Identification of the Metabolites of Bromacil in Rat Urine

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The predominant metabolic compound isolated from the urine of rats fed bromacil at a dietary level of 1250 p.p.m. for one month was 5-bromo-3-*sec*-butyl-6-hydroxymethyluracil. Structural assignment was based on thin-layer chromatographic, infrared spectrophotometric, mass spectrometric, and nuclear magnetic resonance analyses of the material actually isolated from the urine. An authentic sample of this metabolite was synthesized chemically for comparison, and a quantitative analytical method developed to detect it in urine. Five of six minor metabolites of bromacil found in rat urine have been identified by similar analytical techniques. No 5-bromouracil was detected as a metabolite of bromacil by a selective gas chromatographic analysis.

B romacil, 5-bromo-3-*sec*-butyl-6-methyluracil, is an effective herbicide for the control of annual and perennial weeds and is marketed as a wettable powder containing 80% active ingredient under the trade-name Hyvar X bromacil weed killer by E. I. du Pont de Nemours & Co. Bromacil is used for selective weed control in pineapple and for control of certain annual and perennial weeds in citrus. It is widely used as a nonselective weed killer on sites where bare ground is desired.

In chronic feeding studies with rats and dogs and three-generation reproduction studies with rats, Sherman (1968) showed that bromacil and its metabolites in these animal species are of low mammalian toxicity. In studies on mice and *Escherichia coli* 15T by McGahen and Hoffman (1963a, b), there was no evidence of incorporation of these materials in DNA. It was also of interest to establish the chemical identity of the metabolites of bromacil. This paper reports on the metabolic fate of this compound when ingested by rats as a dietary component for one month. The fate of bromacil in soils and plants is described by Gardiner *et al.* (1969).

EXPERIMENTAL DETAILS AND RESULTS

Bromacil Feeding. Male rats (Charles River, CD strain) were maintained on a diet containing 1250 p.p.m. of bromacil in their rations, which consisted of ground Purina Lab Chow to which 1% corn oil had been added. The rats were maintained on this diet for one month, and the urine was collected daily during the third and fourth weeks. In addition, control urine was similarly collected from another group of rats fed ground Purina Lab Chow with 1% corn oil added.

Extraction of Bromacil and Metabolites from Rat Urine. One liter each of control and treated urine was concentrated by evaporation in a laboratory hood to about 100 ml., 500 ml. of absolute ethanol was added, and the insoluble materials were removed by centrifugation. The solution was concentrated to about 50 ml. and adjusted to 200 ml. with water. The aqueous solution was washed with 100 ml. of hexane and extracted three times with 100-ml. portions of ethyl acetate. The combined ethyl acetate extracts were washed with 50 ml. of water and evaporated to dryness in a hood. The residues were dissolved in about 2 ml. of ethyl acetate.

Thin-Layer Chromatography (TLC) of Bromacil and Metabolites. For preliminary TLC cleanup of the extracts, the residues were streaked onto TLC plates coated with 600-micron layers of kieselgel with an incorporated phosphor.

Two plates (20 × 20 cm.) were used for each extract. Each plate was developed three times to 15 cm. in water. All of the adsorbent, 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 as before. These plates were developed three times to 15 cm. in ethyl acetate. Again the adsorbent, from just above the origin to the solvent front, was removed from the plate and eluted with methanol. The volume of the methanol was reduced to about 1 ml. In this way, samples were sufficiently prepared for the final TLC separation of metabolites.

One hundred microliters of each extract was applied to each of ten 250-micron kieselgel TLC plates. A bromacil reference spot (20 μg.) was also applied to each plate. The plates were developed twice to 15 cm. in chloroform and once to 10 cm. in ethyl acetate. Observation of the plates under ultraviolet light showed five streaks of ultraviolet-absorbing materials in the treated sample which were not present in the control. The R_f value for bromacil under these conditions was 0.63. The R_f values for the streaks observed in the treated sample are listed in Table I. The adsorbent containing each of these streaks was removed from the plates and eluted with methanol. When necessary, the residue from each streak was separated into its individual compounds by using H_2O as a developing solvent (Table I). Each compound was further purified by several additional TLC steps following the procedures described. Seven metabolites were isolated in

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

Compound	Chemical Name	A[a]	B[b]
Bromacil	5-Bromo-3-*sec*-butyl-6-methyluracil	0.63	—
Metabolite			
I	5-Bromo-3-*sec*-butyl-6-hydroxymethyluracil	0.54	—
II	5-Bromo-3-(2-hydroxy-1-methylpropyl)-6-methyluracil	0.38	0.44
III	5-Bromo-3-(2-hydroxy-1-methylpropyl)-6-hydroxymethyluracil	0.27	0.65
IV	3-*sec*-Butyl-6-hydroxymethyluracil	0.27	0.56
V	5-Bromo-3-(3-hydroxy-1-methylpropyl)-6-methyluracil	0.27	0.50
VI	3-*sec*-Butyl-6-methyluracil	0.38	0.29
VII	Unknown bromine-containing compound of mol. wt. 339	0.72	—

[a] A. Developed twice to 15 cm. in chloroform and once to 10 cm. in ethyl acetate on 250-micron kieselgel plates.
[b] B. Developed to 10 cm. in water on 250-micron kieselgel plates.

