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STUDIES ON THE MECHANISM OF ACTION OF HYDRAZINE-INDUCED METHYLATION OF DNA GUANINE

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

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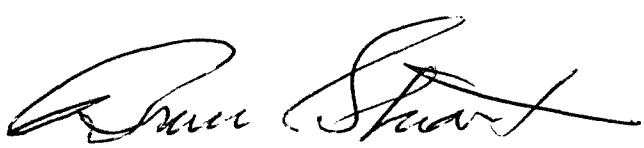

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Hydrazine is a strong reducing agent and is widely used by industry and by the military as a rocket propellant; it is a potent hepatotoxin and a weak carcinogen. Hydrazine administration to rodents results in methylation of DNA guanine. The methyl moiety in these aberrant bases can be derived from either methionine or formate; however, earlier studies could not provide evidence to support monomethylhydrazine as an important intermediate in this unusual methylation of DNA. It was proposed that formaldehyde hydrazone, CH_2=N-NH, which in turn could be metabolically oxidized to the potent methylating agent, diazomethane ($^-CH_2$-N^+=N). Several in vivo studies were carried out to determine the role of aldehydes in the alkylation of DNA in response to hydrazine administration. While tissue levels of acetaldehyde could readily be altered by administration of ethanol, formaldehyde levels were nearly refractory to several techniques designed to raise the concentration of the one-carbon unit in

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rodent liver. Pretreatment of rats and hamsters with disulfiram, an inhibitor of aldehyde dehydrogenase, sucessfully raised formaldehyde or acetaldehyde levels following administration of methanol or ethanol.

Pretreatment of rats and hamsters with disulfiram or cyanamide raised formaldehyde or acetaldehyde levels following administration of methanol or ethanol but led to lower than expected levels of DNA methylation after hydrazine administration. DNA guanine was methylated when incubated with hydrazine and formaldehyde, but only when liver S-9, cytosolic, or microsomal fractions were present. Monomethylhydrazine produced little 7-methylguanine. These results do not strongly support formation of formaldehyde hydrazone and diazoalkane as important intermediates in DNA methylation in hydrazine-treated animals, but do suggest that aldehyde dehydrogenase is important in the activation of a hydrazine-form-aldehyde reaction product. Pyrazole blocked hydrazine-induced DNA methylation, suggesting that alcohol dehydrogenase was important in hydrazine-induced methylation of DNA. A new hypothesis for this methylation reaction suggests that hydrazine reacts spontaneously with endogenous formaldehyde to form formaldazine, which could form methylazomethanol and methylazoxymethanol.

A two year study was begun to correlate DNA methylation levels at 6-month intervals with development of liver cancer. The first six month interval revealed dose-independent levels of 7-methylguanine and dose-dependent levels of O^6-methylguanine in liver DNA; no 7-methylguanine was detected in DNA from kidney and lung, but O^6-methylguanine was measured in DNA from these tissues, suggesting some degree of accumulation of this promutagenic base in these lesser target organs.

Hamsters were given 90 mg hydrazine/kg body wt p.o., i.p., or s.c., and 7-methylguanine and O^6-methylguanine were detected from liver, kidney, and lung but not brain; route of administration had little effect on extent of alkylation.

Hydralazine and isoniazid were adminstratered to rats and mice; twenty-four hours later liver DNA from mice treated with either drug contained 7-methylguanine as did liver DNA from rats given isoniazid (but not hydrazine).

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism Part 1: Carcinogenesis and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract Number F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1983 through May 1984.

