Application of γ-Irradiation Technique in the Identification of Some Metabolites of Maneb in Mice

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The metabolism of manganous ethylenebis(dithiocarbamate) (Maneb) in adult male mice was studied in this work. Ten percent of the orally administered radioactive [^{14}C]Maneb was present in urine after a 22-h collection time as ethylenethiourea (ETU), 2% as 5,6-dihydro-3*H*-imidazo[2,1-*c*]-1,2,4-dithiazole-3-thione (EBIS), 2% as 2-imidazolidin-2-ylsulfenate (ETU-*S*-monoxide), 1% as ethyleneurea (EU), and 2% as 4,5-dihydro-1,3,6-oxadiazepine-2-thione (DOT), a previously unknown metabolite. Elemental sulfur was also identified. In the feces ETU and EBIS could be detected. The application of the γ-irradiation technique as an aid in the identification of metabolites of Maneb in mice is discussed.

Ethylenebis(dithiocarbamates) including Maneb, Zineb, Mancozeb, Nabam, and Amobam are commonly used fungicides for controlling crop diseases. Ethylenethiourea (ETU) is a degradation product of these fungicides (Bontoyan and Looker, 1973) and a primary metabolite in environmental degradation and in test organisms (Engst and Schnaak, 1974; Watts et al., 1974; Marshall, S., 1977). While the parent compounds are relatively nontoxic to animals (Engst and Schnaak, 1974), ETU has been reported to be goitrogenic (Seifter and Ehrich, 1948; Graham and Hansen, 1972; Graham et al., 1973; 1975), carcinogenic (Innes et al., 1969; Ulland et al., 1972; Graham et al., 1973), mutagenic (Seiler, 1974), and tetratogenic (Khera, 1973; Ruddick and Khera, 1975; Lu and Staples, 1978).

Other degradation products and metabolites of Maneb are 5,6-dihydro-3*H*-imidazo[2,1-*c*]-1,2,4-dithiazole-3-thione (EBIS), also referred to as ethylenethiuram monosulfide (ETM) (Bontoyan and Looker, 1973; Hylin, 1973; Seidler et al., 1970) and ethylenethiuram disulfide (ETD) (Czeglédi-Janko, 1967; Seidler et al., 1970; Hylin, 1973). EBIS has been reported to produce perinatal effects and maternal limb paralysis in rats (Chernoff et al., 1979). When the toxicity of pesticide metabolites is evaluated in a living organism, the first task is to identify them. After purification there is usually a microgram or less of the metabolites for use for structural analysis. In these cases in vitro studies with tissue, cellular and subcellular preparations are a common practice. Alternatively prediction of the biological fate has to be made in order to be able to synthesize the compound. In this way the synthetic products or metabolites can be included in the in vivo evaluation of the compound under consideration. The principal modes of detoxification are hydrolysis, oxidation, and conjugation (Smith, 1964). γ-Irradiation of dilute aqueous solutions of organic compounds leads inter alia to hydrolysis and oxidation, too. A large molecule degrades in a various ways, but if the compound contains a very reactive group or atom as sulfur in ethylenebis(dithiocarbamates), the number of radiolysis products is significantly restricted. Even though the two systems, biochemical metabolism and γ-irradiation, are totally different in both cases, the attack will normally take place in the most favorable site of the molecule. Therefore, a high probability exists that the same degradation products will be found in both cases. The primary reactive radiolytic species in γ-irradiated dilute aqueous organic solutions are those originating in water, namely, hydrogen atoms, solvated electrons, and hydroxyl radicals. Their production rates, the *G* values, and reactivities are well-established so that the reaction parameters, dose rate, total dose, and solute concentration, are quite easy to optimize. Usually a few percent of the organic compound in water has to be transformed in order to get a few milligrams of the main radiolysis compounds. The degradation products can be separated and purified by using, e.g., preparative thin-layer chromatography, high-performance liquid chromatography, or gas chromatography. A milligram quantity is nowadays enough for structure analysis and mutagenicity tests. The γ-irradiation technique used for preparing the suspected metabolites is straightforward and very easy to perform and has been used successfully earlier by the authors (Savolainen and Pyysalo, 1979) in synthesizing the main metabolite (2-imidazolidin-2-ylsulfenate) of ethylenethiourea (ETU) in mice. While this product had been postulated earlier (Marshall and Singh, 1977) and the chemical syntheses of various thiourea *S*-monoxides have been presented (Walter and Randau, 1969), the chemical oxidation of ETU to the level of monoxide has not been successful (Marshall and Singh, 1977; Savolainen and Pyysalo, 1979). On the other hand, the 2-imidazolidin-2-ylsulfenate was easily prepared by γ-irradiation.