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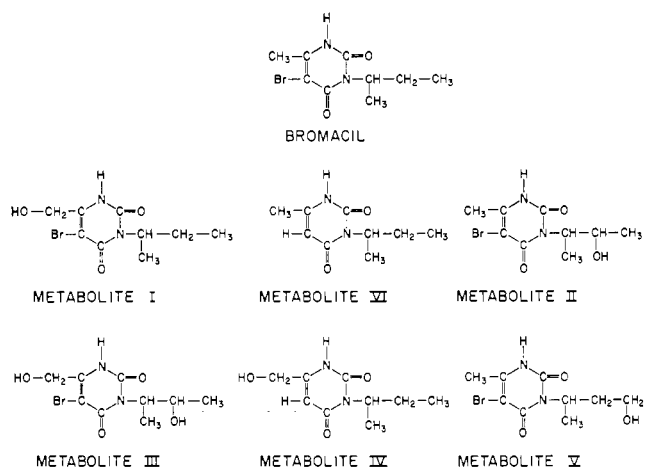
Figure 1. Bromacil metabolism in rats

this manner for structural identification. These compounds, along with their identities, are listed in Table I and shown in Figure 1.

Identification of Bromacil Metabolites. Bromacil metabolites were identified from their infrared, ultraviolet, mass, and nuclear magnetic resonance spectra as determined directly on the isolated materials. Mass spectra were obtained on a Bendix Time-of-Flight mass spectrometer Model 12-107 and the nuclear magnetic resonance spectra on a Varian Model A-60. All NMR spectra were obtained using deuterochloroform as the solvent. Structural assignments for three of these metabolites, including the predominant species, were confirmed by comparison of their spectra with those of synthetic reference materials. The characteristic mass spectral fragmentation patterns of bromacil and a series of other sub-substituted uracils, as described by Reiser (1969), have been used extensively in these direct structural identifications.

The mass spectrum of bromacil (Figure 2) shows many doublet peaks due to the natural isotopic distribution of bromine with the molecular ion (M) doublet at m/e 260 and 262. The series of fragmentation doublet peaks at M-55,
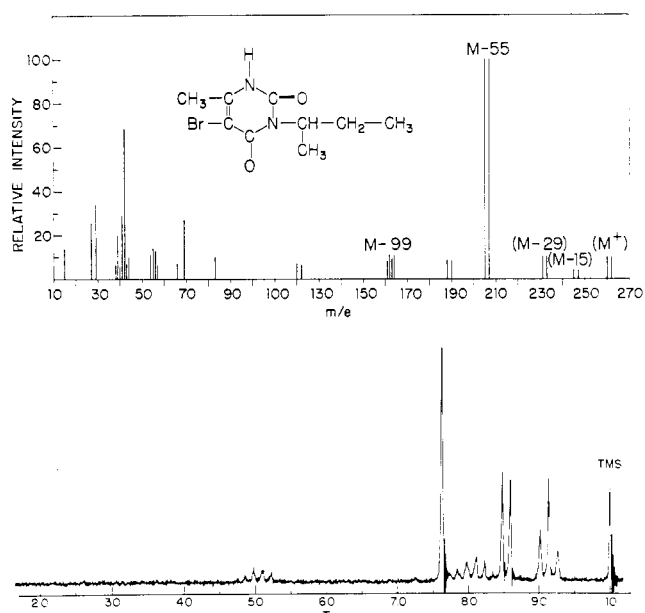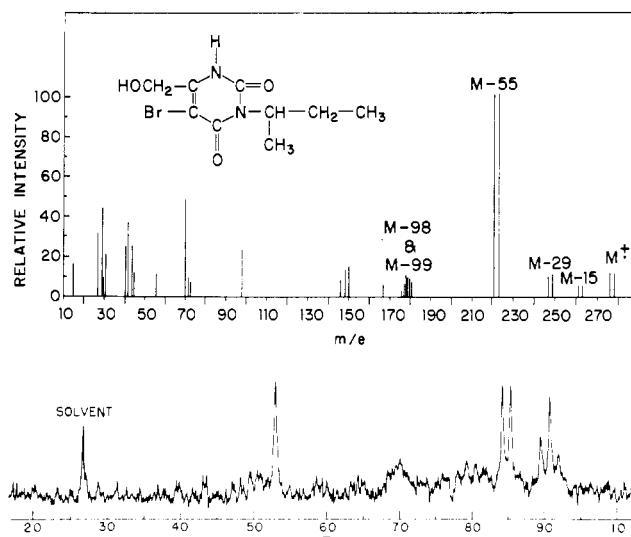

Figure 3. Spectra of metabolite I

Upper. Mass spectrum
Lower. NMR spectrum

M-29, and M-15 are characteristic of the *sec*-butyl group at the 3-position. The nuclear magnetic resonance spectrum of bromacil (Figure 2) exhibits a singlet at 7.65τ, which is characteristic of the protons of the 6-methyl substituent group; a triplet centered at 9.15τ and a doublet centered at 8.55τ characteristic of the terminal methyl protons in the *sec*-butyl group; and complex patterns at about 8.0τ and 5.0τ which

arise from the —CH₂— and the —CH protons in the *sec*-butyl

group.

