Oxidative Conversion of Daminozide to Methylating and Acylating Agents

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Oxidation of the plant growth regulator daminozide [Alar; succinic acid, mono(2,2-dimethylhydrazide)] with *m*-chloroperoxybenzoic acid (MCPBA), sodium hypochlorite, or hydrogen peroxide forms not only a methylating species (probably a diazo hydroxide) that converts carboxylic acids to methyl esters but also the acylating agent succinic anhydride. Additional products are dimethylnitrosamine and methanol on MCPBA or hydrogen peroxide oxidation and 0.6 equiv of nitrogen on treatment of daminozide with 2 equiv of MCPBA or hypochlorite. Reactive intermediates formed on oxidation of 1,1-dimethylhydrazine with 2 equiv of MCPBA also evolve nitrogen and methylate 3-chlorobenzoic acid. Metabolites of [^{14}C]daminozide bind to hemoglobin, liver protein, and a liver DNA fraction of treated mice and to human hemoglobin in vitro via hydrogen peroxide oxidation, in each case with equal labeling from the [^{14}C]methyl and [^{14}C]succinyl moieties. Reactions of daminozide in these chemical oxidation models may be relevant to those in biological systems.

Daminozide [Alar; succinic acid, mono(2,2-dimethylhydrazide)], an important plant growth regulator, is reported to cause tumors of blood vessels and lungs in female and male mice (Toth et al., 1977), adenocarcinomas and leiomyosarcomas of the uterus in rats, and possibly hepatocellular carcinomas in male but not female mice (National Cancer Institute, 1978). Daminozide undergoes relatively little metabolism on oral administration to rats and dairy cows, which excrete 93–95% of the dose as the parent compound (St. John et al., 1969; Environmental Protection Agency, 1985). However, even small amounts of hydrolysis or oxidation may lead to metabolites of toxicological significance. Hydrolysis of daminozide will yield the purported carcinogen 1,1-dimethylhydrazine (Environmental Protection Agency, 1985). Metabolic N-oxidation via *N*-oxide intermediates may yield products of high reactivity, on analogy with studies on monoalkyl- and monoacylhydrazines and dialkylhydrazines and nitrosamines (Druckrey, 1973; Nelson et al., 1976; Ziegler, 1980).

This study examines the oxidation reactions of daminozide to produce reactive species (Figure 1). The chemical oxidants selected for use are *m*-chloroperoxybenzoic acid (MCPBA), hydrogen peroxide, and hypochlorite, each of which is known to oxidize *N*-methyl substituents to *N*-oxide derivatives or to otherwise mimic biological oxidations (House, 1972; Brown et al., 1983). It also considers possible covalent binding of oxidatively activated daminozide to biological macromolecules.

MATERIALS AND METHODS

Spectroscopy. NMR spectra were recorded on a Bruker AM-500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. For integration of peaks in ^{1}H NMR, 30° flip angle pulses were used, with 4-s delays between pulses. Products were identified and quantified by adding standards and integrating peak areas in ^{1}H NMR. Data were acquired over 32K data points and Fourier transformed by 0.2-s (^{1}H) or 5-s (^{13}C) exponential multiplication. Electron impact (EI) (70 eV) or chemical ionization (CI) mass spectrometry (MS) used a Hewlett-Packard 5985B instrument with a methyl silicone high-performance gas chromatography (GC) capillary column (10 m) operating with helium as the carrier gas (1 mL/min) and methane (0.8 Torr) as the ionizing gas or with direct sample introduction into the source. Nitrogen gas was identified and its purity determined by GC–EI-MS.

Chemical Oxidation. Daminozide (10 mg, 62.5 μmol) was treated in acetone-d_6 or acetonitrile-d_3–D_2O (4:1) (0.5 mL) in an NMR tube at room temperature with up to 4 equiv of either solid MCPBA (purified by a bicarbonate extraction of a methylene chloride solution to remove 4-chlorobenzoic acid) or an aqueous solution of sodium hypochlorite (5.25%) or hydrogen peroxide (30%). For determination of nitrogen gas evolution, daminozide (500 mg, 2.94 mmol) in water (100 mL) was slowly treated with MCPBA (1.01 g, 5.87 mmol) in methanol (20 mL) with stirring in a closed system to trap and measure evolved gases.