R. C. Shank, Ph.D., was principal investigator for the subprogram. Acknowledgement is made to Steven Tovani for his technical assistance. M. Pinkerton, Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Hydrazine (NH_2-NH_2) is a strong reducing agent that is widely used in industry and by the military. The compound is acutely toxic to the liver, kidney and central nervous system; hydrazine at 60 mg/kg body wt rapidly produces a fatty liver in fed animals and, in addition, is necrogenic to the liver at higher doses in fasted animals (Witkin, 1956; Amenta and Johnston, 1962; Shank, 1981; Scales and Timbrell, 1982). Hydrazine is rapidly oxidized in the body to nitrogen (N_2) and diimide (HN=NH) and is acetylated to mono- and diacetylhydrazine ($CH_3CONHNHCOCH_3$); approximately 35% of the hydrazine is expired as nitrogen and 30-40% is excreted in the urine as unreacted hydrazine and hydrolyzable conjugates (Dost et al., 1979; Nelson and Gordon, 1980; Springer et al., 1981).

Under appropriate conditions, hydrazine exposure can result in an increase in cancer incidence in the test animal population. One exposure condition that appears necessary for cancer induction is chronic irritation or toxicity. In a chronic inhalation study on hydrazine in three rodent species, squamous cell carcinomas of the nasal turbinates were detected only in the group with the highest exposure, which produced severe rhinitis with focal hyperplasia and squamous metaplasia early in the experiment (MacEwen et al., 1981). Upon oral administration at a dose which was growth-depressing, hydrazine induced hepatocellular carcinomas and lung adenocarcinomas in male rats (Severi and Biancifiori, 1968), hepatocellular carcinomas (Biancifiori, 1970), and pulmonary adenocarcinomas (Severi and Biancifiori, 1968; Biancifiori and Ribacchi, 1962), and an increased incidence of lung adenomas (Yamamoto and Weisburger, 1970) in mice.

Most chemical carcinogens examined so far have proven to form highly reactive electrophiles in target tissue, resulting in covalent binding of the carcinogen to DNA and other macromolecules (Miller and Miller, 1977). DNA adducts are potentially damaging to the genetic material and, through a variety of suggested mechanisms, thought to lead to mutation in the somatic target cells. Investigators have not been able to detect adduct formation between hydrazine and DNA under near physiological conditions, but formation of methylated guanines in DNA following hydrazine administration has been demonstrated (Barrows and Shank, 1978; 1981; Quinter-Ruiz et al., 1981), and such adducts are thought to be relevant to carcinogenesis (Lawley, 1976). There has been some suggestion in the several studies that the methylation of liver DNA in hydrazine-treated animals may be causally linked to the hepatotoxicity of the chemical agent (Barrows and Shank, 1981; Becker et al., 1981; Bosan and Shank, 1983). If it can be proven that DNA methylation resulting from hydrazine exposure is indeed linked to hydrazine-induced hepatotoxicity, and that DNA methylation is causally linked to carcinogenesis, then it may be proposed that exposures to hydrazine which are below those which cause toxicity would be unlikely to induce cancer.

Hydrazine is not the only compound for which an association appears between toxicity and carcinogenicity. Rats (Reuber and Glover, 1967; 1970), mice (Edwards, 1941; Edwards and Dalton, 1942; Eschenbrenner and Miller, 1945) and hamsters (Della Porta et al., 1961) develop liver tumors after receiving liver-necrotizing doses of carbon tetrachloride repeatedly for several weeks but not when the exposure is to non-necrotizing doses. Vinylidene chloride at renal-necrotizing doses produces adenocarcinomas in mouse kidney but not in rat or hamster kidney when these animals are given non-necrotizing doses (Maltoni, 1977; Maltoni et al., 1977). Rats exposed by inhalation to formaldehyde vapors at 15 ppm, 6 hours/day, 5 days/week for 18 months developed highly irritated nasal epithelial tissue including papillary hyperplasia and squamous atypia and then squamous cell carcinomas (Swenberg et al., 1980). Rats exposed to 2 or 6 ppm formaldehyde developed less severe nasal irritation and no cancer; mice exposed to the same concentrations of formaldehyde

failed to produce any nasal tumors. A similar experiment with hydrazine using rats, mice, and hamsters (cited above) also produced carcinomas of the nasal turbinates only in rats at only that dose which was severely irritating to the nasal epithelium; exposure at lower doses, which were less irritating, failed to produce the tumors (MacEwen et al., 1981). The studies with hydrazine, then, may serve as a model in biochemical investigations of the causal relationship, if any, between toxicity and carcinogenicity.

