Pauli, O.; Frank, G. "Proceedings of the 2nd International Symposium on Biodeterioration of Materials"; Walters, A. H., Ed.; Applied Sciences Publishers, Ltd.: Barking, England, 1972; p 52.
Shefer, W.; Ammann, C. *Textilveredlung* **1974**, *9*, 107.
Simmons, P. B.; Branson, D. R.; Moolenaar, R. J.; Bailey, R. E. *Am. Dyest. Rep.* **1977**, *Aug*, 21.
Snow, C. M.; Chairman. *J. Am. Oil Chem. Soc.* **1965**, *42*, 986.
Spain, J. C.; Pritchard, P. H.; Bourquin, A. W. *Appl. Environ. Microbiol.* **1980**, *40* (4), 726.
Voets, J. P.; Pipyn, P.; VanLancker, P.; Verstrate, W. *J. Appl. Bacteriol.* **1976**, *40*, 67.

Received for review June 13, 1983. Accepted January 19, 1984.

Species and Strain Comparison of the Metabolism of Gentian Violet by Liver Microsomes

Jerome J. McDonald,* Cloyd R. Breeden, Betty M. North, and Robert W. Roth

Comparative metabolism of hexamethylpararosaniline chloride, a poultry feed additive known as gentian violet, was examined in vitro in the presence of uninduced liver microsomes prepared from both sexes of four mouse strains, three rat strains, hamster, guinea pig, and chicken. Metabolites, isolated by solvent extraction and HPLC, were identified by comparison of reverse-phase HPLC retention times and electron impact mass spectral fragmentation patterns with those of standards prepared by unambiguous synthetic routes. The major metabolites were pentamethylpararosaniline and the isomeric *N,N,N',N'*- and *N,N,N',N''*-tetramethylpararosanilines. The pattern of demethylated metabolites was found to be comparable among the species with little difference between the sexes. The extent of demethylation was least with mouse microsomes, and formation of *N,N,N',N'*-tetramethylpararosaniline by uninduced guinea pig microsomes was barely detectable.

Gentian violet (**1a**, Figure 1) is an intensely colored, basic triphenylmethane dye that has had direct human application both topically as an antifungal agent and internally as an antihelminthic agent. Although still used, it has largely been superseded by more effective disinfectants. Its principal use now resides in agriculture, particularly as a mycostatic agent in poultry feeds. Reports of its genotoxic potential, reviewed by Combes and Haveland-Smith (1982), have led to some concern over residues that may be found in edible tissues of treated animals.

Little is known about the metabolic transformation of triphenylmethane dyes. Some recent work has measured the biliary excretion and tissue distribution of the acidic dyes Guinea Green B (Minegishi and Yamaha, 1974), Benzyl Violet 4B (Minegishi and Yamaha, 1977), and Brilliant Blue FCF (Phillips et al., 1980). Taylor (1977) applied a colorimetric method to determine total residue of gentian violet in chick tissues. None of these studies was designed to detect and identify metabolites derived from the dyes.

The work described here is a first step in elucidating the metabolism of a major group of dyes and describes the synthesis and isolation of in vitro metabolites of gentian violet. In addition, comparisons have been made among several rodent species to observe how closely mammalian and avian metabolism are related in this case.

EXPERIMENTAL SECTION

Materials and Synthetic Procedures. Chemicals and solvents of standard reagent and HPLC grades were obtained from commercial suppliers. Gentian violet (hexamethylpararosaniline chloride) was obtained as crystal violet from Aldrich Chemical Co. and contained >98% of a single component absorbing at 546 nm, as determined by HPLC.

Pentamethylpararosaniline Chloride, **1b**. The preparation of the pentamethyl homologue from **2a** (2.67 g, 10.3 mmol), *n*-butyllithium (2.2 M hexane solution, 6.3 mL, 13.9 mmol) and 4,4'-bis(dimethylamino)benzophenone (2.5 g, 9.3 mmol) was carried out essentially as described for the tetramethyl homologues: yield, 500 mg (16%) of **1b** as a dark green powder; purity >98% (HPLC, 300-nm detection); UV_{max} (98% CH_3CO_2H) 578 nm (ϵ 100 000); 60-MHz NMR (CD_3OD) δ 3.0 (s, 3, $NHCH_3$), 3.2 [s, 12, $N(CH_3)_2$], 6.67–7.25 (m, 12, aromatic).

