Identification of the Metabolites of Terbacil in Dog Urine

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When a dog was dosed with 2-C^{14}-terbacil, the predominant compound isolated in the urine was 3-*tert*-butyl-5-chloro-6-hydroxymethyl-2-C^{14}-uracil. Structural assignment is based on elemental analysis combined with high resolution mass spectrometric and nuclear magnetic resonance analyses of the material isolated from a dog fed terbacil at a dietary level of 7000 p.p.m. Four additional minor metabolites of terbacil have been identified by similar analytical techniques. No 5-chloro-2-C^{14}-uracil was detected as a metabolite of 2-C^{14}-terbacil by thin-layer chromatographic and liquid scintillation counting techniques.

Terbacil, 3-*tert*-butyl-5-chloro-6-methyluracil, is useful as a selective herbicide for control of many annual and some perennial weeds in crops such as sugar cane, apples, peaches, citrus, and peppermint. It is marketed as a wettable powder containing 80% active ingredient under the trade name Sinbar terbacil weed killer by E. I. du Pont de Nemours & Co. This paper reports on the metabolic fate of terbacil when ingested by dogs. The metabolic fate of a related substituted uracil herbicide, bromacil, in rats (Gardiner *et al.*, 1969a) and the fate of these two compounds in soils and plants (Gardiner *et al.*, 1969b) have been described.

EXPERIMENTAL AND RESULTS

Conventional Analysis. ANIMAL FEEDING. Female beagle dogs were maintained for 9 months on diets containing terbacil at levels of 2500 to 7000 p.p.m. in their rations, which consisted of Purina Dog Chow. Feeding was initiated at the dietary level of 2500 p.p.m. and increased to 3000 p.p.m. after 6 months. The level was then increased incrementally, reaching 7000 p.p.m. at the time the urine was collected. At these levels of feeding there was no clinical evidence of toxicity.

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Extraction of Terbacil and Terbacil Metabolites from Urine. Three hundred milliliters of female dog urine from the 9-month feeding study was adjusted to pH 5 with 6*N* hydrochloric acid. Two milliliters of β-glucuronidase–arylsulfatase enzyme preparation was added to hydrolyze possible conjugates of the metabolites, and the solution was incubated at 35° C. for 24 hours. Five milliliters of 10*N* sulfuric acid was added and the solution was continuously extracted with diethyl ether for 72 hours. The ethereal solution was taken to dryness and the residue was dissolved in about 2 ml. of methanol.

Thin-Layer Chromatography (TLC) of Terbacil and Metabolites. The methanol solution of the residue from the dog maintained on terbacil for 9 months was streaked onto TLC plates coated with 600-micron layers of kieselgel with an incorporated phosphor and developed to 10 cm. in ethyl acetate. All of the absorbent, except that at the origin, was removed from the plates and eluted with methanol. The volume of the methanol was reduced to about 1 ml. and this was streaked on TLC plates and developed as before. Examination of the plates under ultraviolet light showed four streaks of ultraviolet-absorbing materials in the treated sample which were not present in the control sample. The R_f value for terbacil under these conditions was 0.50. The R_f values for the streaks observed in the treated samples are listed in Table I. The absorbent containing each of these streaks was removed from the plate and eluted with methanol. The resi-

Table I. Thin-Layer Chromatographic R_f Values of Terbacil and Its Metabolites

Compound	Chemical Name	R_f[a]	R_f[b]
Terbacil	3-*tert*-Butyl-5-chloro-6-methyluracil	0.55	0.50
Metabolite			
A	3-*tert*-Butyl-5-chloro-6-hydroxymethyl uracil	0.52	0.48
B	6-Chloro-2,3-dihydro-7-(hydroxymethyl)-3,3-dimethyl-5*H*-oxazolo-(3,2-*a*)pyrimidin-5-one	0.44	0.34
C	6-Chloro-2,3-dihydro-3,3,7-trimethyl-5*H*-oxazolo(3,2-*a*)pyrimidin-5-one	0.05	0.05
D	3-*tert*-Butyl-6-hydroxymethyluracil	0.16	0.16
E	3-*tert*-Butyl-6-formyluracil	0.10	0.10
F	Unknown chlorine-containing compound of mol. wt. 293	0.48	—

[a] Developed to 15 cm. in mixture of ethyl acetate–hexane-methanol (10:10:1). [b] Developed to 15 cm. in ethyl acetate.

