO_2 Absorbent Scintillator (B & J). The organs and tissues were homogenized with a three–five-fold excess of water, except for blood which was taken direct, for combustion and analysis as described for feces. All samples were assayed in triplicate. Data computations were made with computer programs which apply the appropriate corrections for background, counting efficiency, combustion efficiency, and sample aliquot size.

Extraction of Urine. A. A 5.0-mL sample of the pooled urine from the multi-dose rat was placed in a 15-mL screw-cap centrifuge tube and the pH adjusted to ~11 with 1 N NaOH, then extracted three times with 5-mL volumes of diethyl ether. The combined extracts produced a base extract. The pH of the aqueous solution was then adjusted to pH 7 with 2 N HCl and reextracted with diethyl ether to produce a neutral extract. The pH of the aqueous solution was then adjusted to 1–2 with 2 N HCl and reextracted again with diethyl ether to produce an acid extract. Each extract and the aqueous residue was sampled in triplicate for radioactive assay. The results are summarized in Table III, part A.

B. A 50.0-mL aliquot of the pooled urine was acidified to pH 1.5 with 1 N HCl and extracted four times with 100-mL volumes of diethyl ether. The combined extracts were dried over anhydrous sodium sulfate, then evaporated to dryness with a Büchi flash evaporator. The residue was then taken up in 5.0 mL of acetone for further analysis. The concentrated extract and the aqueous residue were sampled in triplicate for radioactive assay.

Extraction of Feces. A 51.7-g aliquot of the pooled fecal homogenates from the multi-dose rat was lyophilized for 72 h to yield 4.14 g of a fine brown powder. A 1.0-g sample of the powdered residue was placed in a 15-mL centrifuge tube and extracted three times each with 10-mL volumes of diethyl ether, methanol, and acidic methanol (1 N HCl). The percent of the radioactivity extracted by each solvent system appears in Table III, part B. The two methanol extracts were combined and divided in half for acid and base hydrolysis: Acid hydrolysis, 3-h reflux in 1.0 N HCl; base hydrolysis, 1-h reflux in 0.1 N NaOH. The hydrolysates were then extracted with diethyl ether at pH 1–2 and pH 10–11, and the radioactive content of the extracts was measured. The results appear in the extraction flow chart, Figure 2. The extracts were flash evaporated to dryness, and the residues were reconstituted in 1.0 mL of acetone for further analysis.

Analysis of Excreta Extracts. A. *Sample Purification of Urine Extract.* The urine extract was chromatographed on a 10 mm × 300 mm column packed with silica gel (E. Merck, SG-60). A gradient elution from 100% hexane to 89:10:1 hexane–2-propanol–acetic acid was used. This provided one minor fraction and two major fractions for TLC and GLC analysis.

Table I. Cumulative Excretion of Radioactivity of Single-Dose Rats Expressed as Percent of Dose

	urine		feces		total	
day	M	F	M	F	M	F
1	55.8	42.9	9.6	1.0	65.4	43.9
2	71.2	76.3	13.1	7.9	84.3	84.2
3	75.7	80.5	13.9	8.9	89.5	89.4
4	76.8	81.8	14.1	9.3	90.9	91.1
5	77.3	82.3	14.2	9.5	91.6	91.8
6	77.5	82.6	14.3	9.6	91.8	92.1
7	77.6	82.7	14.3	9.6	91.9	92.3
8	77.7	82.9	14.3	9.7	92.0	92.5
9	77.7	82.9	14.3	9.7	92.0	92.6
10	77.8	83.0	14.3	9.7	92.1	92.7

B. *TLC Histograms.* A 5–20-μL aliquot of each sample was applied as a single spot to a 5 × 20 cm TLC plate, 250 μ silica gel GF (Analtech). A mixture of four standards (II, III, IV, V) was also applied to each plate, just to the left of the sample spot. Duplicate plates were prepared for each sample for chromatographic development in two solvent systems: solvent 1, 95:5 benzene–diethylamine; solvent 2, 94.6:5.0:0.4 hexane–2-propanol–acetic acid. The plates were developed to a height of 10.0 cm above the origin. The R_f values of the standards were noted under UV light and the plates vertically segmented into 2-mm bands using a Camag automatic plate scraper. Each segment was channeled into a counting vial and 15 mL of Diotol scintillation cocktail added for radioactive assay. TLC autoradiograms of additional TLC plates, developed as described above, were also obtained. (The R_f values for the standards were: solvent 1, (II) 0.40, (III) 0.10, (IV) 0, (V) 0; solvent 2, (II) 0.70, (III) 0.65, (IV) 0.92, (V) 0.70.)