N,N,N',N'-Tetramethylpararosaniline Chloride, **1c**. Lithiation of *N,N*-bis(trimethylsilyl)-4-bromoaniline (1.07 g, 3.4 mmol), **2b** (Broser and Harrer, 1965), in ether was accomplished by addition of an excess of 2.2 M *n*-butyllithium (2.1 mL, 4.5 mmol) in hexane while maintaining the reaction temperature at 25 °C. After 10 min, 4,4'-bis(dimethylamino)benzophenone (0.89 g, 3.4 mmol) was added and the reaction mixture stirred for 3 h at room temperature. After the solvents were removed in vacuo, the residue was dissolved in 1 M NaOH and the dye base was extracted into ether. The ether solution was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The residue was treated with 0.06 M HCl on a steam bath for 2 h. The resulting dye was precipitated by addition of NaCl to the cooled solution. The material was further purified by dissolving the precipitate in absolute ethanol followed by filtration. The dye (**1c**) was reprecipitated as a deep blue-green powder in 19% yield by addition of ether. Purity, as determined by HPLC with peak integration at 300 nm, was >98%: UV_{max} (98% CH_3CO_2H) 576 nm (ϵ 72 300); 60-MHz NMR (CD_3OD) δ 3.25 (s, 12, CH_3), 6.75–7.5 (m, 12, aromatic).

N,N,N',N''-Tetramethylpararosaniline Chloride, **1d**. A mixture of *N*-methyl-4-bromoaniline (7.00 g, 37.6 mmol) and oil-free sodium hydride (1.35 g, 56.5 mmol) in 50 mL of tetrahydrofuran was warmed under argon until hydrogen evolution ceased. Chlorotrimethylsilane (7.2 mL, 56

Division of Carcinogenesis Research, National Center for Toxicological Research, Jefferson, Arkansas 72079 (J.M., C.B., and B.N.), and Department of Bioorganic Chemistry, Midwest Research Institute, Kansas City, Missouri 64110 (R.R.).

0021-8561/84/1432-0596$01.50/0

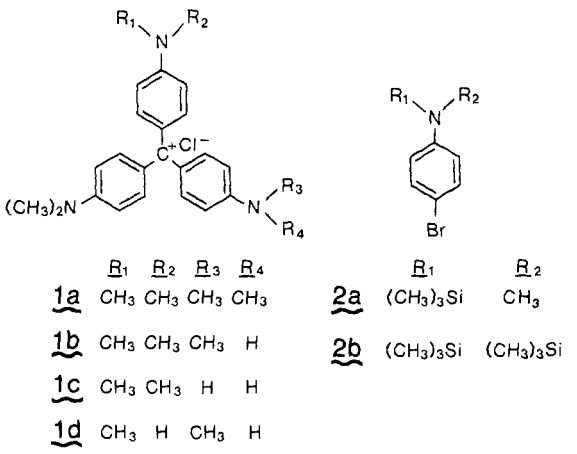

Figure 1. Structures of substituted pararosanilines and blocked reagents.

mmol) was added and the mixture refluxed for 3.5 h. Inorganics were removed by centrifugation, and the supernatant was distilled to give 5.6 g (58%) of *N*-methyl-*N*-trimethylsilyl-4-bromoaniline, **2a**, bp 80–85 °C (0.8 mmHg): 60-MHz NMR ($CDCl_3$) δ 0.15 (s, 9, trimethylsilyl), 2.75 (s, 3, CH_3), 6.65 (d, 2, C_2H and C_6H), 7.18 (d, 2, C_3H and C_5H).