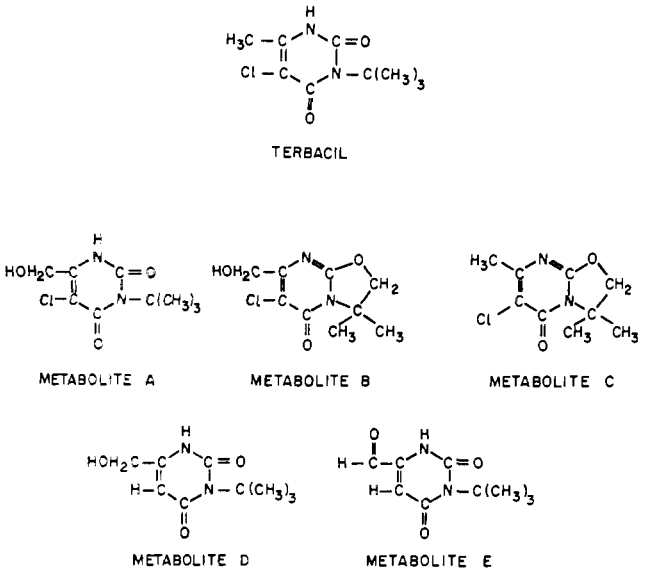

Figure 1. Terbacil metabolism in dogs

due from each streak was purified by several additional TLC steps employing a 10:10:1 mixture of hexane–ethyl acetate–methanol as the developing solvent.

The band of ultraviolet absorbing compounds at R_f 0.48 (ethyl acetate development) separated into two bands in the second solvent system, yielding metabolites A and F. The band at R_f 0.10 (ethyl acetate) separated into three bands, yielding metabolites C, D, and E (Table I). The seven materials isolated in this manner were subjected to structural identification (Figure 1).

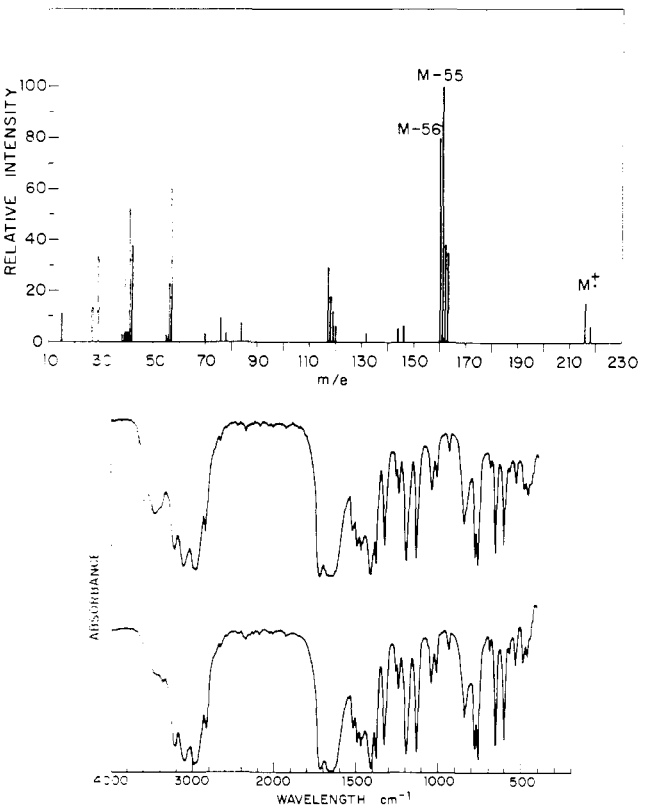

Figure 2. Spectra of terbacil

Upper. Mass spectrum
Middle. Infrared spectrum of terbacil isolated from urine extract
Lower. Infrared spectrum of terbacil reference

Identification of Terbacil Metabolites. Terbacil was identified by comparison of the infrared and mass spectra of the isolated compound with those of a reference sample of terbacil (Figure 2). Structural assignments for the metabolites were based primarily on spectral data obtained directly on the isolated materials.