The mass and nuclear magnetic resonance spectra of metabolite I are shown in Figure 3. The presence of doublets in the mass pattern shows that the metabolite retained the bromine at the 5-position. The molecular ion doublet at m/e 276 and 278 is 16 atomic mass units greater than that of bromacil, which indicates that this metabolite contains one oxygen atom more than the parent molecule. The relatively abundant fragment at M-55 plus fragments at M-15 and M-29 shows that the *sec*-butyl group has not been altered. This finding suggests that the additional oxygen atom is present in the 6-methyl substituent. The nuclear magnetic resonance spectrum of the isolated metabolite, which was obtained using tetramethylsilane as an external standard, contains a triplet centered at 9.1τ and a doublet centered at 8.5τ. The ob-

Figure 2. Spectra of bromacil

Upper. Mass spectrum
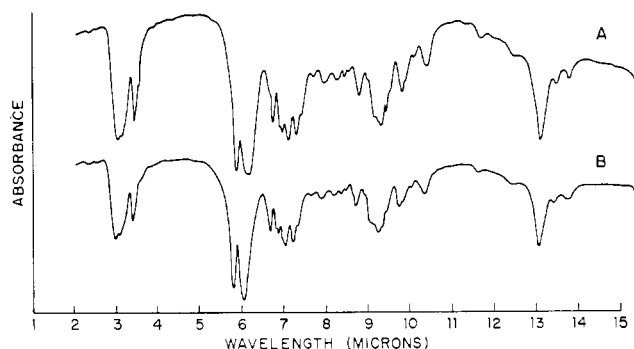*Lower.* NMR spectrum

Figure 4. Infrared spectra

A. Isolated metabolite I
B. Synthetic 5-bromo-3-*sec*-butyl-6-hydroxymethyluracil

Figure 5. Spectra of metabolite II
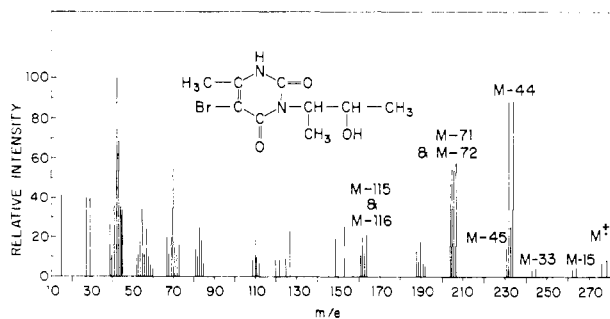
Upper. Mass spectrum
Lower. NMR spectrum

Figure 6. Spectra of synthetic 5-bromo-3-(2-hydroxy-1-methyl-propyl)-6-methyluracil

Upper. Mass spectrum
Lower. NMR spectrum

servation of these absorptions confirms the presence of the unaltered sec-butyl group in the 3-position. However, the singlet at 7.65τ that is characteristic of the 6-methyl protons in the parent molecule does not appear in the spectrum of the metabolite. Instead, a new singlet is observed at 5.3τ which integrates for 2 protons relative to 3 protons each for the doublet and triplet of the sec-butyl protons. These data are consistent with the idea that there is a hydroxymethyl group at the 6-position of the metabolite. Structural assignment as 5-bromo-3-sec-butyl-6-hydroxymethyl uracil is confirmed by direct comparison with the mass, nuclear magnetic resonance, and infrared absorption spectra of an authentic synthetic reference sample. The latter comparison is shown in Figure 4.

The mass and nuclear magnetic resonance spectra of metabolite II are shown in Figure 5. Many of the peaks in the mass pattern appear as characteristic doublets due to the natural isotopic distribution of bromine. The molecular ion, a doublet at 276 and 278 m/e, is 16 atomic mass units above bromacil, which again indicates the presence of an additional oxygen in the metabolite relative to the parent molecule. However, the lack of observation of a fragment at M-55, characteristic of the loss of the sec-butyl group, and the ob-servation of M-71 and M-72 fragments show that the extra oxygen is associated with the sec-butyl group. The observa-tion of a relatively abundant fragment of M-44 indicates that the additional oxygen is attached to the No. 2 carbon in the sec-butyl group. The NMR spectrum, by analogy with bromacil, contains a singlet at 7.6τ characteristic of the intact 6-methyl substituent. However, the triplet-doublet patterns characteristic of the sec-butyl group are not observed. The absorptions in the 8.4- to 8.9-τ region, which consist of two doublets, show that the substituent at the 3-position on the ring now contains two —CH₃ groups, each adjacent to a carbon atom bearing a single proton. This combined with the mass spectral data confirms the location of the hydroxyl group on the No. 2 carbon atom of the sec-butyl substituent. Figure 6 shows the NMR and mass spectra of a synthetic reference compound. These spectra are similar to those of metabolite
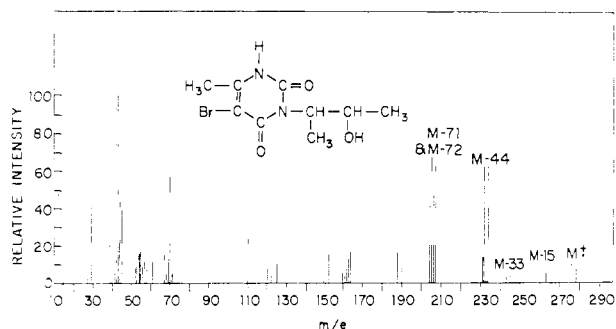
II and form the basis for the same structural assignment. However, the relative ratios of certain peaks differ slightly, which leads to the conclusion that the two compounds differ in stereochemical configuration. This results from the two asymmetric carbon atoms in the hydroxylated sec-butyl substituents. This difference in the stereochemistry of these hydroxylated sec-butyl groups arises from the random course of formation of the synthetic sample vs. the relatively stereo-specific hydroxylation that occurs in biological systems.