The aim of the present work was to study the application of the γ-irradiation technique as an aid in the identification of metabolites of Maneb in mice.

EXPERIMENTAL SECTION

Irradiation of Maneb. The commercial Maneb was purified before irradiation by extracting twice with methanol followed by two extractions with dry chloroform. A solution of Maneb, containing some suspended material, in nitrogen-flushed deionized water (0.5 g/L) was irradiated with ^{60}Co γ-rays (dose rate 200 krad/h, total dose 3.0 Mrad, irradiation temperature +20 °C). The irradiated solution ca. half a litre was concentrated in a rotating vacuum evaporator.

Purification of Radiolysis Products. For the purification of radiolysis products preparative thin-layer chromatography (TLC) was carried out on silica gel plates (Merck, 1.0 mm, F_{254} fluorescence indicator). The plates were first developed in a benzene–acetone (9:1) (D) solvent system. The compounds detected by using UV methods were eluted with chloroform and rechromatographed in chloroform–butanol–methanol–water (100:5:1:0.5) (A) solvent system. When necessary, the further purification was carried out by high-performance liquid chromatography (LC). The compounds were detected by a Waters M-440 absorption detector at 254 nm after chromatography on a 4 mm × 30 cm μBondapak C_{18} column. The eluting solvent was 10 mmol of ammonium acetate, pH 7.0, delivered on the column by a Waters Model M-6000 pump

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at a flow rate of 2 mL/min. The fractions were concentrated and lyophilized (about 2 mg).

Labeled Compounds. [^{14}C]Maneb was prepared by synthesizing first ^{14}C-labeled Nabam (specific activity 15.6 μCi/mmol) from ^{14}C-labeled carbon disulfide (sp act. 62 mCi/mmol) and ethylenediamine according to the method described by W. Marshall (1977). ^{14}C-Labeled Maneb (sp. act. 13.3 μCi/mmol) was synthesized from [^{14}C]Nabam as described by Seidler et al. (1970). [^{14}C]Maneb was purified twice by extraction with methanol followed by two extraction with dry chloroform just prior to administration to the mice. The chemical purity of the final product was higher than 95% as confirmed by ^{1}H NMR and the radiochemical purity higher than 97% as confirmed by TLC in solvent system A (R_f = 0 for Maneb) and autoradiography.

Animals. [^{14}C]Maneb suspended in olive oil was administered orally by a stomach tube to clinically healthy male NMRI mice (30 g, Oy Orion, Finland). The administered dose was 85 mg/kg and a total number of 80 mice were treated with Maneb. Immediately after treatment the mice were placed in metabolic cages for the collection of urine and feces accumulated during the 22 h. The animals received normal laboratory feed and water ad libitum.

Thin-Layer Chromatography. Thin-layer chromatography was carried out on silica gel plates (Merck, 0.25 mm, with F_{254} fluorescence indicator) developed in the following four solvent systems: (A) chloroform–butanol–methanol–water (100:5:1:0.5); (B) ethyl acetate–ammonia–methanol (90:6:6), (C) butanol–ethanol–water (120:57:33); (D) benzene–acetone (9:1). For the detection of radioactive metabolites the plates were covered with X-ray film (Kodak PE 4006) for 2 months. The metabolites were also visualized by using fluorescence quenching, Ehrlich's reagent, and I_2 vapor.

Measuring Radioactivity. For the quantification of the metabolites in the TLC plates the spots were scraped off and put into counting vials containing 10 mL of Instagel (Packard). The radioactivity of the fractions was measured with a LKB Ultrobeta 1210 liquid scintillation counter. For the measurement of the radioactivity of urine a 50-μL sample was mixed with 10 mL of Instagel and measured as mentioned above. The radioactivity of feces was measured as above by mixing 0.5 g of a homogenized feces–water (1:1 v/v) suspension and 1 mL of Lumasolve, maintained at 50 °C for 0.5 h (Lumac System AG) and adding 10 mL of Lipoluma (Lumac system AG).

