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.

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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.

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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.

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the absence of hops. The role of stabilizers on the fate of ETU/PTU residues in beer is also described in this paper.

EXPERIMENTAL SECTION

Apparatus. A Lauda thermostat series NB was used for temperature maintenance of a water bath for fermentation. A Carlo Erba Fractovap Model 2101 AC equipped with a FPD was used for GC analysis. The GC conditions were as follows: capillary quartz column coated with Carbowax 20 M (Hewlett-Packard); column length 8 m; 0.25-mm i.d.; carrier gas, helium; flow rate 4 mL/min; temperature programmed from 60 to 220 °C at 30 °C/min; detector temperature, 250 °C. This unit was connected with a Hewlett-Packard Automation System 3385 A for data evaluation.

A Berthold liquid scintillation counter (BF betaszint 5000/300) with external standardization was used to assay radioactivity. Thin-layer chromatography plates were scanned for radioactive substances on a scanner supplied by Berthold-Frieseke GmbH, Karlsruhe, West Germany.

Reagents. Maneb-^{14}C was synthesized in our laboratory (Attar et al., 1982). Ethylenethiourea-^{14}C (99% pure) was kindly supplied by the Bayer AG (West Germany). Hydroluma, a scintillation liquid (I. T. Baker Chemia) was used for assaying ^{14}C in various samples. Ethylenethiourea (inactive) was purchased from Dr. Sand and I. Ehrenstorfer (Augsberg, West Germany). Ready-made silica gel plates (Merck, 0.25 mm thick) were used for thin-layer chromatography analysis. Extrelut columns (Merck No. 11737) were used for the cleanup procedure of all extracts. The column contained 14 g of Kieselguhr having a corn-shaped structure and high porous volume. The column was loaded with a maximum of 20 mL of aqueous solution. The principle of operation was liquid–liquid extraction. For the optimum extraction of ETU and PTU from aqueous medium, addition of KF (salting out) and adjustment of the pH with NH_4Cl were found essential.

Procedure. Air-dried hops and/or pellets (0.5–1 g) (procured from commercial firms, 1978) were heated for 1 h in 200 mL of H_2O to which was added a solution of 8 g of $SnCl_2$ in 20 mL of H_2O and 20 mL of HCl (concentrated). The gases formed were entrained by suction through two traps: the first contained 10 mL of aqueous NaOH (6.5%) and 5 mL of toluene to trap any H_2S evolved, while the second contained 12.5 mL of diethanolamine (1.25 g) and cupric acetate (0.6 mg) in ethanol, which reacted with the CS_2 evolved. The resulting dithiocarbamate was assayed spectrophotometrically at 435 nm (Keppel, 1969). Each analysis was done in duplicate.

The preanalyzed hops (4 g/L) were heated with 2 L of original wort obtained from the brewhouse Weihenstephan, Freising, West Germany, for 45 min. The original wort was filtered off the hops. The hops after drying at room temperature were anlayzed for dithiocarbamate as mentioned above. Simultaneously, hops (4 g/L) were heated with tap water, and after filtration both wort and hops were analyzed for dithiocarbamate. Wort with 12.2% sugar content was treated with ETU (for 30 min), filtered, cooled (5–7 °C), and transfered into a fermentation glass tube (2-L capacity) and aerated with compressed air for nearly 15 min. The wort was then incubated with yeast and allowed to ferment in a thermostat at 5 °C for 8 days. After this period the young beer was filtered and stored in flasks in the thermostat at 5 °C for 4 days and 3 weeks at 1 °C.

To throw some light on the behavior of ETU during the complete brew process, two experiments, one with different concentrations of ETU (inactive) and the other with ^{14}C-labeled ETU, were carried out. To study the influence of copper and cupric ions on ETU in water and in wort, experiments with CuO (5 ppm), $CuSO_4$ (5 ppm), and copper chips (1 g/L) under reflux for 2 h were undertaken. Two samples, each after an interval of 1 h, were taken for analysis.

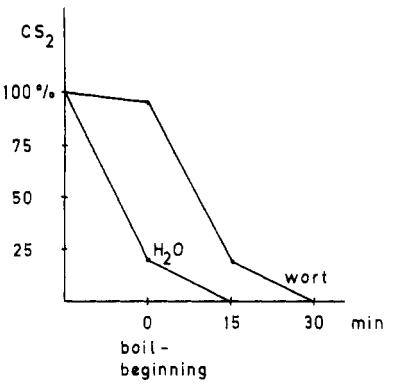

Figure 1. Decomposition of propineb during heating in wort and in water (expressed as percent of CS_2 evolved).