Reference compounds are not available for metabolites III, IV, and V; however, all spectral evidence is consistent with the assigned structures. Figure 7 shows the mass and in-frared spectra of metabolite III. The mass spectrum of metabolite III shows a molecular ion doublet at m/e 292 and 294, 32 mass units above bromacil. This increase indi-cates the addition of two oxygens to the parent molecule. The lack of observation of an M-55 fragment that is shown by bromacil itself and the presence of M-71 and M-72 fragments show that one of the oxygens is added onto the sec-butyl group. The observation of the relatively abundant M-44 fragment indicates that this oxygen is substituted onto carbon 2 in the butyl group. An NMR spectrum was obtained on this material. It shows a singlet at 5.3τ, which by analogy with the NMR spectrum of metabolite I places the other oxy-gen in a 6-hydroxymethyl group.

The mass spectrum of metabolite IV (Figure 8) shows that the isolated material has a molecular weight of 198 and con-tains no bromine. The spectrum shows peaks at M-15, M-29, and M-55 that are characteristic of the intact sec-butyl group. The compound, therefore, appears to be the uracil that is derived from bromacil by the loss of the bromine and the addition of an oxygen to the 6-methyl group. NMR data obtained on this material again indicated a 6-hydroxymethyl group in the compound.

Figure 9 shows the mass and infrared spectra of metabolite V. The molecular weight of metabolite V was found to be 16
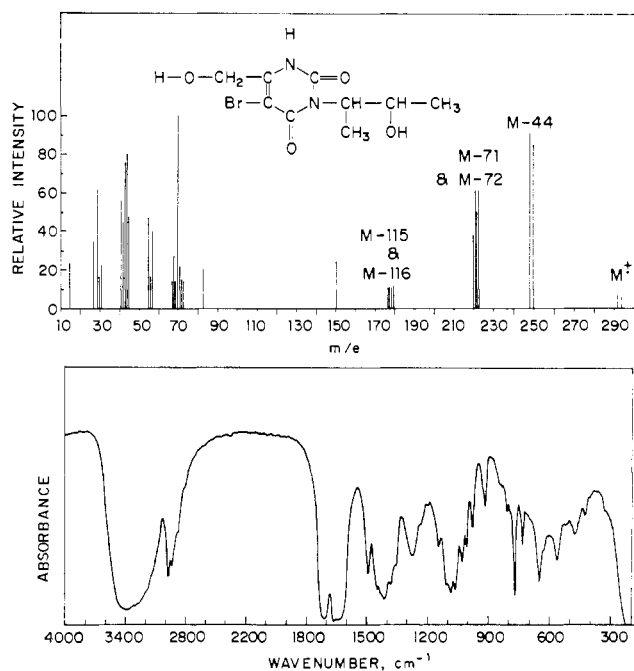
Figure 7. Spectra of metabolite III

Upper. Mass spectrum
Lower. Infrared spectrum

mass units above that of bromacil. The relatively abundant fragments at M-71 and M-72 show that oxygen is substituted on the *sec*-butyl group. The occurrence of a fragment of M-31 and a relatively less abundant fragment at M-44 (compared to that of metabolite II) indicates substitution of the oxygen on the No. 3 carbon of the butyl group. The NMR spectrum of this material could not be obtained because of the very limited amount of material isolated. The assignment
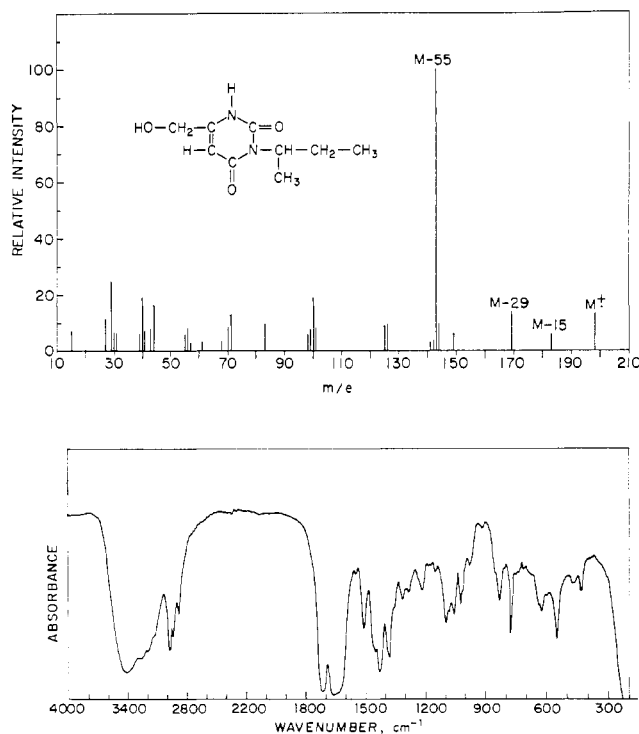