Animals and Treatments. Male Swiss-Webster mice were treated intraperitoneally with [^{14}C]daminozide (1.1 mg/kg; 17.3 mCi/mmol methyl label; 0.83 mg/kg, 11.8 mCi/mmol succinate label; each in 60 μL of ethanol) purified immediately prior to use by thin-layer chromatography (TLC) on silica gel plates (60 F-254, 20 cm × 20 cm × 0.25 mm) developed with methylene chloride–1-propanol–formic acid (88% aqueous)–water (60:120:8:1) (daminozide at R_f 0.5), yielding >99% radiopurity. Controls consisted of mice treated with ethanol (60 μL). After 4–6 h approximately 0.5 mL of blood was removed via the occipital orbit and centrifuged (2000*g*, 30 min) and the supernatant removed. The pellet was washed twice each with ether, methanol, and acetone, dried (average weight 85 mg), and combusted on a Packard Tricarb sample oxidizer, and bound radioactivity was determined by liquid scintillation counting (LSC) with a Beckman LS 100C.

Isolation of Mouse Liver Fractions. A crude DNA fraction was isolated as previously noted (Dashwood et al., 1986) by homogenizing livers from mice treated as described above in 10 mL of lysing medium [sodium phosphate (250 mM, pH 6.8), containing urea (8 M), EDTA (10 mM), and sodium dodecyl sulfate (1%)] four times for 3-s intervals at 0 °C. Protein was precipitated by mixing the homogenate with chloroform–isoamyl alcohol–phenol (24:1:25; 10 mL) and separated by centrifugation (500*g*, 30 min). After this procedure was repeated, the recovered protein layer was washed three times with methanol to remove any unbound radiolabeled compounds. The

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Table I. ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) Chemical Shifts of Daminozide and Its Oxidation Products in Acetonitrile-*d*-Deuterium Oxide (4:1)

compound	nuclei	NMR resonances, δ	
		succinyl	*N*-methyl
daminozide	^{13}C	175.4, 170.8, 29.4, 29.1, 28.7, 26.9	47.4, 46.5
	^1H	2.74 (t), 2.58 (t), 2.34 (t)	2.50 (s), 2.55 (s)
succinic anhydride	^{13}C	173.3, 28.7	
	^1H	2.98 (s)	
succinic acid	^{13}C	175.0, 28.4	
DMN	^1H		3.75 (s), 3.05 (s)

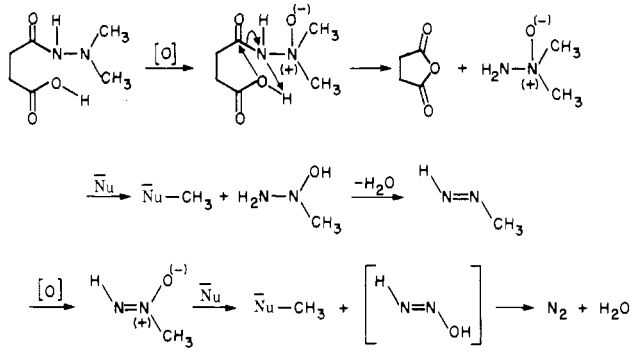

Figure 1. Proposed oxidation pathway of daminozide to succinic anhydride and a methylating agent formed via dimethylhydrazine *N*-oxide. Nu is DNA or another macromolecule or nucleophile. Other products are methanol and dimethylnitrosamine from further hydrolysis and oxidation reactions.

aqueous fraction was extracted with ether to remove residual phenol and dialyzed against distilled water for 12 h to remove the urea and any residual unbound radiolabeled materials. It was then adjusted to 0.2 M NaCl, cooled on ice, and treated with 2 vol of ice-cold ethanol. The precipitated DNA strands were carefully wound up on a glass rod, pressed to remove excess solvent, and washed repeatedly with ethanol, and their purity was determined by the 280/260-nm absorbance ratio (0.515 ± 0.010, $N = 10$) (Dashwood et al., 1986). The average recovery of DNA fraction was 9.2 mg and of protein (dried) 80 mg per liver (average fresh weight 1.30 g). Radiocarbon bound to crude liver DNA fraction or protein was determined by dissolving a sample in Soluene (0.2–0.5 mL), cooling for 48 h, and counting by LSC. Quenching was determined by adding a known amount of [^{14}C]toluene to liver and DNA samples. Results are expressed as means ± SE with three or four replicates.