A solution of **2a** (2.74 g, 10.6 mmol) in 10 mL of ether under argon was lithiated by slow addition of 2.2 M *n*-butyllithium in hexane (6.5 mL, 14.3 mmol) followed by stirring for 6 h. A solution of ethyl 4-(dimethylamino)-benzoate (0.82 g, 4.2 mmol) in 25 mL of ether was added dropwise and stirring continued for 48 h. The dye base was extracted and treated with HCl as described above to yield 550 mg (34%) of **1d** in 91% purity after additional precipitation from ethanol–ether: UV_{max} (98% CH_3CO_2H), 578 nm; 60-MHz NMR (CD_3OD) δ 2.95 (s, 6, CH_3), 3.2 (s, 6, CH_3), 6.7–7.4 (m, 12, aromatic).

Animals. Both sexes of Fisher 344, Sprague-Dawley, and CD rats and $B6C3F_1$, C3H/MTV-, BALB/c, and C57BL/6N mice were obtained from colonies maintained at the NCTR. Syrian golden hamsters and Hartley guinea pigs were purchased from Charles River Laboratories, Wilmington, MA, and held in quarantine until use. Rock Cornish chickens were obtained from USDA Laboratories, Fargo, ND. Mice were 11–14 weeks old, other rodents were 14–16 weeks old, and chickens were 9 weeks old at sacrifice.

Microsomes. Rodents were fasted overnight. Following decapitation, the livers were excised, weighed, minced under 3 volumes of ice-cold 0.25 M sucrose/5 mM Tris-HCl (pH 8.0), and homogenized by three passes in a Teflon–glass homogenizer. After centrifugation for 20 min at 10000*g*, the supernatant was sedimented at 100000*g* for 60 min. The microsomal pellet was washed twice by resuspension in sucrose/Tris buffer followed by centrifugation. The washed microsomes were finally suspended in 40 mM potassium phosphate (pH 7.4) containing 20% glycerol. The protein concentration was determined by the method of Lowry et al. (1951) and adjusted to 20–23 mg/mL, and the suspension was used immediately or frozen at −70 °C for a maximum of 7 days before use. Preparations of microsomes from female Sprague-Dawley rats were tested for demethylase activity immediately after preparation and after 3 and 18 days of storage in phosphate/glycerol at −70 °C. No change in activity was observed.

Gentian Violet Demethylase Activity. An NADPH/NADH-regenerating system containing 0.4 μmol of NADP, 0.3 μmol of NAD, 5.0 μmol of glucose 6-phosphate, 5.0 μmol of EDTA, and 2 units of glucose-6-phosphate dehydrogenase (Sigma Type XXI) in 0.8 mL of 0.125 M phosphate buffer (pH 7.8) was placed in a shaking incubator for 2 min at 37 °C. Dye substrate (0.1 mL in water) and the washed microsomal suspension (0.1 mL) were then added, and the incubation was continued for 30 min. The reaction was quenched with 0.3 mL of acetone and the microsomes were pelleted by centrifugation. The decanted supernatant was extracted with 1-butanol and the extract analyzed by HPLC.

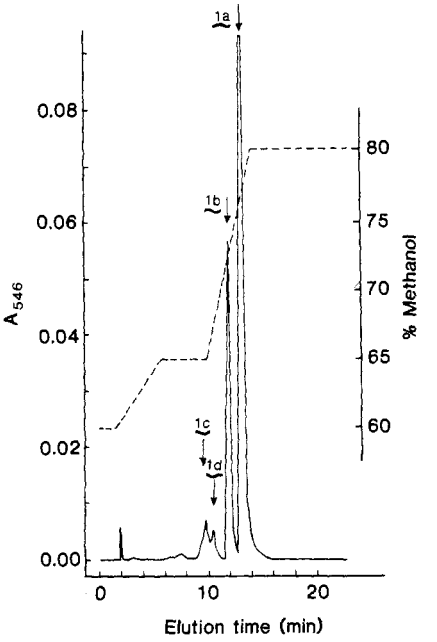

Figure 2. HPLC elution gradient and product mixture from demethylation of gentian violet by female CD rat liver microsomes.

One demethylation control consisted of incubation of the substrate in the complete system without microsomes. A second control consisted of the complete system with substrate and heat-inactivated microsomes.