All mass spectra were obtained on a Bendix Model 12-107 Time-of-Flight mass spectrometer and the NMR spectra on a Varian A-60 NMR spectrometer. Deuteroacetone was used as the solvent for all NMR spectra.

Metabolite A was obtained in sufficient quantity to be recrystallized from ethanol to yield white needles with a melting point of 165–66° C. Elemental analyses for C, H, N, and Cl agree with the calculated values for the molecular formula.

Calculated for $C_9H_{13}N_2O_3Cl$: C, 46.48; H, 5.63; N, 12.00; Cl, 15.23. Found: C, 46.55; H, 5.72; N, 11.73; Cl, 14.68.

The nuclear magnetic resonance, infrared, and mass spectra of metabolite A are shown in Figure 3. The mass spectrum shows that its molecular weight is 232, 16 atomic mass units greater than terbacil, indicating insertion of one oxygen atom in the parent compound. The strong M-55 fragment is

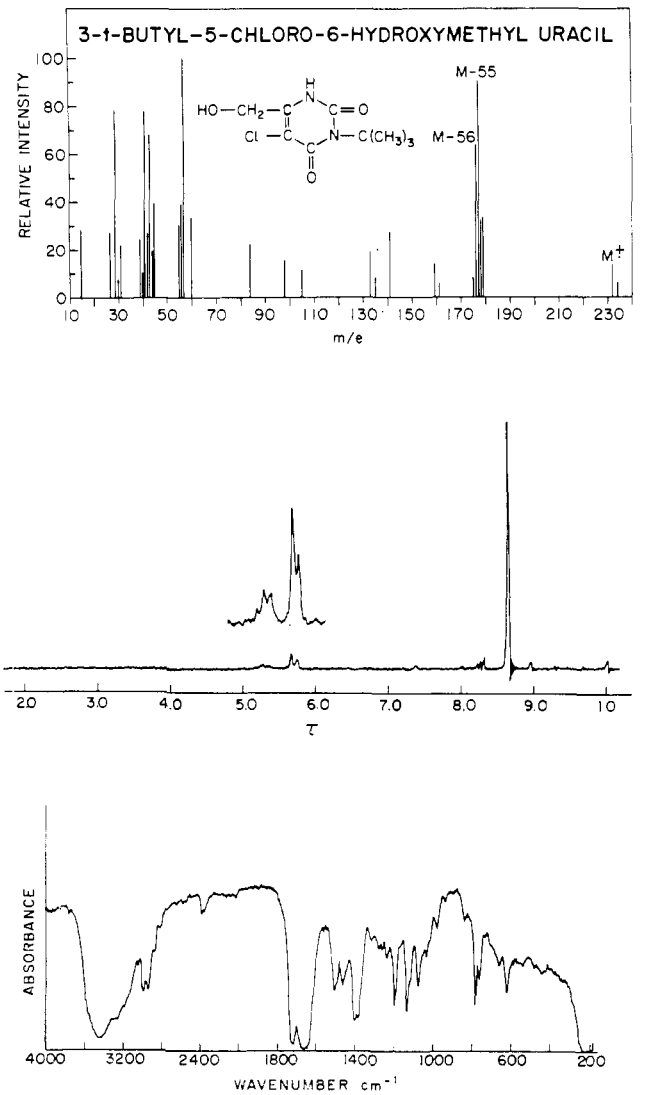

Figure 3. Spectra of metabolite A

Upper. Mass spectrum
Middle. NMR spectrum
Lower. Infrared spectrum

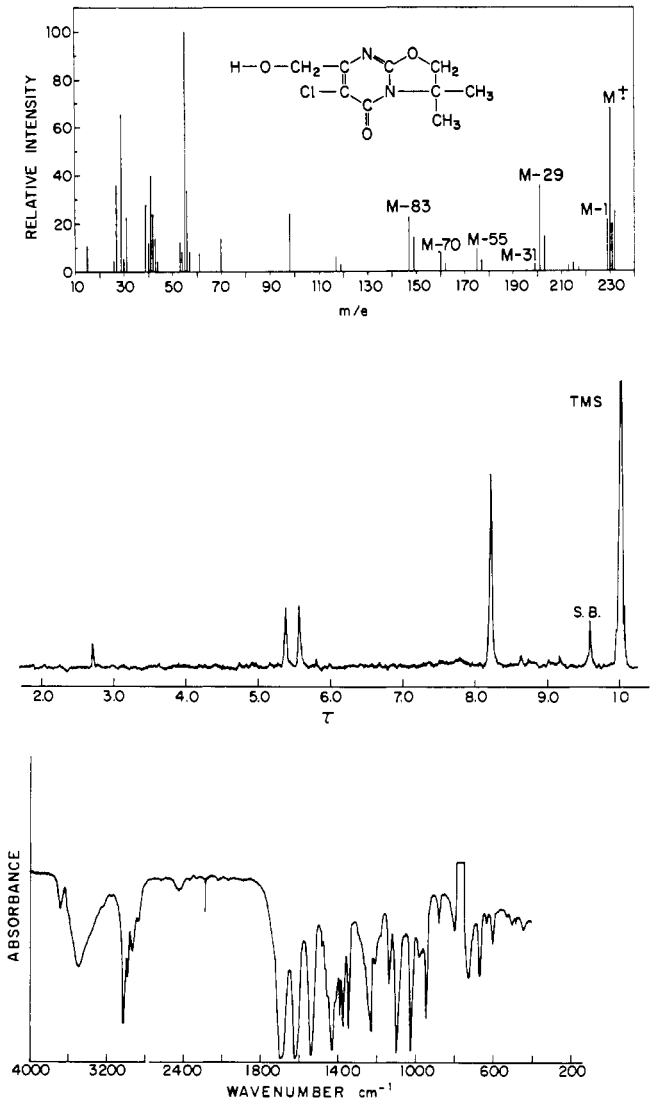

Figure 4. Spectra of metabolite B

Upper. Mass spectrum
Middle. NMR spectrum
Lower. Infrared spectrum