Figure 8. Spectra of metabolite IV

Upper. Mass spectrum
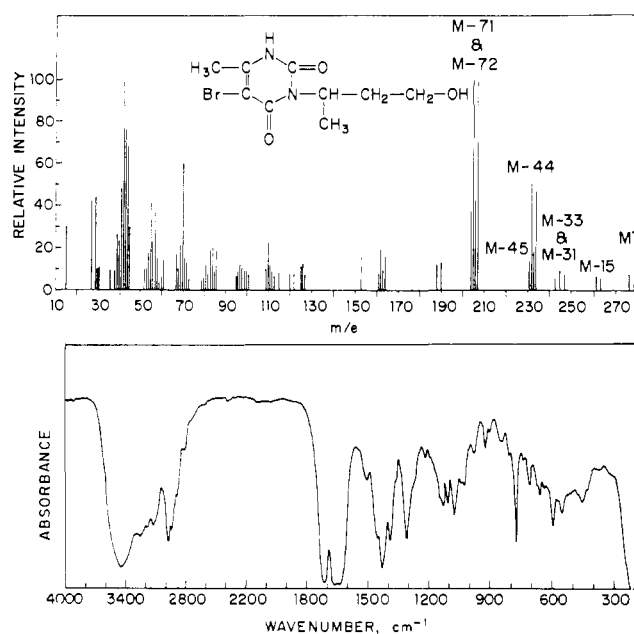*Lower.* Infrared spectrum

Figure 9. Spectra of metabolite V

Upper. Mass spectrum
Lower. Infrared spectrum

of the position of the —OH group is therefore based on an interpretation of mass spectral data alone.

The identification of metabolite VI, a very minor metabolite, was based on comparison of TLC R_f values with an authentic reference compound and was confirmed by mass spectrometry, which gave a molecular ion peak at m/e 182 and a principal fragment at m/e 127. This fragmentation is the same as that observed for the synthetic compound.

The mass spectrum of metabolite VII indicated a bromine-containing compound with molecular weight 339. Principal fragments of M-31, M-48, and M-86 were observed. This compound is unidentified. It is thought to be an addition product of an unknown conjugate and bromacil or possibly one of the bromacil metabolites.

The relative concentrations of these materials in the original rat urine were estimated by ultraviolet analysis of dilute methanol solutions of the materials isolated by TLC procedures. It was assumed that the molecular extinction coefficients of the metabolites were the same as bromacil. The results of these estimates are given in Table II.

Quantitative Examination of Urine. To obtain a more meaningful indication of the level of the urinary excretion products, samples were analyzed for bromacil and its prin-

Table II. Estimated Concentration of Bromacil Metabolites in Rat Urine

Compound	UV-λ_{max}, A	ϵ	Concn., P.P.M.
Bromacil	2770	7850	20[a]
Metabolite			
I	2780	7150	21[b]
II	2770	7850[c]	7
III and V (mixture)	2760	7850[c]	4
IV	2680	7850[c]	4
VI	2620	7850[c]	0.3
VII	2890	7850[c]	<10

[a] Analyzed by microcoulometric GC method for bromacil (Pease, 1966, 1968).
[b] Analyzed by GC.
[c] Extinction coefficient assumed same as bromacil.

Table III. Effect of Enzymatic Hydrolysis on Concentration of Bromacil and Metabolite I in Rat Urine

Sample	Enzyme Added	Enzyme Activity/Ml. of Urine[a]	Bromacil Concn., P.P.M.	Metabolite Concn., P.P.M.[b]
Treated	β-GLU/AS[c]	5000 2000 (AS)	20	146
Treated	None	—	20	21
Control	β-GLU/AS	5000 2000	N.D.[d]	N.D.

[a] Enzyme activity—β-glucuronidase activity measured in Fishman units, arylsulfatase activity measured in Whitehead units.
[b] Concentration of 5-bromo-3-sec-butyl-6-hydroxymethyluracil corrected for 80% recovery.
[c] β-glucuronidase–arylsulfatase, Boehringer and Mannheim Corp., New York.
[d] None detected.

cipal metabolite, 5-bromo-3-sec-butyl-6-hydroxymethyluracil, using selective gas chromatographic methods. Determinations were made on fresh, intact urine and a sample which had been enzymatically hydrolyzed to free possible urinary conjugates. Hydrolysis was carried out on 10-ml. samples by adjusting to pH 5 by addition of 6N hydrochloric acid. β-Glucuronidase–aryl sulfatase enzyme solution was added to the urine sample as shown in Table III and samples were incubated for 40 hours at 30°C.

Hydrolyzed and nonhydrolyzed urine samples were analyzed for bromacil according to the gas chromatographic procedure described by Pease (1968). The results (Table III) show that little, if any, bromacil is present as conjugates.