In Vitro Incubations. [^{14}C]Daminozide (succinyl or *N*-methyl labeled; 100 μM) was incubated with rat liver microsomes (0.8 mg of protein) with or without NADPH (2 mg), by itself or with 1.5 mg of calf thymus DNA or human hemoglobin in buffer solution (sodium phosphate, 100 mM, pH 7.4, 2.5 mL) for 20 min at 37 °C. Under similar conditions, [^{14}C]daminozide was incubated by itself or with horseradish peroxidase (0.4 μg), hydrogen peroxide (200 μM), or both for 20 min at 25 °C. [^{14}C]Daminozide (20 μM methyl label, 15 μM succinate label) was incubated with *d*-amino acid oxidase (0.5 mg) and human hemoglobin (1 mg) by itself or in the presence of one or both of catalase (0.42 unit to decompose the hydrogen peroxide product) and *d*-alanine (25 μM as the substrate). Protein was precipitated with trichloroacetic acid (2 vol, 20% aqueous) and DNA by adjusting the solution to 0.2 M NaCl and adding 2 vol of cold ethanol.

RESULTS

Chemical Oxidation. Model reactions in which daminozide is oxidized with 2 equiv of MCPBA, sodium hypochlorite, or an excess of hydrogen peroxide quantitatively produce succinic anhydride or succinic acid (presumably via hydrolysis of the anhydride) and numerous oxidation products from the *N*-methyl moiety. NMR chemical shifts of the major products are given in Table I. Oxidation always leads to cleavage of the succinyl and dimethylhydrazine moieties; i.e., no oxidation product retaining both moieties is ever observed. Thus, daminozide in acetone with 2 equiv of MCPBA yields succinic anhydride (100%), methyl 3-chlorobenzoate (5%), dimethylnitrosamine (DMN) (2%), and methanol (1.3%). GC–MS (80 °C) shows a peak with a retention time (R_t) corresponding to that of DMN (1.3 min) [m/z 74 (EI), 75 (CI-MS)]. TLC of the reaction mixture developed with hexane–ether (1:1) yields methyl 3-chlorobenzoate (R_f 0.8) identified by ^1H NMR [δ 8.1–7.3 (m, 4 H), 3.92 (s, 3 H)]. The oxidation is accompanied by vigorous bubbling of 0.60 equiv of nitrogen gas during the addition of 2.0 equiv of MCPBA or sodium hypochlorite. A 1-equiv portion of oxidant produces 0.3 equiv of nitrogen gas and leaves 0.5 equiv of daminozide. Oxidation of DMH with 2 equiv of MCPBA also shows vigorous bubbling of nitrogen gas and a reactive species that methylates 3-chlorobenzoic acid, indicating a possible common intermediate with daminozide oxidation. DMN and methanol are also produced from daminozide on MCPBA or hydrogen peroxide oxidation, but formaldehyde or formic acid is not detected. DMN is stable to MCPBA oxidation under these conditions.

Daminozide oxidized with 2 equiv of sodium hypochlorite in methanol-d_4 with an excess of [*O*-^2H]-4-chlorobenzoic acid yields methyl 4-chlorobenzoate (9%) [^1H NMR: δ 7.70 (AB q, 4 H), 3.92 (s, 3 H)] without any incorporation of the deuterium atom in the methyl group of the ester [GC–CI-MS confirms methyl 4-chlorobenzoate: m/z 171 (M + 1, 1 Cl), 199 (M + 29), 211 (M + 41); R_t 5.4 min; methyl silicone column, 80–240 °C programmed at 20 °C/min]. Methanol and DMN are not detected under these conditions. Daminozide in phosphate buffer (pH 6.8, 100 mM) treated with an excess of hydrogen peroxide for 24 h showed complete disappearance of starting material to quantitatively form succinic acid (^{13}C NMR), presumably via hydrolysis of succinic anhydride. The neutral/basic fraction in chloroform-*d* reveals DMN as the only product (^1H NMR), accounting for 0.4% of the starting material. Daminozide alone in buffer was stable during the same time period. Oxidation under similar conditions by hydrogen peroxide in acetone in the presence of 3-chlorobenzoic acid produces methyl 3-chlorobenzoate (neutral/basic fraction, GC–CI-MS).

Covalent Binding and Enzymatic Oxidation. Experiments with [*succinyl*-^{14}C]- and [*methyl*-^{14}C]daminozide show that both portions of the molecule covalently bind to liver protein, to the crude DNA fraction, and to hemoglobin of treated mice (Table II). The hemoglobin-bound or erythrocyte-associated radiocarbon is 14–50% of the total contained in the whole blood. Attempts to detect covalently binding species via enzymatic oxidation of [^{14}C]daminozide using either *d*-amino acid oxidase or rat

Table II. Covalent Binding of Oxidative Activation Products Derived from [*methyl*-^{14}C]- and [*succinyl*-^{14}C]Daminozide to Liver Protein and DNA Fraction and to Hemoglobin of Treated Mice and to Human Hemoglobin via Oxidation with Hydrogen Peroxide

sample	covalent binding, pmol/mg	
	N-methyl	succinyl
	In Vivo	
liver protein	0.12 ± 0.04	0.33 ± 0.13
liver DNA fraction	0.30 ± 0.13	0.24 ± 0.03
hemoglobin	1.0 ± 0.2	1.1 ± 0.3
	In Vitro	
hemoglobin/H_2O_2	26.8 ± 3.4	22.3 ± 1.4

liver microsomes fortified with NADPH were not successful.