Determinations were performed on microsomes prepared from three animals of each sex of the rats, hamsters, and guinea pigs. Duplicate determinations were performed on microsomes prepared from three pooled livers of each sex of the mice.

For determination of the optimum incubation period, the mixture of cofactors and additions were increased to 5.0 mL and 0.5-mL aliquots were removed at intervals, quenched with acetone, and analyzed.

To provide sufficient material for mass spectral analysis of metabolites, the incubation mixture was increased to 50 mL and the incubation period was extended to 60 min. After quenching with 13 mL of acetone and centrifugation, the supernatant was extracted with 1-butanol/hexane (4/1). The extract was evaporated and the residue was redissolved in methanol for preparative HPLC.

Chromatographic Analysis. The methanol/10 mM phosphate buffer (pH 3.0) solvent system with a 3.9 mm i.d. × 30 cm Waters Associates μBondapak C_{18} reverse-phase column (Rushing and Bowman, 1980) was used with a Waters 440 dual-wavelength detector and an Altex microprocessor-controlled pump system to generate a gradient capable of resolving the tetramethyl isomers. For most of the analyses, the following gradient (Figure 2) at an elution rate of 1 mL/min was used: elution was begun at 60% CH_3OH in buffer; after 4 min, a concave gradient increased CH_3OH to 64.8% over 4 min; elution continued for 4 min at 64.8% when CH_3OH was increased to 80% over 4 min and held there until elution of components was complete.

For preparative chromatography, fractions were collected from a 7.8 mm i.d. × 30 cm μBondapak C_{18} column

Table I. Electron Impact Mass Spectra of Cationic Standards and Metabolites

sample	cation M_r	base peak	other significant ions (% rel intensity)
1a, standard	372	373 (M + 1)	374 (24), 372 (51), 358 (3), 356 (12), 253 (67), 252 (33), 237 (23), 186 (8)
metabolite		372	374 (15), 373 (66), 357 (22), 356 (38), 253 (54), 252 (31), 239 (13), 237 (23), 186 (51)
1b, standard	358	356 (M − 2)	359 (70), 358 (65), 357 (92), 342 (9), 340 (12), 253 (32), 252 (10), 239 (43), 238 (26), 237 (18), 223 (10), 178 (44)
metabolite		356	359 (61), 358 (66), 357 (90), 342 (13), 340 (11), 253 (23), 252 (8), 239 (43), 238 (26), 237 (14), 223 (18), 179 (37), 178 (12)
1c, standard	344	343 (M − 1)	346 (16), 345 (91), 344 (88), 328 (18), 253 (62), 239 (8), 237 (21), 225 (49), 224 (35), 223 (14), 171 (56)
metabolite		345	346 (16), 344 (64), 343 (55), 328 (14), 253 (48), 239 (10), 237 (19), 225 (57), 224 (38), 223 (19), 172 (19)
1d, standard	344	342 (M − 2)	346 (9), 345 (39), 344 (37), 343 (93), 328 (16), 239 (24), 238 (10), 225 (10), 224 (10), 223 (10), 171 (16)
metabolite		342	345 (59), 344 (55), 343 (92), 328 (7), 239 (46), 238 (14), 237 (16), 225 (17), 224 (17), 223 (26), 172 (35)

eluted with 80% methanol/20% buffer. Peaks were concentrated in vacuo to remove CH_3OH and were extracted with 4/1 1-butanol/hexane. The fraction containing the tetramethyl isomers was rechromatographed with a higher buffer concentration to separate the two. Butanol extracts were dried and redissolved in CH_3OH for electron impact mass spectrometry (EIMS).

Instrumentation. Mass spectra were obtained on the Finnigan 4023 mass spectrometer operated in the electron impact mode via the solid probe inlet at 70 eV. The source temperature was 270 °C and the probe temperature was programmed to rise from 35 to 240 °C. Standards and isolated metabolites were applied to the probe in CH_3OH solution. Solvent was driven off under a stream of nitrogen at room temperature. UV/vis spectra were recorded on a Varian Superscan 3 instrument and NMR spectra on a Varian EM 306A spectrometer.