Several mechanisms for methylation of DNA guanine resulting from administration of hydrazine have been proposed and investigated. One such mechanism suggested the hepatotoxin induced a direct chemical methylation of nucleophilic sites in DNA by S-adenosylmethionine (Barrows, 1980; Becker et al., 1981). S-adenosylmethionine can methylate DNA nonenzymatically, producing 7-methylguanine and possibly O^6-methylguanine and 3-methyladenine (Barrows and Magee, 1982; Rydberg and Lindahl, 1982). This may also offer an explanation for the presence of enzymes in normal mammalian cells for the specific removal of these aberrant bases from DNA. Quantitatively, however, methylation of DNA guanine in response to hydrazine administration is much greater than could be accounted for by a chemical methylation by endogenous S-adenosylmethionine (Barrows and Magee, 1982). It was also suggested that hydrazine may increase the levels of endogenous S-adenosylmethionine or alter the intracellular distribution of this methylating intermediate in the cell; increasing the endogenous levels of S-adenosylmethionine 30-fold by injecting guinea pigs with methionine, however, did not increase the levels of methylguanines in liver DNA (Shank and Bosan, 1983).

It has also been suggested that hydrazine administration may result in a loss of specificity of DNA methyltransferase that normally transfers the methyl moiety from S-adenosylmethionine to the 5-position carbon atom in cytosine, producing the only normal methylated base in mammalian DNA (Becker et al., 1981). The N-7 position of guanine closely resembles the 5-position of cytosine and both positions are in the major groove of the DNA helix; also most 5-methylcytosine residues occur in the dinucleotide sequence 5'CpG (Wigler et al., 1981) providing close proximity of the N-7 position of guanine and the normal substrate site for DNA methyltransferase. Little evidence has been obtained so far to support or refute this hypothesis, but the simultaneous methylation of the O-6 atom in guanine, a site not similar to the 5-position in cytosine, is difficult to explain by the suggested loss of specificity of DNA methyltransferase and would seem to require a radical alteration in substrate structure or enzyme specificity to accommodate the experimental observations obtained so far.

It was suggested (Barrows and Shank, 1980; Barrows, 1980; Quinter-Ruiz et al., 1981) that hydrazine may be methylated in vivo by S-adenosylmethionine to form monomethylhydrazine, which is a known DNA methylating intermediate but not a hepatotoxin (Hawks and Magee, 1974). Since 75% of the hydrazine administered to animals has been accounted for as expired nitrogen gas and as excreted acetylhydrazines and unsubstituted hydrazine, only a quarter of the administered compound could be available for methylation to monomethylhydrazine. Administration of hydrazine together with ^{14}C-labeled formate (Quinter-Ruiz et al., 1981) or S-adenosylmethionine (Barrows and Shank, 1980; 1981) results in the formation of ^{14}C-labeled methylguanines in liver DNA in rats and mice. On an equal molar basis, however, hydrazine is more efficient in producing methylguanines in liver DNA of mice than is monomethylhydrazine, and in the hamster administration of monomethylhydrazine even at doses exceeding the LD_{50} failed to produce methylguanines in liver DNA although hydrazine in the hamster is effective at doses approaching one-tenth the LD_{50} (Bosan and Shank, 1983). Monomethylhydrazine is rapidly absorbed through skin and distributed in the body water (Smith and Clark, 1969) and thus it is unlikely that administration of hydrazine to

animals could result in higher intracellular levels of monomethylhydrazine than in animals given monomethylhydrazine directly. Current methods capable of quantitatively measuring both hydrazine and monomethylhydrazine in biological fluids are unable to distinguish between the two compounds with adequate sensitivity, precluding direct measurement of intracellular methylation of hydrazine. In addition, monomethylhydrazine does not appear to be carcinogenic to the rat or mouse (Kelly and O'Gara, 1965; Roe et al., 1967; Kelly et al., 1969; Mirvish et al., 1969, Hawks et al., 1974). The experimental evidence to date, then, does not support the hypothesis that monomethylhydrazine is an important intermediate in the methylation of liver DNA in rodents given hydrazine.