DISCUSSION

Figure 1 shows a proposed oxidation pathway accounting for both the succinyl and *N*-methyl moieties of daminozide forming approximately equal amounts of covalently binding species, the requirement of 2 oxidizing equiv to yield 1 equiv of nitrogen gas, and the formation of small amounts of methanol. Cyclization to form succinic anhydride via oxidation is precedented by the oxidation reaction of monoacyl hydrazides by chlorine in the presence of chloride ion to yield the corresponding acid chloride and nitrogen gas (Carpino, 1957). Succinic anhydride formed in situ at the cellular level via oxidation of daminozide in vivo could be an efficient acylating agent for biological macromolecules. The intermediate DMH *N*-oxide may be a moderately reactive methylating agent, and its hydrolysis could be a source of the methanol. The leaving group for this reaction is analogous to the dialkylhydroxylamine leaving group observed in the Cope elimination reaction. Dehydration yields the diazene intermediate proposed for oxidative activation of other monoalkyl- or monoacylhydrazines (Nelson et al., 1976; Druckrey, 1973). Diazomethane formation seems unlikely since the methyl ester product of [*O*-^{2}H]-4-chlorobenzoic acid does not show the characteristic oxygen–deuterium insertion. Daminozide may be a poor substrate for oxidases either because of the hydrophilic nature conferred by the carboxylate moiety or because of its unique structural features. However, human hemoglobin reacts with daminozide via direct oxidation with hydrogen peroxide, giving approximately equal incorporation with either the [^{14}C]succinyl- or [^{14}C]methyl-labeled material. This equivalent incorporation seen in vitro is also seen in vivo with labeling of liver protein, liver DNA fraction and hemoglobin, and can be rationalized by the chemical oxidation scheme in Figure 1, which yields reactive species from both the succinyl and *N*-methyl moieties. Labeling of the crude DNA fraction is probably due in the most part to derivatization of DNA itself rather than a small amount of protein possibly present since similar binding levels are found for the protein and DNA fractions.

Possibly the porphyrin in the hemoglobin catalyzed the hydrogen peroxide oxidation of daminozide, although ferric salts and EDTA did not accelerate the oxidation by hydrogen peroxide. In vivo binding of daminozide may be due to direct oxidation with endogenous hydrogen peroxide. Steady-state peroxisomal hydrogen peroxide concentrations have been estimated to be approximately 0.02–0.05 mM in the presence of suitable substrates such as glycolate or benzphetamine (Jones et al., 1981) compared to 0.2 mM used in the present study.

The 3- to 5-fold ratio of covalent binding to hemoglobin versus crude liver DNA fraction can be compared to a 3- to 8-fold ratio for the alkylation of hemoglobin versus DNA of liver, spleen, and testes of mice exposed to ethylene oxide (Segerbäck, 1983). Covalent binding to hemoglobin in vivo is proposed as a generic dose monitor for carcinogens (Pereira and Chang, 1981). The covalent binding index (micromole of chemical bound per mole of nucleotide divided by millimole of dose per kilogram of body weight) of 15–16 for daminozide compares to 17 000 for aflatoxin B_1, 2310 for DMN, 525 for vinyl chloride, 47 for 1,2-dichloroethane, and 10 for benzene (Lutz, 1979; Arfellini et al., 1985).

Oxidation of daminozide to the diazo hydroxide yields an efficient methylating intermediate responsible for methylation of 3- or 4-chlorobenzoic acid. Analogous bioxidations may be involved in the derivatization of biological macromolecules.

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Registry No. MCPBA, 937-14-4; DMN, 62-75-9; daminozide, 1596-84-5; sodium hypochlorite, 7681-52-9; hydrogen peroxide, 7722-84-1; succinic anhydride, 108-30-5; methanol, 67-56-1; nitrogen, 7727-37-9; succinic acid, 110-15-6; 1,1-dimethylhydrazine, 57-14-7.