characteristic of an intact *tert*-butyl group, suggesting strongly that oxygen has been added to the 6-methyl substitution. The NMR spectrum shows that metabolite A exhibits absorption at 8.66 (sharp singlet, relative area 24), 5.68, and 5.78 τ (doublet, relative area 5.3) and a triplet at 5.20, 5.30, and 5.40τ (relative area 2.6). The extra tracing above the 5.0 to 6.0τ region in the NMR spectrum was obtained by increasing the amplitude of the signal in order to increase the detail of the fine structure. The absorption at 8.66τ falls well within the region expected for a *tert*-butyl group. The doublet at 5.68 and 5.78τ is in the region of absorption of protons attached to carbon that additionally bears oxygen or nitrogen and is coupled with the hydroxylic proton. The triplet at 5.20, 5.30, and 5.40τ is due to a hydroxylic proton, which is coupled with the adjacent methylene protons. The relative areas of the absorptions indicate that there are nine methyl protons, two methylene protons on a carbon atom that is additionally attached to either oxygen or nitrogen and is coupled with one adjacent proton, and one hydroxylic proton coupled with an adjacent methylene group. This evidence, coupled with the mass spectral data and elemental analyses, clearly supports the structural identification of metabolite A as 3-*tert*-butyl-5-chloro-6-hydroxymethyluracil.