It was suggested (Biancifiori and Severi, 1966) that hydrazine might react with endogenous formaldehyde to form a hydrazone which could then be oxidized to the strong methylating agent, diazomethane:

$$HCHO + H_2N\text{-}NH_2 \longrightarrow \left[HC(OH)NH\text{-}NH_2\right] \longrightarrow CH_2{=}N\text{-}NH_2 \longrightarrow {}^{-}CH_2\text{-}N^{+}{\equiv}N$$

This hypothesis is consistent with the observation that co-administration of either ^{14}C-formate (Quinter-Ruiz et al., 1981) or ^{14}C-methyl(methionine) (Barrows and Shank, 1978) with hydrazine results in ^{14}C-labeled 7-methylguanine and O^6-methylguanine. This proposed mechanism involving the formation of a hydrazone intermediate in the hydrazine-induced methylation of DNA guanine is the focus of the present report; in addition, study of this novel response by hydrazine has been expanded to investigations on the accumulation of these aberrant methylguanines in hamsters exposed chronically to hydrazine in their drinking water, to occurrence of the methylation response in tissues other than liver, and to the response by the hydrazino drugs, hydralazine and isoniazid.

RESEARCH PROGRAM

Methods

Animals

All animals were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. The animals were housed over sterilized hardwood chips and maintained on commercial chow diets and deionized water 1-3 weeks before use. All animals were fasted overnight before treatment; hydrazine was administered between 8:00 am and 10:00 am. Animals were killed by decapitation or, in the case of the chronic study, by halothane overdose; tissues were rapidly removed and stored at -70°C.

DNA Isolation and Chromatographic Analysis

DNA was isolated and purified from fresh or frozen tissue by the phenolic extraction method of Kirby (1962) as modified by Swann and Magee (1968). The DNA was dissolved in 10 mM sodium cacodylate pH 7.0 and heated to 100°C for 35 min to preferentially release 7-methylguanine from the polymer; the partially apurinic DNA was precipitated from cold neutral thermal hydrolysate with HCl and hydrolyzed in 0.1 M HCl (5 mg DNA/mL) at 70°C for 45 min to yield pyrimidine oligonucleotides and free purine bases (Becker et al., 1981). Individual purines were separated from the hydrolysates on a Whatman Partisil 10 strong cation exchange column (25 cm x 4.6 mm I.D.) with a mobile phase of 0.5-1.0 M ammonium phosphate with 0-5% methanol, pH 2.0 at 2 mL/min using a Perkin Elmer Series 4 liquid chromatograph. Elution of 7-methylguanine and O^6-methylguanine was detected using a Perkin Elmer Model 630S fluorescence spectrophotometer with an excitation wavelength of

290 nm and emission wavelength of 360 nm (12 nm slits). Guanine concentrations of the acid hydrolysates (less than 1% of the total DNA guanine was released from the polymer during neutral thermal hydrolysis) were determined by absorbance at 275 nm (Micromeritics Model 780 variable wavelength ultraviolet spectrophotometer). Quantification of each base was made by comparison to calibration standards as described by Becker et al. (1981).

Quantitative Determination of Aldehydes in Animal Tissues

To study the possible role of formaldehyde in the methylation of liver DNA guanine of rodents given hydrazine, attempts were made to alter the levels of endogenous formaldehyde in the liver by pretreatment of animals with formate, methanol, disulfiram, etc.; however, it was not possible to determine the extent to which the formaldehyde levels were actually altered for want of a reliable method.