Gas Chromatography. *GC/RAM.* The extracts were analyzed on a Hewlett-Packard GC (Model 402) equipped with FID and a radioactive monitor (gas proportional counter-Nuclear Chicago) and fitted with a 0.25 in. × 3 ft glass column packed with 3% OV-17 (Applied Science). A temperature program from 125 to 160 °C at 5 °C/min was used. Carboxylic acid standards, e.g., IV and V, were derivatized with diazomethane in ether prior to GC. The extracts were analyzed both before and after treatment with diazomethane.

GC/MS. The samples were analyzed on an LKB-9000 GC/MS interfaced with an IBM 1800 data system. The GC conditions were identical with those just described. Mass spectra were generated by electron ionization operated at 70 eV.

RESULTS AND DISCUSSION

Excretion Patterns. The cumulative excretion of radioactivity in the excreta is recorded in Table I. Within 72 h of treatment, the rats had excreted 90% of the dose, of which 75–80% was found in the urine. The herbicide was thus rapidly absorbed and readily excreted, primarily by the urinary tract. The concentration of radioactive residues in various tissues 10 days posttreatment is shown in Table II. The liver contained the highest residue level as might be expected, followed by the blood. The radioactive content in the entire carcass accounted for 0.5% of the dose for the male rat and 1.1% of the dose for the female rat. In no case did any one tissue content exceed 0.1% of the administered dose.

The residue concentrations for the multi-dose rat at 24-h posttreatment are also presented in Table II. The radioactive content in the entire carcass accounted for 5.0% of the cumulative dose. The high residue levels in the intestines, 9.2 and 23.1 ppm (2.3% of the cumulative dose), are attributable to the last dose administered only 24 h prior to necropsy. Otherwise, the liver and kidney contained the highest levels: 5.2 ppm or 0.4% of the cumulative dose and 7.5 ppm or 0.1% of the cumulative dose. These results do not appear to indicate a significant or site specific residue accumulation, though much more data are certainly required to clarify this point.

Table II. Concentration of Radioactivity (Herbicide I Equivalence) in Rat Tissues

	ppm found		
	single dose rats (10 days posttreatment)		multi-dose rat[a]
tissue	male	female	male
liver	0.051	0.116	5.2
kidney	0.014	0.026	7.5
large intestine	0.007	0.011	9.2
small intestine	0.006	0.011	23.1
stomach	*b*	0.009	1.1
blood	0.029	0.053	1.5
heart	*b*	0.027	1.5
bladder	*b*	*b*	1.3
muscle	*b*	0.019	0.9
fat	*b*	0.017	1.2

[a] The tissues were collected 24 h after the last treatment. [b] The scintillation counting data for these tissues were not statistically different from the background as defined by 3σ.

Table III. Extraction of ^{14}C from Rat Excreta

A. urine at various pH	
pH 10–11 extract	5.9%
pH 6–7 extract	1.6%
pH 1–2 extract	84.1%
aqueous residue	8.5%
B. lyophilized feces	
ether extract	14.5%
CH_3OH extract	48.0%
CH_3OH (+1 N HCl) extract	24.8%
unextracted residue	13.4%

Metabolism. The extraction of 92% of the urine radioactivity into diethyl ether was accomplished by simply adjusting the urine pH to 1–2. A preliminary extraction on a small urine sample at various pH values, Table IIIA, indicated that only 8% was extractable at neutral and basic pH. Thus, the bulk of the extractable radioactivity was of an acidic nature. This was further suggested from chromatographic results; an acetic acid component in the mobile phase was required for sample migration. One further point of interest was that the urinary metabolites were apparently unconjugated compounds since simple pH adjustments would not cleave the usual types of urinary conjugates, such as glucuronides and sulfates.

The results of the extraction and metabolite identification in the rat feces are summarized in Figure 2. The feces, which had been homogenized with water, were lyophilized to a fine dry powder, then suspended in various solvents. Only a small quantity (15%) of the feces radioactivity could be extracted into ether (Table IIIB), and in this extract, no known compounds were found. Most of the radioactivity could be extracted into methanol, which to us suggested that the fecal metabolites were probably polar conjugates. The methanol soluble material was then subjected to both acid and base hydrolysis. Compounds I and II, which survive the acid hydrolysis conditions, would have been detected, if present, as acid-cleavable conjugates or residues. The extraction data clearly show that only acidic compounds were extracted.

The identification of the extracted metabolites was rather straightforward. Preliminary identifications were

Table IV. Metabolites of I in Rat Urine and Feces

metabolite	% in urine	% in feces	total, % of dose[a]
II	2.8		1.9
III	7.7		5.1
IV	40.6	37.6	36.0
V	21.9	17.6	18.8
Total	73.0	55.2	61.8

[a] Based on the distribution of 66% of the radioactivity excreted in the urine and 24.6% excreted in the feces.