The nuclear magnetic resonance, infrared, and mass spectra of metabolite B are shown in Figure 4. The conventional mass spectrum of metabolite B shows that its molecular weight is 230, 14 above terbacil. This was confirmed by a high resolution mass spectrum obtained on a CEC-21-110B high resolution mass spectrometer, which showed the molecular weight of the compound to be 230.04483, corresponding to a molecular formula of $C_9H_{11}N_2O_3Cl$. The very intense molecular ion (relative intensity ca. 0.85) observed for metabolite B indicates that the compound has a more stable structure than terbacil or metabolite A under the disruptive forces of the mass spectrometer. The latter have weaker molecular ions (relative intensity ca. 0.15). The M-55 fragment, which is very intense for compounds with an unchanged *tert*-butyl group on the 3-nitrogen, is weak in this spectrum, indicating that the *tert*-butyl group has been altered. The M-29 fragment was shown by high-resolution measurements to be due to loss of CHO, and is most likely due to loss of CO from the M-1 fragment. This is analogous to what is obtained in the mass spectrum of benzyl alcohol (Budzikiewicz *et al.*, 1967), and is indicative of a hydroxymethyl group attached to a conjugated ring. This fragment was not obtained in the spectrum of metabolite A, since its hydroxymethyl group is not attached to a conjugated ring system. The infrared spectrum shows a strong absorption for aliphatic hydroxyl at 3500 cm^{-1}. The NMR spectrum shows that metabolite B exhibits absorption at 8.23 (sharp singlet, relative area 9.2), 5.57 (sharp singlet, relative area 3.0), 5.38 (sharp singlet, relative area 3.0), and a broad band from about 7 to 8.1τ that is most likely due to the hydroxylic proton indicated by the infrared spectrum. The absorption at 8.23τ falls well within the region expected for methyl protons when the methyl group is attached to a tertiary carbon. The 5.57 and 5.38τ peaks are in the region of absorption of protons attached to carbon atoms that additionally bear oxygen or nitrogen. The lack of coupling shows that all absorbing groups are isolated by at least one atom from other proton-bearing atoms. The relative areas of the absorptions, together with the other structural information, indicate that there are six methyl protons and two sets of two equivalent protons on carbon atoms that are additionally attached to either oxygen or nitrogen. The protons in the structure assigned to metabolite B meet all the requirements of the observed nuclear magnetic resonance data. Furthermore, the assigned structure has the proper molecular weight, and its formation is reasonable to expect through oxidation of the alkyl substituents at the 3- and 6-positions, followed by abstraction of a mole of water.

Assignment of these absorptions at 5.57 and 5.38τ to the methylene groups at the 2- and 7- positions, respectively, is well within the normal regions where absorptions for these types of groups are to be expected.

The lesser metabolites (C, D, and E) were tentatively identified on the basis of their mass spectra alone because insufficient quantities were obtained for nuclear magnetic resonance studies. Their mass spectra are shown in Figures 5, 6, and 7.

The molecular weight of metabolite C was 214 by mass spectroscopy, two less than terbacil. The mass spectrum also showed the presence of one chlorine atom and the absence of a *tert*-butyl group on the 3-nitrogen. Formation of this structure by a mechanism similar to that proposed for metabolite B, without oxidation of the alkyl substituent in the 6-position, appears reasonable.

The molecular weight of metabolite D was 198 by mass spectroscopy. The mass spectrum also showed that the molecule contained no chlorine atoms and showed a major fragment which corresponds to loss of 55 mass units, which is

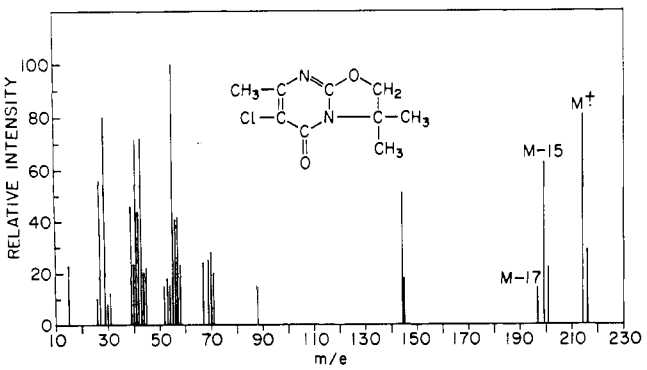

Figure 5. Mass spectrum of metabolite C

characteristic of the *tert*-butyl group on the 3-nitrogen. The spectrum is very similar to a bromacil metabolite, 3-*sec*-butyl-6-hydroxymethyluracil (Gardiner *et al.*, 1969a).

The mass spectrum of metabolite E showed that the compound had a molecular weight of 196 and that the molecule contained a *tert*-butyl group on the 3-nitrogen but no chlorine atoms. This spectral evidence suggests further oxidation of the substituent in the 6-position of metabolite D.