The first attempt to develop a new method for formaldehyde determination in liver homogenate was based on an assay reported by Chrastil and Wilson (1975). This method reacts tryptophan in the presence of sulfuric acid and iron with formaldehyde after precipitation of protein from homogenates with 1% HCl in ethanol. The method was tried repeatedly but proved to have poor sensitivity. The method relied upon measuring light absorption by a chromophore against a high background in liver extract and could not detect a doubling of the formaldehyde content over basal levels in rodent liver. This was no better than previous trials with the Nash reagent method.

The second method tried was described by Radford and Dalsis (1982) and is based on reaction of formaldehyde in tissue homogenate immediately with 2,4-dinitrophenylhydrazine (DNP) to form the aldehyde hydrazone and extracting with toluene. The toluene extract is concentrated almost ten times and is then applied to a 5 μm ODS (reverse phase) 4.6 x 1500 mm HPLC column. The aldehyde hydrazone and acetone hydrazone, added as an internal standard, are eluted with 2:1 methanol:water and detected in the eluate by UV absorption at 348 nm.

The method was modified slightly and the sensitivity improved greatly. Methylene chloride, HPLC grade, was obtained from Baker Chemical Co. DNP reagent was prepared by dissolving 50 g trichloroacetic acid in 100 mL water, adding 1 g DNP, and diluting to 1 liter with water. The solution was extracted with methylene chloride before use to remove impurities. Formaldehyde 2,4-dinitrophenylhydrazone was prepared by adding 2 mL conc. sulfuric acid to 1 g DNP in 20 mL methanol; after filtration, 2 mL formaldehyde were added. Crystals of presumed formaldehyde dinitrophenylhydrazone were collected and recrystallized from ethanol twice: melting point, 116°C (lit. m.p. 116°C). Methylethylketone 2,4-dinitrophenylhydrazone was prepared in the same manner: melting point, 128° (lit. m.p. 128°C); this replaced use of the acetone analog as the internal standard as acetone frequently occurs in liver in response to toxic insult but could not be determined in the presence of an acetone hydrazone internal standard.

Minced liver is homogenized in 6 volumes DNP reagent in the cold; the acidic DNP reagent is presumed to convert endogenous acid-labile adducts of formaldehyde (principally S-formylglutathione and N^5,N^{10}-methylene-tetrahydrofolate) as free formaldehyde (Heck et al., 1982). The homogenate is shaken for 30 min with methylene chloride (half the DNP reagent volume). Two milliliters of the methylethylketone dinitrophenylhydrazone standard (0.15 g per liter) in methylene chloride is added. The mixture is centrifuged at 18,000 x g in the cold for 30 min (GSA rotor, 12,000 rpm, Sorval RC-5 centrifuge). The methylene chloride layer at the bottom of the centrifuge tube (below the solids layer) is removed with a pipet and passed through a Millipore Swinney FH filter.

The methylene chloride extract is fractionated on a Beckman 5 μm Ultrasphere ODS 4.6 x 1500 mm reverse phase column with 2:1 methanol:water, isocratically at 1.1 mL per min; detection of eluting hydrazones is accomplished with a Micromeritics Chromonitor UV spectrophotometer at 348 nm, 0.01 AUFS. The methylene chloride extract (2 uL, unconcentrated) is injected; the formaldehyde hydrazone eluted at 4.4 min., acetaldehyde hydrazone at 6.2 min., and acetone hydrazone at 9.5 min. The standard curve for formaldehyde hydrazone was linear over the range of 3.5 to 280 ng applied to the column (all amounts tested):

Amt. F-hydrazone applied to column (ng)	Elution time (min)	Area units for elution peak
280	4.46	1,080,256
140	4.35	550,856
70	4.39	261,255
7	4.32	19,143
3.5	4.32	(no integr.)*

*height of this elution peak was half that obtained with 7 ng stnd.