RESULTS AND DISCUSSION

Metabolites of **1a** were identified by comparison with synthetic standards by means of retention times from HPLC and by fragmentation patterns from EIMS. In an adaptation of a literature method (Grocock et al., 1973), authentic standards of **1b–d** (Figure 1) were prepared by reaction of aryllithium reagents derived from *N*-trimethylsilyl-protected (Brosser and Harrer, 1965) 4-bromoanilines (**2a,b**) with either 4,4′-bis(dimethylamino)benzophenone (Michler's ketone) or ethyl 4-(dimethylamino)benzoate. The tetramethyl isomer **1d** proved refractory to complete purification by either recrystallization or HPLC but was of sufficient purity for use as an unambiguous marker compound. The UV/vis and NMR spectra of **1b–d** were consistent with those of assigned structures.

Elution of synthetic standards from a reverse-phase column in a system adapted from Rushing and Bowman (1980) proceeded with retention time related directly to the degree of amino group substitution. The position of elution of standards is indicated by the arrows in Figure 2, which illustrates the separation of in vitro metabolites from a microsomal incubation.

McEwen et al. (1977) have described the EIMS of commercial **1a** as having a base peak at m/e 373. They attribute this M + 1 ion to thermal reduction of the cation on the probe. Among the standard compounds in our study, the M + 1 ion appears as the base peak only for the fully substituted **1a** while the standards for the demethylated congeners display base peaks for ions in varying stages of dehydrogenation (Table I). However, M + 1 ions at m/e 359 and 345 are prominent in the spectra for **1b** and **1c** and **1d**, respectively. The characteristic clustering of ions from M − 2 to M + 2 limits the effectiveness of the molecular ion in establishing the true molecular weight for this series of dyes. The fragmentation patterns are revealing, however. Strong ions are observed at m/e 253 for **1a**, at m/e 239 for **1b**, and at m/e 225 for **1c**, representing the loss of a dimethylanilino fragment, $C_6H_4N(CH_3)_2$, of mass 120 from the M + 1 ion. The appearance of fragment ions of progressively lighter masses, which reflects the clustering of masses near the molecular ion and the fragment ion, is also observed in the mass spectrum of **1d**, but in this case, the loss of a monomethylanilino fragment, $C_6H_4NHCH_3$, of mass 106 from the M + 1 ion is the more abundant process, leading to the ion at m/e 239. Loss of the anilino moiety of mass 92 is evident in the prominant ion at m/e 253 formed during the fragmentation of **1c**. Thus, the major mode of fragmentation is the loss of an entire ring with substituent.

The fragmentation patterns of metabolites **1a–1d** exhibit a high degree of congruency with those of the standards with regard to the relative abundance of ions from loss of ring moieties and to the clustering of ions around the molecular ion and fragments (Table I). For example, the ratio of abundances 225/239 is 5.7 and 6.1, respectively, for the metabolite and standard of the tetramethyl congener **1c**. The values of the same ratio for the other isomer **1d** drop to 0.38 and 0.42, indicative of the preferred loss of mass 106 for this isomer.

However, the base peaks for metabolites **1a** and **1c** are not the same as those for their standards, although the same ions are present in the cluster. The differences in detail of the cluster around the molecular ion are probably due to the matrix from which the sample is vaporized. Synthetic standards prepared as chloride salts were applied to the probe in methanol solution. The metabolites were subjected to several chromatographies in acidic phosphate buffer and were undoubtedly isolated as phosphate salts. Trial extractions of standards in phosphate buffer with butanol/hexane did not duplicate metabolite spectra in detail but showed sensitivity to thoroughness of drying to remove extraction solvents.