The molecular weight of a sixth metabolite (F) was 293 by mass spectroscopy, 77 higher than terbacil. The mass spectrum also shows that the molecule contains one chlorine atom and the intact 3-*tert*-butyl group. It is assumed to be similar to an unidentified metabolite of bromacil (Gardiner *et al.*, 1969a) with a molecular weight 78 greater than the parent compound.

Radiochemical Analysis. ANIMAL FEEDING. A male beagle dog was initially fed a diet containing 500 p.p.m. of unlabeled terbacil in his rations, which consisted of Purina Dog Chow. Over a 3-week period, the feeding level was gradually increased to 2500 p.p.m. of terbacil. At the end of the 3-week period, the dog was dosed with 24.3 mg. of 2-C^{14}-terbacil (Gardiner *et al.*, 1969b) (0.895 mc. per mmole) in a gelatin capsule and maintained on the high level diet. Daily urine samples were collected and pooled for 3 days after C^{14}-treatment. The dog was sacrificed 72 hours after treatment and all tissues were frozen and retained for total C^{14} analyses.

Radioassay of All Tissues. The urine, feces, blood, and all tissues from the dog dosed with 2-C^{14}-labeled terbacil were analyzed for total C^{14} according to the procedure of Smith *et al.* (1964). Each tissue sample was homogenized in a blender and 1-gram aliquots were analyzed. The results of these analyses (Table II) show that essentially all of the radioactivity detected was eliminated in the urine and feces, and that elimination was essentially complete after 72 hours.

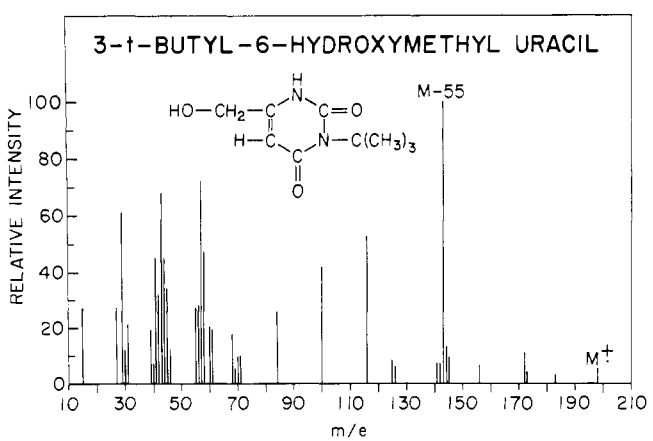

Figure 6. Mass spectrum of metabolite D

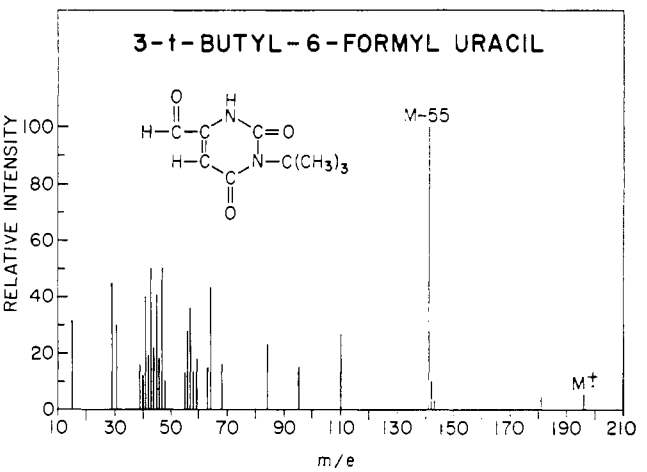

Figure 7. Mass spectrum of metabolite E

Concentration of Metabolites in Urine. To establish the relative concentrations of the various metabolites in urine, 50 ml. of the first day's urine collection were enzymatically hydrolyzed and extracted as in the conventional analysis. The extract was taken to dryness and the residue made to 2.0 ml. with methanol. About 35% of the total C^{14}-activity in the original urine aliquot was present in the 2.0 ml. of concentrated extract. One hundred microliters of the concentrated extract was applied to a TLC plate as a streak next to a terbacil reference spot, and the plate was developed to 15 cm. in a 10:10:1 mixture of hexane–ethyl acetate–methanol. Exposure of the TLC plate to x-ray film showed the presence of seven C^{14}-labeled compounds. The R_f values of these compounds corresponded to those of the materials identified in Table I. The absorbent containing each compound was removed from the plate into a scintillation counting vial and