Table I. Distribution of Radioactivity (ETU-^{14}C) in Wort after Heating and in Beer after Brewing and after Storing

time	μCi
0 h in wort	0.58
after $^1/_2$ h of heating and filtered (wort)	0.52
7 days after fermentation (beer)	0.60
7 weeks after storage and filtered (beer)	0.59
filtered over silica gel (beer)	0.59

Commercial beers are usually stabilized by treating with silica gels (e.g., stabifix and stabiquick), PVPP, and bentonite. Their influence in reducing the residues of ETU in beer was studied. Table III records the amount of each stabiizer, volume of beer, ETU content in beer, and the contact time between beer and the stabilizer. The beer containing a definite amount of ETU-^{14}C was shaken with a stabilizer and filtered, and the filtrate was then analyzed for ETU content.

RESULTS AND DISCUSSION

Air-dired hops and hop pellets from 1978 crops were analyzed for carbamates by the common procedure (Keppel, 1969), and ETU/PTU was analyzed by the reported method (Nitz et al., 1982). The amount of carbamates in pellets (185 ppm) was relatively small as compared to the amount analyzed in hops (490 ppm). The difference in value can be due to the decomposition of carbamate to other products during pellet formation. The amount of ETU/PTU in hops was found to be 3 and 6 ppm, respectively. The commercial beers (1980) were found free of EBDC's on analysis. EBDC's are heat unstable (Newsome and Laver, 1973) and are converted to thiourea derivatives in the range of 40–80% depending upon the experimental conditions. The results of decomposition of propineb (Figure 1) upon heating with and without wort showed that within $^1/_2$ h it decomposed into PTU, which could appear in the commercial beer. However, decomposition of the carbamate was slowed down in the presence of wort as compared to pure water.

In the case of ETU-^{14}C more than 80% of the applied activity after fermentation was found in the beer (Table I). The dissolved ^{14}C substance was extracted from beer by the method reported earlier (Nitz et al., 1982). The extracted radioactivity upon TLC and GLC analysis was found mainly to be ETU (Table II). Storing beer for 4 weeks neither changed the quantity of ETU nor effected any chemical change. In order to see the relevance of the results obtained on a laboratory scale (that ETU remains

Table II. Residues of ETU/PTU during the Brew Process

substance	concn, ppm	before heating, ppm	after heating,[b] ppm	after fermentation,[c] ppm	after storing,[d] ppm
ETU	100	100 (20 L)[a]	99	99	95
ETU	10	9.5 (20 L)[a]	8.8	8.7	8.7
ETU	1.0	1.19 (2 L)[a]	1.0	0.95	1.07
PTU	1.0	1.0 (2 L)[a]	1.0	1.0	1.2
ETU-^{14}C	10	8.33	7.38	8.1	9.09

[a] L = liters of wort. [b] 45 min. [c] After filtration. [d] Young beer after filtration.

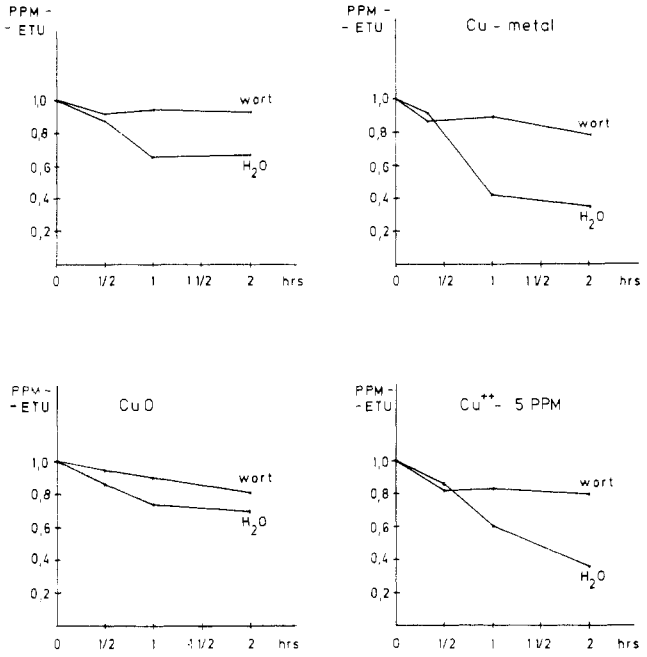

Figure 2. Change of ETU content in H_2O and wort after heating in the presence of copper chips, CuO, and $CuSO_4$.

unchanged both qualitatively and quantitatively during the brewing process) compared to commercial-scale manufacture of beer in the presence of ETU, 20 L of beer was prepared with different concentrations of ETU. It was observed that the fate of ETU in a small-scale preparation of beer is the same as in a large-scale preparation (Table II).