Demethylation of gentian violet was observed with all microsomal preparations (Table II). The major component in the incubation mixture was the substrate, **1a**, followed by the pentamethyl homologue **1b**. The two tetramethyl homologues, **1c** and **1d**, were well resolved by HPLC using gradient elution. Incomplete resolution was obtained in earlier work using an isocratic system for analysis of product mixtures from microsomes of chicken and three mouse strains (Table II). Nevertheless, the HPLC profile from these preparations, when compared to the standards, indicate that comparable amounts of **1c** and

Table II. Demethylation of 0.1 mM Gentian Violet by Liver Microsomes

animal	sex	% composition of metabolite mixture				
		1a	1b	1c	1d	1c + 1d
mouse[a]						
B6C3F$_1$	M	83.2 ± 0.5	13.5 ± 0.5			3.4 ± 0.9[d]
	F	81.9 ± 0.1	16.8 ± 0.1			1.3 ± 0.1[d]
C3H/MTV−	M	77.9 ± 1.8	18.1 ± 2.5			4.0 ± 0.6[d]
	F	80.6 ± 1.5	16.6 ± 0.3			2.8 ± 0.2[d]
BALB/c	M	75.8 ± 0.2	17.2 ± 0.1			7.0 ± 0.2[d]
	F	77.6 ± 0.1	16.3 ± 0.2			6.0 ± 0.1[d]
C57BL/6N	M	74.3 ± 0.1	18.2 ± 0.4	3.1 ± 0.2	4.4 ± 0.3	7.5
	F	71.8 ± 0.8	20.2 ± 0.4	2.5 ± 0.5	5.4 ± 0.2	7.9
rat[b]						
Sprague-Dawley	M	64.2 ± 0.9	22.4 ± 0.3	8.9 ± 0.8	4.4 ± 0.1	13.3
	F	61.9 ± 2.3	24.2 ± 1.7	9.5 ± 1.3	4.5 ± 0.8	14.0
CD	M	57.5 ± 3.5	24.8 ± 0.9	10.2 ± 2.1	7.4 ± 0.7	17.6
	F	65.7 ± 3.4	22.2 ± 1.7	7.2 ± 1.3	5.0 ± 1.3	12.2
Fischer 344	M	54.6 ± 2.0	26.0 ± 0.6	9.4 ± 0.6	10.1 ± 1.1	19.5
	F	60.2 ± 4.1	24.2 ± 1.4	8.4 ± 0.4	7.2 ± 0.5	15.6
hamster[b]	M	67.5 ± 1.8	19.6 ± 1.1	2.6 ± 1.1	10.3 ± 0.9	12.9
	F	71.0 ± 1.2	17.5 ± 0.5	3.4 ± 1.0	8.0 ± 0.6	11.4
guinea pig[b]	M	61.1 ± 0.5	23.6 ± 0.2	0.2 ± 0.2	15.2 ± 0.1	15.4
	F	61.7 ± 0.3	23.8 ± 1.0	0.0 ± 0.0	14.5 ± 1.2	14.5
chicken[c]	M	59.8 ± 5.9	29.1 ± 5.6			11.1 ± 2.9[d]

[a] Entries represent average ± range for duplicate determinations on pooled livers. [b] Mean ± standard deviation for three animals. [c] Mean ± standard deviation for four animals. [d] Incomplete resolution from use of an isocratic elution system.

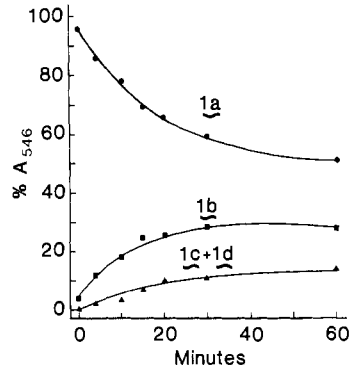

Figure 3. Kinetics of demethylation of gentian violet by female CD rat liver microsomes.

1d were present. A standard incubation time of 30 min was chosen as a result of the kinetic analysis graphically presented in Figure 3.

Comparisons of the demethylation of gentian violet between preparations were complicated by the strong binding of the dye and its homologues to the microsomes themselves. While 99% of the gentian violet can be extracted from an incubation mixture without microsomes, the recovery of total dye products falls drastically in the presence of either active or heat-inactivated microsomes. Microsomes pelleted after incubation with 0.1 mM gentian violet are colored deep blue. The recovery of dye molecules on a molar basis from the supernatant of active-microsome incubations is only 65–75% of that recovered from the incubation mixture without microsomes. Recovery of quantified products on molar basis drops to 30–35% from incubation mixtures with active microsomes containing 0.01 mM gentian violet.