Table II. 2-C^{14}-Terbacil Dose Found in Dog Tissues

Tissue	% of Original Dose Found	% of Total Activity Detected
Urine (0–24 hr.)	50.14[a]	64.8
Urine (24–48 hr.)	11.04	14.3
Urine (48–72 hr.)	1.16	1.5
Feces (0–24 hr.)	10.57	13.7
Feces (24–48 hr.)	3.92	5.1
Feces (48–72 hr.)	0.41	0.5
Liver	0.06	0.1
Kidney	< 0.005	—
Blood	0.001	—
Stomach	0.01	—
GI tract	0.02	—
Brain	< 0.005	—
Lung	< 0.005	—
Heart	< 0.005	—
Bladder and prostate	< 0.005	—
Testes	< 0.005	—
Thyroid	< 0.005	—
Adrenals	< 0.005	—
Pancreas	< 0.005	—
Spleen	< 0.005	—
Bone marrow	< 0.005	—
Fat	< 0.001	—
Muscle	< 0.001	—
Total	77.34	100.0

[a] Part of first day's urine sample splashed outside metabolism cage and was lost.

Table III. Relative Concentrations of Terbacil and Metabolites in Urine Extract

Compound	Relative Concn.
Terbacil	1
Metabolite	
A	100
B	10
C	1
D	5
E	2
F	>1
Unknown[a]	7

[a] Activity remained at origin of TLC plate.

the samples were counted to obtain the relative concentration of each metabolite in the urine extract.

Examination of Dog Urine for 5-Chlorouracil. Even though no evidence of the presence of 5-chlorouracil had been obtained, urine samples from the dog dosed with 2-C^{14}-labeled terbacil were re-examined specifically for this suspect mutagen. Fifty-milliliter aliquots of the urine from the first and second day following dosage with radio-labeled terbacil were analyzed, both before and after enzymatic hydrolysis (Table IV). Enzymatic hydrolysis was carried out as previously described. The 50-ml. samples to be analyzed were adjusted to pH 5 and extracted continuously with diethyl ether in liquid-liquid extractors for 48 hours. The extracts were evaporated to dryness in a hood and the residues were dissolved in 2 ml. of methanol.

Twenty-five microliters of each extract was spotted in the corner of a 20 × 20 cm. TLC plate. Approximately 20 μg. of nonlabeled 5-chlorouracil was overspotted on the extracts to serve as a reference. The plates were developed in ethyl acetate for 15 cm., then rotated 90°, and developed for 15 cm. in water. The location of the 5-chlorouracil spot was found by viewing the plate under an ultraviolet light. Approximate R_f values for 5-chlorouracil in the systems used were 0.33 in ethyl acetate and 0.83 in water. The developed plates were exposed to Ansco Nonscreen x-ray film for 3 weeks. The resulting radioautogram is shown in Figure 8. The circled area indicates where the ultraviolet absorbing 5-chlorouracil coreference spot was found on the plate; it is obvious that no radioactivity was detected in the 5-chlorouracil spot, showing that 5-chlorouracil-2-C^{14} is not present.

To estimate the sensitivity of this qualitative examination, the areas of adsorbent containing the 5-chlorouracil reference spots were removed from the plates and placed in 20-ml. liquid scintillation vials. Fifteen milliliters of the scintillator solution was added and the samples were placed in the dark for 3 to 4 days, then counted for 100 minutes. Silica from unused portions of the TLC plates was counted to give background counting levels. The data (Table IV) show only insignificant radioactivity in the samples scraped from the areas of the plates which would have contained 5-chlorouracil. There is a slight positive trend in the samples isolated from the 5-chlorouracil areas when compared to background obtained from untreated silica plates. This probably was caused by slight contamination during the handling of the plates, which contained total activities in the 5 × 10^4 to 10^5-d.p.m. range, whereas even the largest of the absolute counts is only 13 counts per minute.