The effect of Cu metal and Cu^{2+} on the behavior of ETU in the presence and absence of wort is shown in Figure 2. From this graph it is obvious that during 2 h of heating the concentration of ETU in water falls to more than 50% while the concentration of ETU practically remains the same in the presence of wort. From these results one can conclude that the biological matrix plays an important role in effecting the chemical reaction between Cu/Cu^{2+} and ETU. Lesage (1980) has shown that the concentration of ETU in the presence of Cu^{2+} also falls and eventually diethylamine and other products are formed. Our efforts to find diethylamine in the reaction mixture were negative.

In order to remove proteins and polyphenols partially from the beer to maintain its stability, silica gel and bentonite were used. It was observed that neither silica gel nor bentonite helped in filtering ETU from the spiked beer (Table III). The effect is probably attributed to the high solubility of ETU in water.

In 1977 FAO (Food Agriculture Organization)/WHO (World Health Organization) recommended that the content of ETU in beer should remain at the level of 0.1 ppm. The results of beer analysis (Nitz et al., 1982) show that the levels of ETU in some commercial beers (1979) were higher than 0.1 ppm. In 1980 a new regulation was enforced in West Germany. According to this regulation ("Merkblätter des Amtlichen Pflanzenschutzdienstes der Bayerischen Landesanstalt für Bodenkunde und Pflanzenbau", 1980) the hops should be sprayed no more than 14 times with the active agent until blossoming and no less than 28 days should be allowed between the last spray and harvest. In order to see the effect of the new regulation on the ETU content in beer, some commercial beers (1983) were analyzed. Table IV records the results of analysis, and it is observed that the levels of ETU are considerably less as compared to results of 1979 (Nitz et al., 1982). These results show that the new regulation produces a drastic reduction of this contaminant in beer.

A new regulation on the spraying of hops with dithiocarbamates has been enforced. According to this regulation ("Merkblätter des Amtlichen Pflanzenschutzdienstes der Bayerischen Landesanstalt für Bodenkunde und Pflanzenbau", 1981) the hops should be sprayed a maximum of 12 times with the fungicide until blossoming and the postharvest interval after the last spray harvest should be at least 35 days.

Table III. Effect of Stabilizers on the Residues of ETU/PTU in Beer

stabilizers	residues of ETU/PTU before addition of stabilizers in beer, ppm		residues in beer, ppm	
	ETU	PTU	ETU	PTU
stabifix (5 min)	1.07	1.2	1.0	1.16
stabiquick (5 min)	1.07	1.2	1.0	1.2
PVPP (24 h)	1.07	1.2	1.05	1.2
bentonite (7 days)	1.07	1.2	1.1	1.2

Table IV. ETU/PTU Residues in Commercial Beer (1980)

beer[b]	ETU residues, ppm	PTU residues, ppm
A (Pils)	0.03	0.01
B (Heller Bock)	0.01	<0.01
C (Pils)	0.05	0.01
D (Hell)	<0.01	<0.01
E (Pils)	<0.01	n.d.[a]
F (Pils)	<0.01	<0.01
G (Heller Bock)	<0.01	<0.01
H (Pils)	<0.01	<0.01
I (Pils)	<0.01	<0.01
K (Pils)	<0.01	n.d.
L (Bock)	<0.01	n.d.
M (Pils)	<0.01	n.d.
N (Pils)	0.03	<0.01
O (Pils)	<0.01	<0.01
P (Vollbier)	<0.01	<0.01
Q (Pils)	0.06	n.d.
R (Guiness Beer)	<0.01	<0.01

[a] n.d. = not detected. [b] The letters A-R are different brands of beer.

CONCLUSIONS

EBDC's in hops during brew technology are mainly changed to ETU/PTU. ETU/PTU's do not undergo any chemical change during the brew process. The common stabilizers such as bentonite, PVPP, stabifix, and stabiquick have no influence in reducing the content of ETU/PTU that has been transferred into the beer during brewing. Metallic copper and copper ions have no significant influence on changing the content of ETU in the

presence of wort. With the new regulations introduced in West Germany in 1981 for spraying hops with EBDC's, the residues of ETU/PTU in beer are considerably reduced.