Attempts to recover more dye from the microsomal pellet by solvent extraction at room temperature with butanol, methanol, or acidified methanol were unsuccessful. Nor did incubation of acetone-precipitated microsomal pellets with 2% Triton X-100 at 37 °C result in solubilization of dye. Therefore, the product distributions were normalized on the basis of percentage of total metabolites detectable at 546 nm in extracts of the supernatant. The major demethylated metabolites and unchanged substrate comprise 95% of the total detected metabolites. A substrate level of 0.1 mM was the high limit beyond which the microsomal suspension would precipitate.

All microsome preparations tested caused the demethylation of gentian violet (**1a**) to various mixtures of the pararosanilines **1b**–**1d**, as recorded in Table II. Substrate **1a** was the major component of the product mixtures under our incubation conditions.

Stepwise demethylation was demonstrated by the incubation of 0.1 mM synthetic **1b** with liver microsomes from female Sprague-Dawley rats. Of the 546 nm absorbing components in the butanol extracts, an average of 63% was due to **1b**, 32% to the demethylation products **1c** and **1d**, and 5% to unknown material. No **1a** could be detected.

The composition of extracts from control incubations without microosomes varies little from that of the substrate itself in that 98% of the material chromatographs with **1a**. The addition of heat-inactivated microsomes to an incubation mixture does little to change the product composition, decreasing the amount of **1a** to 96% of the mixture. In each control, most of the remainder is **1b**.

Two differences are apparent in comparisons of the metabolite composition data in Table II. One difference is in the extent of demethylation. Generally, demethylation is less extensive with microsomes from mice than with those from chicken and the other rodents. Statistical comparisons could not be made between mice and the other species in this study because of differing experimental methodology. Mouse data were obtained from duplicate demethylation assays performed on microsomes prepared from pooled livers from three animals. The data for the other species were obtained from assays on microsomes from individual animals. Nonetheless, the standard conditions used in the assay suffice for a comparison of the relative extents of demethylation. Evidently, the difference is one in the rate of demethylation.

A second difference is apparent in product distribution. Complete demethylation to a primary amino group is not favored in guinea pigs as seen by the almost complete lack of **1c** in the product mixture. Instead, guinea pig microsomes produce the greatest proportion of **1d** of any mi-

crosomes in this assay. Sex differences among the rodents are minimal, as determined by the t test. The value of P did not fall below 0.1 for comparison of the percentage of **1a** for any of the rat strains, hamsters, or guinea pigs. Microsomes from the male chicken exhibited demethylating ability similar in extent to that of the larger rodents.

Demethylation of dimethylamino substituents in aromatic rings is a commonly observed phenomenon involving hemoprotein of the mixed-function oxidase system. A recent example is the demethylation of the antitumor agent, hexamethylmelamine, by mouse liver microsomes (Brindley et al., 1982). The vast majority of cases investigated so far, however, involve neutral molecules. Gentian violet differs in that it is a resonance-stabilized cation at physiological pH. Not only do the metabolites isolated from incubation with microsomes constitute the first identified metabolites of a triphenylmethane dye but also they are evidence for the further oxidative metabolism of an oxidized, charged chromophore.

ACKNOWLEDGMENT

We thank Drs. Pat Freeman and W. Korfmacher for determination of mass spectra.

Registry No. 1, 548-62-9; **1b**, 603-47-4; **1c**, 84215-49-6; **1d**, 89232-79-1; **2a**, 89232-80-4; **2b**, 5089-33-8; *n*-butyllithium, 109-72-8; 4,4′-bis(dimethylamino)benzophenone, 90-94-8; ethyl 4-(dimethylamino)benzoate, 10287-53-3; *N*-methyl-4-bromoaniline, 6911-87-1; chlorotrimethylsilane, 75-77-4; sodium hydride, 7646-69-7.