Isolation of Metabolites. For the separation and identification of the metabolites the urine samples (about 200 mL) were evaporated to 2–3 mL in a rotating evaporator and suspended in ethyl acetate (2 × 20 mL). The ethyl acetate extracts were filtered over Na_2SO_4 and concentrated under a nitrogen flow at 20 °C to 0.1–0.2 mL of volume. The ethyl acetate unextractable portion of the urine was extracted with chloroform (2 × 20 mL). The chloroform extracts were filtered over Na_2SO_4 and concentrated under nitrogen to 0.1–0.2 mL. The chloroform unextractable portion of the original urine was lyophilized to dryness and the residue dissolved in methanol (20 mL) and filtered. The solution was evaporated under nitrogen to about 0.5–1.0 mL. The radioactivity of these fractions was measured as described above and analyzed by TLC in A, B, C, and D solvent systems.

Feces were homogenized with 5 volumes of an ethanol–water mixture (1:1 v/v) and centrifuged. Aliquots of the homogenate were concentrated in a rotating vacuum evaporator to dryness. Thereafter the extraction procedure was continued as described with urine samples.

Table I. Identified γ-Radiolysis Products of Maneb and Their TLC Characteristics

	R_f values						
	solvent systems[c]				detection using[a,b]		
compound	A	B	C	D	E	I_2	Q
ETU	0.35	0.50	0.70	0.08	+	+	+
ETU-*S*-monoxide	0.18	0.35	0.82	0	–	+	+
EBIS	0.75	0.89	0.80	0.30	+	+	+
EU	0.13	0.20	0.54	0	+	–	–
DOT	0.45	0.90	0.70	0.17	–	pale	+

[a] E = Ehrlich's reagent; I_2 = iodide; Q = fluorescence quench. [b] (+) positive reaction. [c] (A) $CHCl_3$–BuOH–MeOH–H_2O (100:5:1:0.5); (B) EtOAc–NH_3–H_2O (90:6:6); (C) BuOH–EtOH–H_2O (120:57:33); (D) C_6H_6–CH_3COCH_3 (9:1).

Spectral Analysis. The mass fragment spectra (75 eV, EI) was determined by a JEOL JMS 01-SG-2 photoplate mass spectrometer equipped with a JEC 980 B data system. A direct inlet probe was used. Proton nuclear magnetic resonance (^{1}H NMR) spectra at 100 MHz were obtained with a JNM-FX 100 instrument. The samples were in a $CDCl_3$ solution in a 10 mm o.d. tube. The chemical shift values are expressed in ppm downfield from internal tetramethylsilane (Me_4Si) standard.

Infrared (IR) spectra were obtained with a Perkin-Elmer 683 instrument (KBr disk).

RESULTS AND DISCUSSION

Six fractions of the radiolysis products of Maneb contained enough material for spectral analysis. On the basis of TLC characteristics (Table I) and mass spectra, four of the compounds, namely, ethylenethiourea (ETU), 5,6-dihydro-3*H*-imidazo[2,1-*c*]-1,2,4-dithiazole-3-thione (EBIS), also referred to as ethylenethiuram monosulfide (ETM), 2-imidazolidin-2-ylsulfenate (ETU-*S*-monoxide), and ethyleneurea (EU), were previously known. The mass spectra of the compounds gave the following prominent ions: for ETU at M^+ = 102 (100%); for EBIS at M^+ = 176 (75%), m/e = 144 (65%), m/e = 72 (100%); for EU (from the radiolysis products) at M^+ = 86; for ETU-*S*-monoxide at m/e = 100 (100%) and m/e = 102 (15%). The compound with R_f = 1.00 in A and D solvent systems were according to the mass spectral studies elemental sulfur with M^+ = 255.7766 (in theory S_8 = 255.7760). In the IR spectrum the compound showed bands at 2900–3000 cm^{-1} and at 1450 cm^{-1} which are typical of an aliphatic chain. In the ^{1}H NMR signals were found with shifts δ = 0.83 and δ = 1.22 downfield from internal Me_4Si standards. For these reasons it is possible that the elemental sulfur in the very final steps of ionization in the mass spectrometer is produced via very unstable sulfur compounds containing aliphatic chains or a ring structure.

