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PRODUCTION OF ETHANE BY RATS TREATED WITH THE COLON CARCINOGEN, 1,2-DIMETHYLHYDRAZINE

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Abstract—Ethane was exhaled by rats treated with the colon carcinogen, 1,2-dimethylhydrazine (DMH). At 1 hr, ethane production (mean ± SD) was 0.2 ± 0.2 nmol/kg (controls) and 5.2 ± 1.3, 13.7 ± 3.4, and 27.7 ± 9.6, respectively, for DMH injections of 0.15 mmol/kg (20 mg/kg of the dihydrochloride salt), 0.45 mmol/kg, and 1.35 mmol/kg. Rates of ethane evolution tapered off after 2 hr, but persisted for up to 3 hr at the lower dose, and up to 5–6 hr at the higher dose. Although ethane is produced *in vivo* during lipid peroxidation, experiments with vitamin E, a potent lipid antioxidant, indicated that lipid peroxidation was unlikely to be the source of ethane in DMH-treated rats: pretreatment with vitamin E had no effect on ethane formation from DMH but did suppress ethane production from rats treated with carbon tetrachloride, an inducer of hepatic lipid peroxidation. When rats were injected with 1,2-diethylhydrazine in place of DMH, large amounts of ethane and ethylene were produced (9800 and 5600 nmol/kg/hr). The hydrocarbon gases exhaled by rats may arise from dimerization of methyl radicals ($\cdot CH_3$) generated during the metabolism of DMH, and from ethyl radicals ($\cdot CH_2CH_3$) generated during the metabolism of 1,2-diethylhydrazine. Previously, it was shown that methane and ethane are formed from methyl radicals *in vitro*. Other investigators have observed formation of hydrocarbon gases during the *in vitro* metabolism of monoalkylhydrazines by microsomes, and ethyl radicals, derived from monoethylhydrazine, have been detected by electron spin-resonance spectroscopy. The results presented here suggest that *in vivo* metabolism of DMH may produce methyl radicals. Methyl radicals are capable of interacting with biomolecules. Their indiscriminate reaction with tissue constituents may be a contributory factor in DMH-induced carcinogenesis.

Remarkable progress in the understanding of colon cancer has been made possible by the discovery of the colon carcinogen, 1,2-dimethylhydrazine (DMH‖). Druckrey [1] showed that DMH and azoxymethane induce colon tumors in rodents. These agents are highly colon specific, and the characteristics of the lesions produced are remarkably similar to those seen in humans. Although DMH has now been widely used for the study of colon cancer, its carcinogenic mechanism, as well as the reason for its high colon specificity, are not clearly understood. Druckrey [1] proposed that DMH is metabolized to the proximate carcinogen, methylazoxymethanol, which is further transformed to an ultimate carcinogen that methylates DNA. As to the carcinogenic mechanism for methylazoxymethanol, there is no general agreement. Miller and Miller [2] suggested that methylazoxymethanol is converted to the methyl carbonium ion, which methylates DNA. Grab and Zedeck [3] found that alcohol dehydrogenase, present in the colon, catalyzes the transformation of methylazoxymethanol to methylazoxyformaldehyde. They proposed that this aldehyde may be the ultimate carcinogen.

Diets high in lipid content promote an increased rate of colon tumorigenesis in DMH-treated rats [4, 5]. The mechanism by which dietary lipids play a role in cancer induction in the colon is not known. However, the spontaneous auto-oxidation of polyunsaturated fatty acids produces highly reactive species such as free radicals, peroxides, and aldehydes. Some lipid peroxidation products have been suggested to be mutagens [6].