Registry No. EBDC, 34731-32-3; ETU, 96-45-7; PTU, 2055-46-1; propineb, 12071-83-9; copper, 7440-50-8.

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Metabolism of Tributyl Phosphate in Male Rats

Takashi Suzuki, Kumiko Sasaki,* Mitsuharu Takeda, and Mitsuru Uchiyama

When rats were given a single oral dose of ^{14}C-labeled tributyl phosphate at 14 mg/kg, 50, 10, and 6% of the applied ^{14}C were excreted in urine, exhaled air, and feces, respectively, within 1 day. On the other hand, when rats were given a single intraperitoneal dose (same amount), 70, 7, and 4% of the applied ^{14}C were excreted in urine, exhaled air, and feces, respectively, within 1 day. After a single intraperitoneal dose of tributyl phosphate at 250 mg/kg, 11 phosphorus-containing metabolites were identified in the 24-h urine. Major metabolites were dibutyl hydrogen phosphate, butyl dihydrogen phosphate, and butyl bis(3-hydroxybutyl) phosphate as well as small amounts of derivatives hydroxylated at the butyl moieties. Administration of a probable intermediate, dibutyl 3-oxobutyl phosphate, gave a metabolic profile similar to that of dibutyl 3-hydroxybutyl phosphate, which is considered to be the major metabolic intermediate. This supports the view that dibutyl 3-oxobutyl phosphate is also on the main metabolic pathway. Furthermore, the metabolites from urine of rat administered butyl bis(3-hydroxybutyl) phosphate and dibutyl hydrogen phosphate were examined. Some of the possible metabolic pathways of tributyl phosphate are proposed.

Tributyl phosphate (TBP) (I) has been widely used as a solvent for extraction of metals and as a plasticizer. Recently it has also been examined for possible usage as a volatilization controller for insecticidal fumigants (Koezuka, 1979). The occurrence of this material in drinking water (Williams and LeBel, 1981), river water (Schou et al., 1981; Meijers and Van der Leer, 1976), and the edible parts of fish (Environmental Agency of Japan, 1978) has been demonstrated. It is toxic to fish (Sasaki et al., 1981), Daphnia (Bringmann and Kuehn, 1982), other types of aquatic organisms (Bringmann and Kuehn, 1980) and mammals (Oishi et al., 1980).

The metabolic fate of this compound has not been examined in detail, though Jones (1970) reported that I was metabolized to butyl-L-cysteine and dibutyl hydrogen phosphate in rodents. We now report the metabolic fate of I in male rats in order to provide a basis for assessing its toxicological impact.

MATERIALS AND METHODS

Treatment of Rats and Collection of Samples. For the identification and determination of the metabolites of I, male Wistar rats (180–210 g) were given a single intraperitoneal (ip) injection of I, dibutyl 3-hydroxybutyl phosphate (II), dibutyl 3-oxobutyl phosphate (III), butyl bis(3-hydroxybutyl) phosphate (IV), or dibutyl hydrogen phosphate (Va) dissolved 10% in corn oil at the dosages indicated in table III or under Extraction and Purification of Some Main Metabolites.

For the radioanalysis, male rats were given a single ip injection or a single oral dose of [^{14}C]TBP (specific activity 0.179 mCi/mmol) at a dosage of 14 mg/kg of body weight, dissolved in 0.1 mL of corn oil. The rats were kept in metabolic cages and supplied ad libitum with diet and water, both of which were proved to be free from TBP. Feces and urine were collected separately at regular intervals. The volume of each sample of urine was determined and aliquots were taken for radioassay. Each sample of feces collected was air-dried, weighed, and ground, and an appropriate quantity was taken for radioassay. The radioactivity fortified to the sample was recovered almost quantitatively.

Radioassay. Radioactivities in organic solvents or in the aqueous layer were determined by mixing the sample with Aquasol-2 (New England Nuclear). Radioactivity in fecal material was determined by using an Aloka ASC-113 automatic sample oxidizer. $^{14}CO_2$ formed was trapped in a scintillation solution, Oxifluor-CO_2 (New England Nuclear), and counted on an Aloka LSC-700 liquid scintilla-

Division of Food, National Institute of Hygienic Sciences, 18-1, Kamiyoga 1-chome, Setagaya-ku, Tokyo 158, Japan.

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