To establish the validity of these isolation procedures, 0.62 μg. of C^{14}-labeled 5-chlorouracil was added to 50 ml. of control urine and carried through the entire extraction, thin-layer, and radiocounting procedure described above. Seventy-one per cent of the original activity was recovered.

Table IV. 5-Chlorouracil-2-C^{14} in Extracts from 50 Ml. of Dog Urine

Sample Counted	Total Counts per Minute: 5-Chlorouracil spot	Background	Net counts[a] per min.
1st day's urine			
Before hydrolysis[b]	36	30	6
Before hydrolysis	43	30	13
After hydrolysis	41	39	2
2nd day's urine	32	29	3
After hydrolysis[c]	40	29	11

[a] Average counting efficiency 74%.
[b] Urine contains 5.53 × 10^5 d.p.m./ml.
[c] Urine contains 7.13 × 10^4 d.p.m./ml.

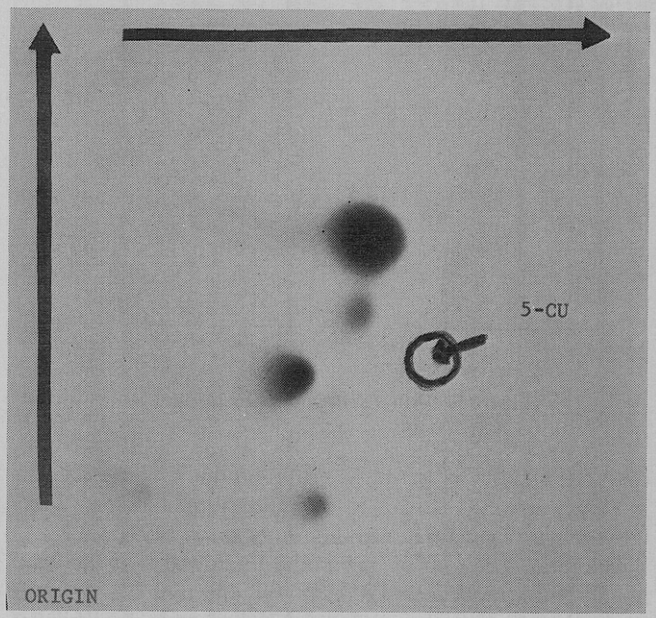

Figure 8. Radioautogram of dog urine extract showing absence of 5-chlorouracil

DISCUSSION

A major metabolite of terbacil in the urine of dogs is 3-*tert*-butyl-5-chloro-6-hydroxymethyluracil. This is analogous to bromacil in rats (Gardiner *et al.*, 1969a), in which 5-bromo-3-*sec*-butyl-6-hydroxymethyluracil is the major metabolite. In general, the metabolic breakdown of terbacil in the dog was very similar to that of bromacil in the rat, which involved primarily oxidation of the alkyl substituents of the 3- and 6-positions of the molecule. However, oxidation of the 3-*tert*-butyl group to the hydroxy compound in terbacil is followed by elimination of water to give 6-chloro-2,3-dihydro-7-(hydroxymethyl) - 3,3 - dimethyl - 5*H* - oxazolo - [3,2*a*] - pyrimidin - 5-one. Three other metabolites have also been isolated and tentatively identified by mass spectrometry, as shown in Figure 1.

Radiochemical analysis of urine showed that the relative concentration of 3-*tert*-butyl-5-chloro-6-hydroxymethyluracil (metabolite A) in the urine extract was 10 times greater than that of any other metabolite.

Analyses of the urine, feces, and all tissues of the dog dosed

with C^{14}-labeled terbacil showed that over 99% of the C^{14} was excreted in the urine and feces. Specific radiochemical analysis of urine from a dog dosed with 2-C^{14}-labeled terbacil conclusively showed that 5-chlorouracil is not a metabolite of terbacil in the dog.

The *tert*-butyl group is easily hydrolyzed from the 3-position of the uracil ring under acidic conditions. Thus, in all this work, care was taken to prevent terbacil or its metabolites from remaining in acid solution for extended periods of time, especially at elevated temperatures.

ACKNOWLEDGMENT

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