We were conducting studies to determine whether or not lipid peroxidation was associated with DMH-induced tumorigenesis. Exhaled ethane was measured as an index of *in vivo* lipid peroxidation. Ethane and other alkanes have been detected as byproducts of lipid peroxidation [7–9]. We observed significant ethane production by rats immediately after the i.p. injection of DMH. This report characterizes the ethane produced by DMH and compares it to hydrocarbon gas production after injection of the ethyl analogue, DEH.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 160–200 g were purchased from Perfection Breeders, Douglasville, PA. Animals were given standard Purina rat chow with free access to water. Food was withheld 20 hr prior to the experiments. 1.2-Dimethylhydrazine dihydrochloride and 1,2-diethylhydrazine dihydrochloride were purchased from the Aldrich Chemical Co., Milwaukee, WI; carbon

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‖ Abbreviations: DMH, 1,2-dimethylhydrazine; DEH, 1,2-diethylhydrazine; CCl_4, carbon tetrachloride.

Table 1. Cumulative ethane production following injection of DMH

Time (hr)	Ethane (nmol/kg)			
	Control	0.15 mmol DMH/kg	0.45 mmol DMH/kg	1.35 mmol DMH/kg
1	0.2 ± 0.2	5.2 ± 1.3	13.7 ± 3.4	27.7 ± 9.6
2	0.4 ± 0.4	9.3 ± 1.1	25.5 ± 6.7	59.8 ± 19.4
3	0.4 ± 0.4	11.6 ± 2.0	32.6 ± 8.2	81.4 ± 26.5
4	1.0 ± 0.5	12.4 ± 2.1	37.0 ± 8.1	92.0 ± 27.8
5	1.2 ± 0.7	13.2 ± 1.6	38.1 ± 8.9	99.7 ± 32.2
6	1.4 ± 0.7	13.8 ± 2.0	37.3 ± 9.8	103.2 ± 33.5

Rats received i.p. injections (0.5 ml) of DMH or saline (controls). Data are the mean ± SD for N = 4, except for 1.35 mmol/kg at 6 hr where N = 3. Each experimental value was significantly greater than the corresponding control ($P < 0.001$).

tetrachloride (CCl_4) from the Fisher Scientific Co., Fair Lawn, NJ; vitamin E (alpha-tocopherol acetate) from the Sigma Chemical Co., St. Louis, MO; and calibration gases (methane, ethane and ethylene) from Alltech Associates, Inc., Deerfield, IL. Light mineral oil and sesame oil were from Cumberland, Smyrna, IN, and JFC International Inc., San Francisco, CA, respectively. All solutions for injection were prepared immediately before use and were injected i.p.

The HCl salts of DMH and DEH were dissolved in distilled water and neutralized to pH 6.5 with NaOH. Animals received the hydrazines by i.p. injection in a volume of 0.5 ml. Control animals received the identical volume of 0.9% (w/v) sodium chloride. Immediately after the injections, animals were placed into breath collection chambers. The methodological details for maintaining the chambers, sampling the atmosphere, and analyzing for hydrocarbon gases by gas chromatography are described elsewhere [10]. Briefly, the chamber air was cycled continuously via an oscillating pump (100–200 ml/min) through a series of traps: 1 N KOH, 1 N H_2SO_4, and a cold-finger in a dry-ice/2-propanol bath. Oxygen entered the system to replace respiratory CO_2. The pump was turned on for 5 min before a zero-time sample (50 ml) was removed by syringe for analysis of hydrocarbon gases. Data for subsequent 50-ml samples were expressed as the increment in hydrocarbon content with respect to zero time. Chromatography was performed with a Hewlett–Packard model 5750 gas chromatograph that was equipped with a gas concentrator (Chemical Data Systems model 310), a 1.8 m steel column packed with Porapak N (60/80 mesh), and a flame ionization detector. One half of the concentrator trap was packed with activated charcoal for adsorbing (at room temperature) short-chain hydrocarbon gases including ethane and ethylene, and the other half with activated alumina for pentane and other contaminants. The trap was programmed to desorb ethane and ethylene into the gas chromatograph at 250°.

Alpha-tocopherol acetate was dissolved in sesame oil as described by Wattenberg [11]. Animals were injected i.p. at a dose of 150 mg/kg (150 mg/ml sesame oil). The controls received equal volumes of sesame oil. Animals were treated once a day for 3 days. The last injection was given 20 hr prior to the experiment.