Difficulties were encountered in chromatographing metabolite I directly, and it was necessary to convert it to a silyl ether prior to determination. Ten-milliliter samples of hydrolyzed and nonhydrolyzed urine were diluted to 90 ml. with water. The solutions were acidified with 10 ml. of 10N sulfuric acid and extracted three times with 100-ml. portions of chloroform. The extracts were taken to dryness and the residues were dissolved in 25 ml. of 1N sodium hydroxide and washed with 50 ml. of n-hexane. The aqueous solutions were extracted twice with 50-ml. portions of ethyl acetate. The combined ethyl acetate extracts were washed with 10 ml. of 0.1N sulfuric acid and evaporated to dryness. The residues were dissolved in 4 ml. of chloroform, and 100 μl. of hexamethyldisilizane and 50 μl. of a 1% solution of trimethylchlorosilane in pyridine were added. The mixtures were heated under reflux for 3 hours and adjusted to 5.0 ml. with chloroform.

The resulting derivative was determined by programmed temperature gas chromatography on a Beckman GC 5 gas chromatograph equipped with a flame ionization detector. The following conditions were employed:

Column	3-foot, 1/4-inch (o.d.) glass column packed with 5% XE 60 and 0.2% Epon 1001 on 80- to 100-mesh Diatoport S
Inlet temp.	285°C.
Detector temp.	270°C.
He carrier flow	100 cc./min.

Five-microliter aliquots of the extract were injected with the column temperature at 125°C. The column temperature was held at 125°C. for 2 minutes, programmed to 180°C. at 12°C. per minute, held for 3 minutes, then programmed to 220°C. at 12°C. per minute. The retention time of the silyl ether derivative of metabolite I was 10.4 minutes.

A calibration curve was prepared by plotting peak area vs.

concentration of standard solutions. Peak areas were determined by the peak height times width at 1/2 peak height method. A recovery study at the 50-p.p.m. level showed that 80% of the added material was recovered by this procedure. The results of these analyses, corrected for 80% recovery, and listed in Table III, show that a large fraction of metabolite I is present in the form of urinary conjugates.

Enzymatically hydrolyzed samples of urine were also qualitatively examined by the previously described thin-layer chromatographic techniques and were examined visually. As expected from the preceding gas chromatographic results, the amount of metabolite I seen on the TLC plates was much greater than was detected in the unhydrolyzed samples. No new metabolites were detected. However, the hydrolysis step appeared to have increased the concentration of the minor metabolites of bromacil by perhaps as much as a factor of 2 as contrasted with a factor of about 7 for metabolite I. Thus, some evidence of conjugation of the other identified metabolites was seen, but analytical methods are not available for the accurate determination of their concentration before and after enzyme hydrolysis to liberate the conjugates. Therefore, this information is presented only to indicate that conjugates of the minor metabolites in the urine do exist and to indicate that apparently conjugation is relatively more important with metabolite I than with the minor metabolites.

Examination of Urine for 5-Bromouracil. Because of the implication of 5-bromouracil as a suspect mutagen (Freese, 1959; McGahen and Hoffman, 1966), a selective gas chromatographic method for this compound was developed and applied to treated urine, even though there was no qualitative evidence of its presence as a metabolite.

Ten-milliliter samples of rat urine (treated, control, and control with 5 p.p.m. of added 5-bromouracil) were diluted to 300 ml. with 0.1N sulfuric acid. These solutions were extracted continuously with diethyl ether for 30 hours. The ether extracts were evaporated to dryness and the residues were dissolved in 1 ml. of methanol.

The residues were streaked on a TLC plate next to 5-bromouracil reference spots and the plates developed twice to 10 cm. in ethyl acetate. The areas of absorbent corresponding to the R_f values of 5-bromouracil were removed from the plates and eluted with methanol. The eluates were taken to dryness under a hood. The residues were dissolved in 1 ml. of methanol and treated with 10 ml. of diethyl ether containing a large excess of diazomethane. The resulting solutions were evaporated to dryness and the residues dissolved in 1.0 ml. of ethyl acetate. One-microliter aliquots of the methylated extracts were analyzed on a MicroTek 2000R gas chromatograph equipped with an electron-capture detector and a 4-foot glass column containing 5% XE 60 and 0.2% Epon 1001 on 80- to 100-mesh Diatoport S. The following conditions were employed:

Column temp.	170°C.
Inlet temp.	210°C.
Detector temp.	190°C.
N₂ carrier flow	200 cc./min.

The retention time of methylated 5-bromouracil was 3.1 minutes.

Analysis of the urine of rats on the bromacil-containing diet showed that 5-bromouracil was not present in the urine. The limit of detection of this over-all procedure was estimated as 2 p.p.m., based on the response of the urine sample containing 5 p.p.m. of added 5-bromouracil.