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Case Study of the Depletion of Sulfamethazine from Plasma and Tissues upon Oral Administration to Piglets Affected with Atrophic Rhinitis

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Administration of 1075 mg of sulfamethazine (SMZ)/kg of feed for 3 weeks to piglets affected with atrophic rhinitis resulted in a plasma level of SMZ during the medication period of 45 ± 6 mg/L, while the extent of N^4-acetylation (N^4-Ac-SMZ) was 10–18%. Furthermore, small amounts of a deaminated metabolite of SMZ have been detected in plasma and tissues. The half-life of SMZ in plasma was 11 h. Elimination of SMZ and N^4-Ac-SMZ from organs and tissues was rapid in the beginning, at high plasma levels ($t_{1/2}$ = 10–14 h), but was much slower at low residual levels ($t_{1/2}$ = 3–9 days). A withdrawal period of approximately 18 days should be considered in order to meet the generally accepted tolerance level for sulfonamide residues of 0.1 mg/kg.

Sulfonamide drugs are widely used for therapeutic and preventive treatment of bacterial infections caused by *Streptococcus spp*, *Staphylococcus spp.*, *Pasteurella multocida*, *Escherichia coli*, *Bordetella bronchiseptica*, or *Haemophilus spp.*

Sulfamethazine (SMZ) is commonly administered to piglets or swine in a concentration of 400 or 200 g/1000 kg of feed, respectively, for treatment of pneumonia, enteritis, or atrophic rhinitis. Atrophic rhinitis (AR), a frequently occurring disease in pigs, is characterized by atrophy of the nasal conchae and excessive space in the nasal cavity. Since the efficacy of the SMZ dosages for preventive treatment of AR has been questioned, administration of higher SMZ doses through feed to piglets and swine is not uncommon in veterinary practice.

However, information on the clinical efficacy of such a treatment and on the depletion of SMZ residues from edible tissues after medication withdrawal is limited. Therefore, a study has been performed with piglets housed on a farm with atrophic rhinitis problems, which were treated with feed containing 1075 mg of SMZ/kg for 3 weeks.

This paper reports the depletion of residues of SMZ and metabolites from plasma and tissues of diseased animals treated with SMZ under practical conditions. This information may be used for the establishment of a proper withdrawal period for the drug before slaughter.

MATERIALS AND METHODS

Twenty-seven 4–5-week-old piglets (great Yorkshire × Large White) were divided after weaning into three groups with comparable body weight, sex, and bacteriological scoring on *Pasteurella multocida* (van Leengoed and Kamp, 1986). The animals were born, weaned, and housed on a farm with a moderate degree of atrophic rhinitis. All animals received creepfeed containing 50 mg/kg apramycin for 5 days after weaning. Subsequently control group A (12 animals, 9.2 ± 2.8 kg body weight) received pelleted feed with a Cu content of 150 mg/kg. Group B (15 animals, body weights 9.5 ± 3.7 kg) received similar feed for 21 days with 1075 mg of SMZ/kg additionally. Premix, medicated, and blank feed samples contained barley, corn, soy flour, wheat, tapioca, linseed meal, calcium carbonate, dicalcium phosphate, sodium chloride, and salts of copper, zinc, manganese, and iron, as determined by microscopic analysis (*Manual of Microscopical Analysis of Feedstuffs*, 1978).

Control feed and medicated feed were analyzed for SMZ content with a simple HPLC method. Five grams of ground feed was extracted in 130 mL of boiling 0.02 M sodium hydroxide. After centrifugation, the aqueous phase was neutralized with hydrochloric acid and filtrated after further centrifugation. After addition of $NaHCO_3$, the solution was filtered and injected onto an HPLC system. A μBondapack C_{18} column (Waters Associates) was used with water/acetonitrile/acetic acid (850/75/8.5) as eluent and detection at 254 nm. With this method SMZ concentrations from 0.5 to 1500 mg/kg of feed could be determined.

In both feeds the absence of the following antibiotics, chemotherapeutics, and growth promotors was established (<2 mg/kg): Zn-bacitracine, spiramycine, virginiamycine, flavophospholipol, tylosine, monensin, avoparcine, amprolium, ethopabate, dinitolmide, dimetridazole, clopidol, decoquinate, robemidine, ronidazol, ipronidazol, methylbenzoquate, arprinocide, lasalocide, narasin, salinomycine, nicarbazin, furazolidone, nitrovin, carbadox, olaquindox, sulfadimethoxine, sulfanilamide, sulfaquinoxaline, sulfadiazine, sulfadoxine. The feeds contained approximately 50 and 0.5 mg of nitrite/kg. The following elements were determined. ICP-AAS technique: Ca (0.84%), Mg

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