CCl_4 was diluted with light mineral oil to make a 30% (v/v) solution. Animals were injected i.p. with a dose of 1.5 g CCl_4/kg. The control animals received corresponding volumes of light mineral oil.

RESULTS

Ethane production following DMH treatment. Table 1 shows the cumulative production of ethane by rats after treatment with DMH and by control animals injected with saline. Control animals produced small amounts of ethane, while those injected with DMH had significantly increased ethane production. Even at the lowest dose studied (0.15 mmol/kg or 20 mg/kg), at which dosage, multiple weekly injections are often used to induce colon carcinomas in rats, DMH produced a more than 25-fold increase in ethane over control rats during the first hour. The amount of ethane produced was dependent upon the dose of DMH injected. With each of the concentrations studied, the rate of ethane formation declined after the second hour. Ethane production persisted for up to 5–6 hr at the highest dose, and for up to 3 hr at the lowest dose. The low levels of ethane evolved by control animals have been reported previously [12] and may represent endogenous lipid peroxidation.

Vitamin E and ethane production. Ethane gas can be produced as a byproduct during lipid peroxidation *in vitro* and *in vivo* [7–9]. Ethane production due to lipid peroxidation is inhibited effectively by the lipid antioxidant, vitamin E [7, 8]. We used vitamin E to determine whether or not ethane formed after injection of DMH was due to lipid peroxidation. CCl_4 was used as a positive control since it is known that CCl_4 stimulates ethane production as a result of hepatic lipid peroxidation and that vitamin E inhibits lipid peroxidation and consequent ethane production.

Figure 1 shows the effect of vitamin E on ethane production after i.p. injections of DMH or CCl_4. Following treatment with CCl_4 (Fig. 1A), ethane was produced. In rats pretreated with vitamin E, significantly less ethane was formed. This is in agreement with previous reports [7, 8]. Figure 1B shows a similar experiment in which DMH was substituted

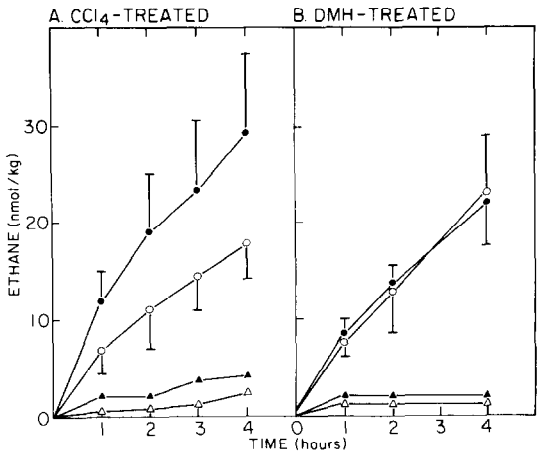

Fig. 1. Ethane production by rats treated with either CCl_4 (A) or DMH (B). Rats received i.p. injections of either CCl_4 (1.5 g/kg) or DMH (0.15 mmol/kg); controls received the appropriate vehicle (light mineral oil or isotonic saline respectively). The animals had been pretreated for 3 days with either vitamin E (alpha-tocopherol acetate, 150 mg/kg) in sesame oil or sesame oil alone. Pretreatment with vitamin E then CCl_4 injection (panel A, $P < 0.01$ at 1, 2, and 4 hr, and $P < 0.02$ at 3 hr); pretreatment with vitamin E then DMH-injection (panel B). Data are the mean ± SD. Panel A: (▲) sesame oil + mineral oil (N = 2); (△) vitamine E + mineral oil (N = 2); (●) sesame oil + CCl_4 (N = 7); and (○) vitamin E + CCl_4 (N = 7). Panel B: (▲) sesame oil + saline (N = 2); (△) vitamin E + saline (N = 2); (●) sesame oil + DMH (N = 8); and (○) vitamine E + DMH (N = 8).

for CCl_4. The dose of DMH was selected to give a comparable rate of ethane production as CCl_4. In contrast to the results with CCl_4, vitamin E had no significant effect on ethane production by DMH-treated rats.