The mass spectrum of the compound R_f = 0.45 in A and 0.17 in D solvent systems showed two prominent peaks at M^+ = 130.0197 (90%) ($C_4H_6O_1N_2S$, calculated M^+ = 130.0203) and m/e = 102.0015 (100%) (C_3H_4OSN, calculated 102.0015). The loss of CH_2N is typical of N-heterocyclic compounds (Benz, 1969). In the ^{1}H NMR spectrum signals were observed with δ = 3.78 (2 CH_2), 5.18 (NH), and 6.40 (=CH—) downfield from the internal Me_4Si standard. The multiplicity of the signals of the methylene protons and that of the vinylic proton was seen as broadening of the signals but the coupling constants could not be accurately measured with the resolution available.

In the IR spectrum strong absorptions were found at 3430 cm^{-1} (NH) and 2930 cm^{-1} (C–H). The above data are

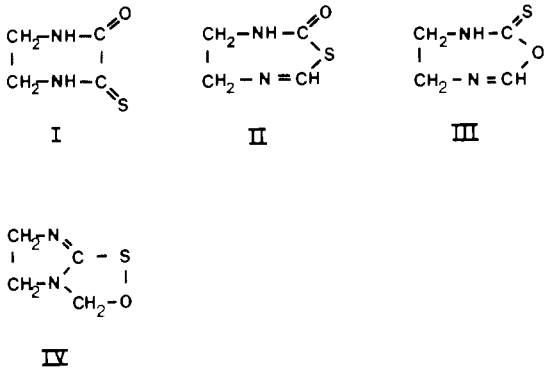

Figure 1. Chemical structures of I, 4,5-dihydro-2-oxo-1,4-diazine-3-thione, II, 4,5-dihydro-2-oxo-1,3,6-thiadiazepine, III, 4,5-dihydro-1,3,6-oxadiazepine-2-thione, and IV, the bicyclic structure.

Table II. Percentages of Radioactivity of Different Metabolites in Urine and Feces of Mice

	urine, %	feces, %
ETU	10	2
EBIS	2	detectable
ETU-*S*-monoxide	2	
EU	1	
DOT	2	
other polar products	83	98

in accordance with the structures 4,5-dihydro-2-oxo-1,4-diazine-3-thione (I), 4,5-dihydro-2-oxo-1,3,6-thiadiazepine (II), 4,5-dihydro-1,3,6-oxadiazepine-2-thione (III) (DOT), and a bicyclic structure (IV) in Figure 1. Compounds I–III also have tautomeric enol and thionel form. The strong band in the IR spectrum at 1630 cm^{-1} was taken as indicative of —C=N— (Thorn, 1960). The absorptions at 1460, 1400, and 1350 cm^{-1} can be assigned to N—C=S. The lack of absorptions at 1650–1700 cm^{-1} (—C=O) and the detected strong absorption at 1100 (C—O—C) support the structure III rather than those of I, II, and IV (Colthup et al., 1964).

Of the radioactivity of [^{14}C]Maneb administered into mice, 50% was excreted within 22 h, 32% in the urine, and 18% in feces. Seidler et al. (1970) reported that the total radioactivity excreted in urine and feces was about 56%, 35% in urine, and 20% in feces of rats within 5 days. In contrast, Jordan and Neal (1979) found that 91% of the radioactivity from [^{14}C]Maneb orally administered to adult male mice was excreted in feces and 9% in urine when the dose was 0.25 mmol/kg. The results presented here cannot be fully compared with those of Jordan and Neal (1979) and Seidler et al. (1970), since here the radioactive label is in a different carbon atom of the molecule, namely, in the carbon atom adjacent to sulfur, their Maneb being labeled in ethylenic carbon atoms. According to Truhaut et al. (1973), Zineb is metabolized to carbon disulfide. This may have taken also place with Maneb. Secondly, the mice were sacrificed 22 h after application, some of radioactivity still remaining in the tissue and intestine.

Some of the metabolites of Maneb in the urine of mice and feces had the same R_f values (in four different solvent systems, Table I), the same reactivities with Ehrlich's reagent and I_2 vapor, and the same retention time in LC as the radiolysis products of Maneb. Table II shows the percentages of radioactivity in the urine and feces present as ETU, ETU-*S*-monoxide, EBIS, EU, and other compounds. Of the radioactivity in urine, about 10% was present as ETU. Jordon and Neal reported 15.8% when the dose was 0.25 mmol/kg and 7.8% when the dose was 0.05 mmol/kg.