Synthesis of Reference Compounds. Metabolite I, 5-bromo-3-sec-butyl-6-hydroxymethyluracil, was prepared by a three-

step synthesis proceeding through the following intermediates:

5-BROMO-3-sec-BUTYL-6-BROMOMETHYLURACIL. In a 500-ml. three-necked, round-bottomed flask equipped with a mechanical stirrer, an efficient condenser, and an adding funnel were placed 26.1 grams (0.10 mole) of 5-bromo-3-sec-butyl-6-methyluracil and 150 ml. of water. While the mixture was stirred at room temperature, 16.0 grams (0.10 mole) of bromine was added dropwise. After 15 minutes more stirring, 40 ml. of hydrobromic acid was added and the mixture heated under reflux for 2 hours.

The mixture was chilled, and the pale yellow solids were collected on a filter, washed with water, and dried. After two recrystallizations from nitromethane, 17.4 grams of white solid was obtained (m.p. 183.5–84.5°C.).

Calculated for $C_9H_{12}Br_2N_2O_2$: C, 31.2; H, 3.6; N, 8.2. Found: C, 32.8; H, 3.7; N, 8.4.

5-BROMO-3-sec-BUTYL-6-ACETOXYMETHYLURACIL. A mixture of 17.0 grams (0.050 mole) of 5-bromo-3-sec-butyl-6-bromomethyluracil and 4.1 grams (0.050 mole) of anhydrous sodium acetate in 50 ml. of glacial acetic acid was heated under reflux for 1 hour. A clear solution was obtained at first, followed by precipitation of a white solid after 10 minutes.

The solids were filtered off and discarded. The filtrate was poured into 250 ml. of cold water, and the excess acetic acid partially neutralized with 50% aqueous sodium hydroxide. The mixture was extracted twice with 250-ml. portions of methylene chloride. The combined methylene chloride extracts were washed with water and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure on the steam bath and finally at 0.2-mm. pressure at 30° to 40°C. for 2 hours. The residue was a very viscous light yellow oil which weighed 16.3 grams.

5-BROMO-3-sec-BUTYL-6-HYDROXYMETHYLURACIL (METABOLITE I). The product obtained as described above from the reaction of 5-bromo-3-sec-butyl-6-bromomethyluracil and sodium acetate was dissolved in 100 ml. of methanol and treated with 2.5 grams (0.050 mole) of hydrazine hydrate at room temperature for 3 days.

The solvent was evaporated at room temperature or below in a current of nitrogen. The yellow oily residue was taken up in 300 ml. of methylene chloride and washed twice with 200 ml. of water. The methylene chloride solution was dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure on a water bath to obtain 11.6 grams of yellow glass.

PURIFICATION OF 5-BROMO-3-sec-BUTYL-6-HYDROXYMETHYLURACIL (METABOLITE I). A chromatographic column was prepared by slurrying 85 grams of silicic acid (100-mesh, analytical reagent, Mallinckrodt) in reagent chloroform, and pouring it on a 25 × 350 mm. column. When the adsorbent had settled, a nearly transparent column was obtained.

To this column was added a solution of 7.2 grams of crude 5-bromo-3-sec-butyl-6-hydroxymethyluracil in 25 ml. of chloroform. As the column developed, two bands were visible as opaque zones on it. The first narrow band together with the clear zone that separated it from the main band was eluted and discarded. The main band was collected as it was eluted from the column. The solvent was evaporated under reduced pressure on the water bath and the residue dried in vacuo overnight. The residue was taken up in methanol, treated with Darco, filtered, and evaporated. The residue consisted of 2.5 grams of yellow glass.

Calculated for $C_9H_{13}BrN_2O_3$: C, 39.1; H, 4.7; Br, 28.8; N, 10.1. Found: C, 39.7; H, 5.2; Br, 27.2; N, 10.1.

Metabolite II, 5-bromo-3-(2-hydroxy-1-methylpropyl)-6-methyluracil, was prepared by a three-step synthesis proceeding through the following intermediates:

1-(2-HYDROXY-1-METHYLPROPYL)UREA. In a 1-liter, four-necked, round-bottomed flask equipped with mechanical stirrer, thermometer, and condenser were placed 103 grams (1.15 mole) of 3-amino-2-butanol, of unknown configuration, and 350 ml. of water. This solution was cooled to −5°C., and 114 grams (1.15 mole) of concentrated HCl was added. The temperature rose to 20°C. The solution was again cooled to −5°C. and 117 grams (1.44 mole) of potassium cyanate was added. The reaction was stirred at −5°C. for 30 minutes, heated on a steam bath with stirring for 30 minutes, then poured into a 2-liter beaker of ice. The resulting solution was reduced to a mixture of solid and oil under vacuum. This mixture was stirred with ethanol at 20°C., the ethanol layer separated by filtration, and the ethanol removed under reduced pressure; 94 grams of a yellow oil remained.

Calculated for $C_5H_{12}N_2O_2$: C, 45.22; H, 9.15; N, 21.20. Found: C, 44.56; H, 9.38; N, 20.04.