Methane, ethane and ethylene production in response to DMH or DEH. In addition to ethane, other short-chain hydrocarbon gases were measured in the chamber environment of rats treated with DMH. As shown in Table 2, methane was increased by 57%, but the increase was not statistically significant. The methane exhaled by control animals indicates the presence of methanogenic bacteria in the colon [13]. DMH did not increase ethylene production.

Animals treated with DEH produced markedly increased amounts of ethane and ethylene. At the same dose of hydrazine compound (0.15 mmol/kg), the amounts of ethane evolved by DEH-treated rats were 1000-fold higher than the amounts produced by DMH-treated rats. At the same time, ethylene production was similarly augmented compared to control or DMH-treated rats.

Azomethane, a metabolite of DMH that appears in expired air [14], generates methyl radicals on heating [15]. Therefore, it was possible that the observed ethane in experiments with rats arose artifactually from the breakdown of azomethane during desorption of bound gases from the concentrator trap of the gas chromatograph at 250°. Fiala *et al.* [14] eliminated azomethane and other gaseous contaminants from expired air with a series of gas-trapping solutions consisting of ethanol at −72°, and 1 N KOH and 1 N H_2SO_4 at room temperature. As these authors indicated, azomethane should be quantitatively removed by the 1 N H_2SO_4, which was also part of our chamber air-scrubbing system. In a separate set of experiments with DMH-injected rats, levels of ethane were essentially the same for animals in chambers scrubbed with the sequence described by Fiala *et al.* [14] versus our scrubbing system, which consisted of 1 N KOH, 1 N H_2SO_4, and a cold-finger trap in a dry-ice/2-propanol bath (data not shown). Therefore, the observed ethane did not derive from exhaled azomethane or other metabolites.

DISCUSSION

Significantly increased ethane exhalation was noted following the administration of DMH to rats (Table 1). The amounts of ethane was dose dependent within the range of DMH doses that are used to induce colon carcinomas. The production of ethane leveled off after 2 hr. When DEH was used in place of DMH, the rats produced very much larger amounts of ethane and ethylene (Table 2). Formation of ethane or ethylene *in vivo* after injection of DMH or DEH has not been described previously.

A number of investigators [7–9] have observed that ethane gas is evolved during *in vivo* lipid peroxidation in experimental animals and that treatment with vitamin E, a lipid antioxidant, reduces ethane formation by inhibiting lipid peroxidation. In our experiments, vitamin E had no effect on ethane production by DMH (Fig. 1B). In parallel experiments, vitamin E did suppress ethane production by

Table 2. Hydrocarbon gas production by rats receiving DMH or DEH

Exptl. group	Hydrocarbons (nmol/kg/hr)		
	Methane	Ethane	Ethylene
Control	10.7 ± 9.3 (7)	0.2 ± 0.4 (4)	1.9 ± 1.8 (8)
DMH (0.15 mmol/kg)	16.8 ± 5.6 (4)	8.2 ± 0.7* (4)	1.5 ± 0.5 (4)
DEH (0.15 mmol/kg)	8.8 ± 5.5 (5)	9839 ± 3093* (5)	5642 ± 1057* (5)

DMH or DEH was administered i.p. (0.15 mmol/kg). Hydrocarbon gas production is the hourly rate (mean ± SD) during the first 2 hr. The number of animals is shown in parentheses.
* $P < 0.001$ compared to corresponding control.

CCl_4, a hepatic lipid peroxidation inducer (Fig. 1A). These results suggest that the ethane produced in DMH-treated rats did not result from lipid peroxidation. Artifactual production of ethane from the breakdown of gaseous metabolites from DMH in the gas chromatograph was also excluded (see Results).