LITERATURE CITED

Brindley, C.; Geshcher, A.; Langdon, S. P.; Broggini, M.; Columbo, T.; D'Incalci, M. *Biochem. Pharmacol.* **1982**, *31*, 625.
Broser, W.; Harrer, W. *Angew. Chem., Int. Ed. Engl.* **1965**, *4*, 1081.
Combes, R. D.; Haveland-Smith, R. B. *Xenobiotica* **1982**, *98*, 101.
Grocock, D. E.; Hallas, G.; Hepworth, J. D. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1972.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
McEwen, C. N.; Layton, S. F.; Taylor, S. K. *Anal. Chem.* **1977**, *49*, 922.
Minegishi, K.-I.; Yamaha, T. *Chem. Pharm. Bull.* **1974**, *22*, 2042.
Minegishi, K.-I.; Yamaha, T. *Toxicology* **1977**, *7*, 367.
Phillips, J. C.; Mendis, D.; Eason, C. T.; Gangolli, S. D. *Food Cosmet. Toxicol.* **1980**, *19*, 7.
Rushing, L. G.; Bowman, M. C. *J. Chromatogr. Sci.* **1980**, *18*, 224.
Taylor, G. W. *U.S. Patent* 4 033 721, 1977.

Received for review August 22, 1983. Accepted January 26, 1984. This study was presented in part at the American Society for Pharmacology and Experimental Therapeutics Meeting, Calgary, Alberta, Canada, Aug 16–20, 1981.

Fate of Ethylenebis(dithiocarbamates) and Their Metabolites during the Brew Process

Siegfried Nitz, Pran Nath Moza,* Jutta Kokabi, Dieter Freitag, Akbar Behechti, and Friedhelm Korte

Hops treated with ethylenebis(dithiocarbamate) (EBDC's) and hops extract (procured from commercial firms, 1978) were analyzed for unchanged dithiocarbamate and ethylenethiourea (ETU) residue. In hops the residues of EBDC (370–490 ppm) were high as compared to those in the hops extract (185 ppm). The residues of ETU and propylenethiourea (PTU) in hops were 3 and 6 ppm, respectively. Thermal decomposition of propineb in pure water started in 15 min, while in the presence of wort the decomposition slowed down. The wort was spiked with ETU-^{14}C (10 ppm, 10 μCi) and was subjected to the brew process. The radioactivity assayed in the drinkable beer was more than 80% of the applied amount and consisted of only ETU. Both metallic copper and stabilizers like bentonite, polyvinyl polypyrolidone (PVPP), stabifix, and stabiquick (silica gels) commonly used in brew technology did not reduce the residues of ETU in wort and in beer.

Ethylenebis(dithiocarbamates) (EBDC's) form one of the most important classes of fungicides for controlling diseases of agricultural crops. Maneb [ethylenebis(dithiocarbamate)], among other fungicides, is used to control *Peronospora* prophylactically in hops. Air-dried hops and/or hops extracts are vital ingredients in the manufacture of beer. Ethylenethiourea (ETU), a major metabolite of EBDC, has been found to have carcinogenic and teratogenic effects in animals (Larsson et al., 1976; Graham et al., 1975; Graham and Hansen, 1972; Innes et al., 1969) and a concern has arisen regarding its possible occurrence in the food supply. Residue analysis of environmental chemicals in foodstuffs is not enough for the assessment of hazards involved, especially for those chemicals that undergo a chemical change during food technological process. A study (Newsome and Laver, 1973) has shown that boiling of foods containing maneb can result in increased levels of ETU. So it was thought to be of interest to study the behavior of maneb/propineb and their main metabolite ETU/propylenethiourea (PTU) during the brew process. Besides this, some light has been shed on the ETU/PTU residue leves in drinkable beer. Since copper vessels are used in heating of the original wort with hops in brewhouses and it has been reported (Lesage, 1980) that the formation of ETU by the thermal degradation of EBDC in aqueous medium is greatly reduced by the addition of copper sulfate, we also examined the behavior of ETU/PTU during the heating process in the presence of copper mtal and copper salt both in the presence and in

Gesellschaft für Strahlen- und Umweltforschung mbH München, Institute für Ökologische Chemie, D-8042 Neuherberg, West Germany, and Technische Universität München, Institut für Chemie, D-8050 Freising-Weihenstephan, West Germany.

0021-8561/84/1432-0600$01.50/0