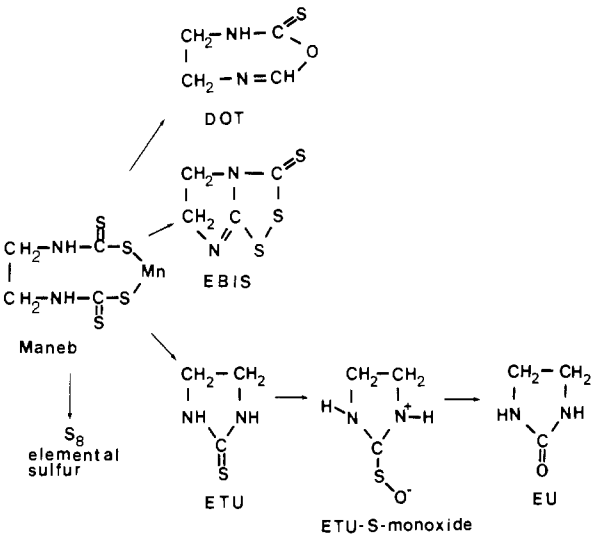

Figure 2. Degradation of Maneb in mice.

About 2% of the radioactivity in urine was excreted as 2-imidazolidin-2-ylsulfenate, the main metabolite of ETU in mice (Savolainen and Pyysalo, 1979), and about 2% as EBIS. EBIS is a fungitoxic degradation product which is metabolized to ethylenebis(isothiocyanate), a product which can bind sulfhydryl groups via the formation of isothiocyanates (Engst and Schnaak, 1974). Chernoff et al. (1979) reported that EBIS caused maternal limb paralysis in the rat but not in the mouse at much higher doses. The radioactivity excreted as EU in urine was about 1%. EU is a metabolite of ETU (Lyman, 1971; Kato et al., 1976; Ruddick et al., 1976a,b; Savolainen and Pyysalo, 1979). EU is probably a harmless metabolite, at least being neither tumorigenic nor teratogenic (Ruddick et al., 1976a,b). One previously unknown metabolite of Maneb in mice had the same R_f value in four different solvent systems as a compound found among the radiolysis products of Maneb. The TLC characteristics of this compound, 4,5-dihydro-1,3,6-oxadiazepine-2-thione, is presented in Table I. Likewise, the mass spectrum of the metabolite after purification by preparative TLC and LC was identical with that of DOT, giving prominent ions at $M^+ = 130$ and $m/e = 102$. The radioactivity excreted as DOT in urine was about 2%.

Elemental sulfur was also found in urine. Truhaut et al. (1973) reported that sulfur was also detected as the metabolite of Zineb in rats. Sulfur is known to inhibit oxygenase enzymes and to degrade cytochrome P-450 (Pélissier et al., 1981).

About 2% of the radioactivity found in feces was as ETU (Table II). EBIS was found in the ethyl acetate fraction of feces, though the amount was low, barely detectable. No EU nor ETU-*S*-monoxide could be detected in feces. The structures of the metabolites of Maneb detected in the urine of mice are presented in Figure 2. The γ-irradiation technique was of great value during this work as all the main metabolites of Maneb in mice could be produced easily in one-step γ-irradiation of dilute deaerated water solution of Maneb. The γ-irradiation technique is useful only in the identification of metabolites when the reactions concerned are hydrolytic or oxidative. The same limitation also concerns the in vitro studies, especially when using microsomal enzymes with NADPH, when predominantly oxidative metabolites are encountered. For example, sulfur-containing drugs can be metabolized through S-dealkylation, desulfuration, sulfoxide formation, and S-alkylation (Hayaishi, 1969). Except in the case of

S-alkylation γ-irradiation is worth trying. Of these reactions, desulfuration and sulfoxide formation are more common among ethylenebis(dithiocarbamates) than S-alkylation. The value of γ-irradiation synthesis in the identification of metabolites thus depends on the compound concerned and on the possible metabolic pathways. The method is at its best when the number of degradation products is small, because then the separation and purification procedure is more easily performed. The number of radiolysis products and their oxidative state can be checked by the reaction parameters. Deaeration of the solution before irradiation diminishes further oxidation. On the other hand, by use of oxygen saturation, oxygen molecules scavenge, reducing primary radicals by transforming them to oxidative species.

Registry No. Maneb, 12427-38-2; ETU, 96-45-7; EBIS, 33813-20-6; ETU-*S*-monooxide, 85150-75-0; EU, 120-93-4; DOT, 85135-72-4.

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