An alternative source for ethane may be the dimerization of methyl radicals during DMH metabolism. A number of reports (e.g. Refs. 16–18) have shown that methane and ethane are formed from methyl radicals *in vitro*. Methane is the result of hydrogen abstraction, whereas ethane is derived by dimerization of methyl radicals. However, the major product is formaldehyde [19].

Other investigators [20, 21] have observed formation of alkane gases during the *in vitro* metabolism of monoalkylhydrazines by microsomal fractions of rat liver. Spin-trapped ethyl radicals have been observed by electron spin resonance spectroscopy during the metabolism of monoethylhydrazine by liver microsomes [22]. The detection of dimerized products, such as diphenyl from *N*-phenyl-*N*-benzoyldiimide *in vitro* [23] and hexachloroethane from CCl_4 *in vivo* [24], has been cited as evidence for the intermediate formation of phenyl radicals and trichloromethyl radicals respectively. The detection of ethyl radicals during the metabolism of monoethylhydrazine by liver microsomes [22] implies that tissue pathways exist for the formation of alkyl radicals from mono- or dialkylhydrazines. The observed formation of both ethane and ethylene from the metabolism of DEH *in vivo* (Table 2) is in keeping with the formation of intermediary ethyl radicals: ethane represents the result of hydrogen abstraction from donor molecules, while ethylene can be formed by hydrogen atom loss. Both ethane and ethylene have been observed during metabolism of monoethylhydrazine by liver or lung microsomes [25].

The amounts of ethane and ethylene evolved by rats after DEH treatment were very much higher than the quantity of ethane produced from DMH at the same dose. This result is expected in view of the kinetic limitation imposed for the formation of ethane from collision of two methyl radicals, compared to the more facile formation from the interaction of ethyl radicals with a variety of hydrogen donors or acceptors. An attempt to measure butane, the dimeric form of ethyl radicals, was impeded by technical difficulties, such as a high background level and multiple non-specific peaks at the required high operating temperature. Methyl radicals react readily with oxygen to produce formaldehyde as an end product [19]; the relative reactivity of ethyl radicals with oxygen has not been clarified. Measurement of formaldehyde has been used to monitor DMH metabolism by human colon microsomes and colon tumor cells in culture [26]; however, these observations do not indicate the formation of methyl radicals, as formaldehyde is a normal product in oxidative demethylation.

Methyl radicals are a reactive species. They may be expected to undergo a number of reactions including abstraction of univalent atoms such as hydrogen atoms or halogens, substitution in heterocyclic bases, and addition to unsaturated bonds [27, 28]. Further investigation is warranted to characterize the nature of the effects that methyl radicals may exert on biomolecules. Via these reactions, methyl radicals could cause alterations in cellular integrity.

Several methylated residues of nucleic acids have been described after DMH administration. Hawks and Magee [29] detected 7-methylguanine from DNA and RNA of liver, kidney, lung, spleen, small intestine, and large intestine after injections of 15 and 200 mg DMH/kg into rats and mice. Rogers and Pegg [30] detected 7-methylguanine, O^6-methylguanine, and 1-, 3-, and 7-methyladenine from DNA of rat liver, colon, and kidney following administration of 200 mg DMH/kg. Methyl radicals are nucleophilic [31] and would not be expected to methylate guanine in the 7-position or the O^6-position. Therefore, it appears that methyl radicals do not play a major role in methylating nucleic acids *in vivo*. However, a number of researchers [30, 32, 33] have suggested that the mechanism for DMH-induced tumorigenesis may involve more than DNA alteration. It may involve modifications of macromolecules such as enzymes or receptor proteins that are important in the regulation of transcription, replication, or repair of genetic material.

The mechanism of cancer induction by DMH may involve multiple factors. The data presented here suggest that methyl radicals may be formed *in vivo* during DMH metabolism. Since methyl radicals are highly reactive, we suggest that this species may contribute to DMH-induced carcinogenesis.

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