3-(2-HYDROXY-1-METHYLPROPYL)-6-METHYLURACIL. A mixture of 66 grams (0.5 mole) of 1-(2-hydroxy-1-methylpropyl)urea, 65 grams (0.5 mole) of ethyl acetoacetate, 2 grams of p-toluenesulfonic acid, and 500 ml. of benzene was refluxed for 4 hours under Dean and Stark conditions. Only 2 ml. of water was collected, so 2 grams more of p-toluenesulfonic acid was added and the reaction refluxed all night. A total of 6 ml. of water was collected. The reaction was cooled to room temperature, 32.4 grams (0.6 mole) of sodium methoxide in a 30% methanol solution was added, followed by a 1-hour reflux, then finally 250 ml. of water was added. The water layer was separated, and the organic layer was extracted with two more portions of water. The water layers were combined, acidified with concentrated HCl, and extracted with 3 portions of methylene chloride. The methylene chloride layers were dried over anhydrous magnesium sulfate and reduced to 5 grams of a semisolid under vacuum. Treatment of the semisolid with acetonitrile yielded a solid which was recrystallized from acetonitrile; 2 grams collected (m.p. 181–83°C.).

Calculated for $C_9H_{14}N_2O_3$: C, 54.53; H, 7.12; N, 14.13. Found: C, 55.07; H, 6.70; N, 14.05.

An additional 1.5 grams of product was obtained by extracting the acidified water layer with 2-butanol, and then removing the 2-butanol under reduced pressure to yield a heavy oil. On standing, the product precipitated from this oil.

5 - BROMO - 3 - (2 - HYDROXY - 1 - METHYLPROPYL) - 6-METHYLURACIL (METABOLITE II). To a solution of 1.5 grams (0.0076 mole) of 3-(2-hydroxy-1-methylpropyl)-6-methyluracil, 0.62 gram (0.0076 mole) of sodium acetate, and 10 ml. of glacial acetic acid was added 1.21 grams (0.0076 mole) of bromine. A precipitate formed. The reaction mixture was poured into ice water. The resulting solution was extracted with 3 portions of 1-butanol, and the solvent taken off under reduced pressure, leaving a solid. The solid was recrystallized from nitromethane: 1.5 grams (m.p. 187–88°C.).

Calculated for $C_9H_{13}BrN_2O_3$: C, 39.05; H, 4.73; Br, 28.82; N, 10.11. Found: C, 39.61; H, 5.00; Br, 28.79; N, 9.89.

Metabolite VI, 3-sec-butyl-6-methyluracil, was prepared by a two-step synthesis as follows:

3-(3-sec-BUTYLUREIDO) CROTONIC ACID, ETHYL ESTER. In a 1-liter three-necked round-bottomed flask equipped with a stirrer, reflux condenser, Dean and Stark trap, and an addi-

tion funnel, was refluxed 500 ml. of benzene containing 58 grams (0.4 mole) of *sec*-butylurea and 1 gram of 85% phosphoric acid. To this mixture was added, in a steady stream, 40 grams (0.3 mole) of ethylacetoacetate. After refluxing overnight, during which time the water produced in the reaction was collected in the trap, the mixture was decanted from the excess urea and the benzene solution was stripped in vacuo.

3-*sec*-BUTYL-6-METHYLURACIL (METABOLITE VI). The product was added, using a small amount of ethanol to facilitate transfer, to a solution of 18 grams (0.32 mole) of sodium methylate in 300 ml. of absolute ethanol. The solution was refluxed for one-half hour, and stripped in vacuo, and the residue was dissolved in 300 ml. of water. The aqueous solution was washed with 2 parts of 100-ml. ethyl ether, adjusted to pH 4 to 5 by the addition of 10% hydrochloric acid, and extracted with 2 parts of 200-ml. ethyl ether. After drying the ether solution over magnesium sulfate, filtering, and stripping off the solvent in vacuo, the crude product was obtained as a solid. Recrystallization from ethanol-water gave 23 grams (m.p. 117.5–20°) and a second crop of 15 grams [m.p. 112–15° (70% of theory)]. A small sample was purified further to m.p. 119–20° by dissolving in aqueous base and precipitating with hydrochloric acid.

Calculated for $C_9H_{13}N_2O_2$: C, 59.7; H, 7.20. Found: C, 58.9; H, 7.66.

DISCUSSION

These studies show that a major metabolite of bromacil in the urine of rats maintained on a diet containing 1250 p.p.m. of bromacil is 5-bromo-3-*sec*-butyl-6-hydroxymethyluracil, and that the metabolite is predominantly in the form of a conjugate in the urine. The concentration of the metabolite in the nonhydrolyzed urine was 21 p.p.m. and was increased to 146 p.p.m. by hydrolysis of the urine with β-glucuronidase–

aryl sulfatase preparation. Five other metabolites have also been isolated and identified; their concentrations were estimated to range from 0.3 to 7 p.p.m.

All metabolites of bromacil were formed by oxidation of the 6-methyl group, oxidation of the 3-*sec*-butyl group, debromination of the 5-bromo group, and combinations of these (Figure 1). Conclusions cannot be drawn on the order in which the metabolism occurs to form the minor secondary metabolites of bromacil. Similar hydroxylation of alkyl-substituted pyrimidine derivatives is reported in the literature—i.e., thymine is converted to 5-hydroxymethyluracil, and 5-*n*-butyl-5-ethylbarbituric acid is converted to 5-ethyl-5-(3′-hydroxybutyl)barbituric acid (Williams, 1959). Specific analysis of the rat urine did not detect 5-bromouracil as a metabolite of bromacil in rats.

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