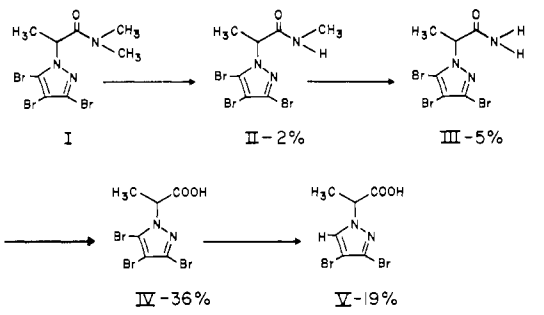


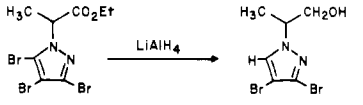
Figure 3. Metabolic route for 3,4,5-tribromo-*N*,*N*,α-trimethyl-1*H*-pyrazole-1-acetamide in the rat.

made by TLC comparison of R_f values with authentic standards. GC/RAM and GC/MS provided the information necessary for validation. Derivatization of compounds IV and V with diazomethane was necessary for successful gas chromatography. The mass spectral fragmentation patterns were easily recognized due to the presence of two or more bromine atoms. Ions containing three bromines displayed the typical M, M + 2, M + 4, and M + 6 quartet pattern (relative intensities 1:3:3:1) whereas ions containing two bromines displayed the typical M, M + 2, and M + 4 triplet pattern (relative intensities 1:2:1).

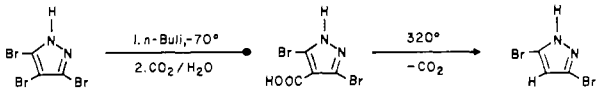
The quantitative results of the metabolite identification are given in Table IV, and the metabolism scheme which results is shown in Figure 3. The herbicide itself was not detected in any excreta sample examined and thus was completely metabolized by the rat, most likely by two successive N-demethylations, first to II, then to the unsubstituted amide III. This type of transformation in biological systems is well kown.

The metabolism of pesticides with the *N*,*N*-dimethylamide group have been studied in soils, plants, and animals. Diphenamid, for example, suffers N-demethylation in soybeans (Krzeminski et al., 1972), strawberries (Golab et al., 1966), and tomatoes (Schoeltz and Tweedy, 1971). The metabolism of Diphenamid in the rat also proceeds via N-demethylation (McMahon and Sullivan, 1965). McMahon and Sullivan have elegantly demonstrated that methyl hydroxylation is the first step and the resultant *N*-hydroxymethyl derivative may either conjugate with glucuronic acid or sulfate, or in a more facile transformation split out formaldehyde to produce nordiphenamid. N-Demethylation of nordiphenamid is apparently slow compared to other potential processes since the unsubstituted amide was detected only in small quantities. Further, diphenyl acetic acid was not detected as even a minor metabolite.

In contrast, the metabolism of the nor-methyl compound, in this case II, was not slow since acid IV, perhaps derived by simple hydrolysis of III, accounted for 36% of the dose and was thus the major metabolite. Perhaps even more significant, however, was the further metabolism to V, the second most abundant metabolite (19% of the dose). This type of transformation, in essence a reductive debromination, is quite unique in a mammalian system. Debrominations in the tribromopyrazoles have been chemically observed. For example, lithium aluminum hydride reduction of VI yields the 3,4-dibromo compound VII (Kornis et al., 1972). However, treatment of tri-



bromopyrazole itself, VIII, with *n*-butyllithium preferentially removes the 4-bromine rather than the 3(5)-bromine (Hüttle and Schör, 1959).



It is not the intention here to discuss the chemical nature of the pyrazoles involved in these observations, but merely to point out that highly active reagents are necessary to bring about debromination. Further, these debrominations are not random, but are controlled by the electronic effects in the pyrazole ring. The metabolism of the pyrazole IV is apparently influenced by these same electronic forces.

There are three important observations concerning the debromination of IV. First, the debromination apparently occurs only on compound IV. No 3,4-dibromo compounds corresponding to I, II, or III were found. Second, only the 5-bromine is displaced. No compounds in either the 3,5-dibromo or the 4,5-dibromo series were found, though most of the authentic standards were available for comparison. Thus, this debromination is apparently compound specific and position selective. Third, nucleophilic substitution of the bromine with such nucleophiles as OH or OCH_3 was not observed. Only a hydrogen displacement of the bromine, i.e., reductive debromination, was found. No suitable explanation for this result is readily apparent.

Our conclusions then are as follows: The herbicide I is rapidly absorbed and extensively metabolized by the rat. The metabolites are readily excreted, primarily through the urinary tract. The metabolic sequence of I has been established and characterized by successive N-demethylations of the side chain, hydrolysis of the resultant amide to the acid, and highlighted by a selective reductive monodebromination of the pyrazole ring in the later stages.

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