The major modification of the method of Radford and Dalsis (1982) was use of methylene chloride instead of toluene for the extraction of the hydrazones from the liver homogenates. Toluene is immiscible with the HPLC mobile phase and gives a wide elution peak which decreases resolution of the hydrazones. Methylene chloride gives a narrow elution peak, greatly increasing resolution.

Analysis of frozen rat liver indicated concentrations of 180 nmol formaldehyde hydrazone per gram liver (lit. values 50 to 500 nmol/g). Normal livers also contained acetone but no detectable acetaldehyde by this method. The limit of detection is estimated to be 7 nmol formaldehyde/g liver.

Effect of the Hepatic 1-Carbon Pool on DNA Methylation in Hydrazine Toxicity: In Vivo

In a review article by Biancifiori and Severi (1966), reference was made to a personal communication from F. L. Rose, who suggested that hydrazine might react with endogenous formaldehyde or a functional derivative, such as hydroxymethylfolic acid, to form a hydrazone which could then be oxidized to the strong methylating agent, diazomethane:

$$HCHO + H_2N\text{-}NH_2 \longrightarrow \left[\begin{matrix} HCHNH\text{-}NH_2 \\ | \\ OH \end{matrix}\right] \longrightarrow CH_2{=}N\text{-}NH_2 \longrightarrow \bar{C}H_2\text{-}\overset{+}{N}{\equiv}N$$

The fact that the methyl source in the hydrazine/DNA methylation response can be labeled by either ^{14}C-formate or (^{14}C-methyl)methionine (Barrows and Shank, 1978; Quinter-Ruiz et al., 1981) is consistent with this hypothesis.

Endogenous Formaldehyde Levels and Hydrazine-induced Methylation of DNA Guanine

Sprague Dawley rats were used in a preliminary experiment to test the above hypothesis. Three rats (177-189 g) were given 800 mg sodium formate/kg body wt intraperitoneally to greatly expand the 1-carbon pool in the liver; 15 min later each rat was given 90 mg hydrazine/kg body wt by stomach tube, and the animals were decapitated 6 hr later. Pretreatment of the animals with formate should have provided a relatively large pool of formaldehyde (relative to untreated animals) with which the hydrazine could react to form diazomethane, as proposed above. It is already known (Becker et al., 1981) that administration of 90 mg hydrazine/kg body wt to rats results in levels of about 800 micromoles 7-methylguanine and 80 micromoles O^6-methylguanine per mole guanine in 6 hr; thus, in the liver DNA of these animals, considerably (possibly an order of magnitude) greater methylation of DNA guanine could be expected; however, the DNA contained no detectable methylguanines.

A second attempt to alter the level of the hepatic 1-carbon pool was based on the following rationale. Methanol was administered to provide a source for the metabolic formation of formaldehyde; disulfiram was given to inhibit aldehyde dehydrogenase and thus prolong the persistence of formaldehyde in the liver; formate was administered in another attempt to increase the level of the 1-carbon pool in the liver. Semicarbazide was given to compete with hydrazine for the available formaldehyde and possibly 'protect' against DNA methylation.

Young adult Sprague-Dawley rats (218-275 g) were fasted overnight and then treated as follows: at T = -4 hr rats were given 500 mg disulfiram/kg body weight po in corn oil (5 mL/kg body wt); at T = -0.5 hr animals were treated with 1 mL methanol (100%)/kg ip, 100 mg semicarbazide HCl/kg in 1 mL water/kg po, and/or 500 mg sodium formate/kg in 2 mL water/kg ip. Hydrazine (90 mg/kg po) was given at T = 0. All animals were killed at T = 24 hr. A summary of the treatment schedule is given in Table 1 and the results are summarized in Table 2.

The combination of hydrazine and methanol proved to be fatal to all animals (Groups 6, 8, and 11); in addition, 1 out of 3 animals died after single administration of disulfiram, semicarbazide, or sodium formate alone (Groups 3, 9, and 12). The high mortality left few animals with which to complete the experiment. The few results that were obtained weakly suggest that semicarbazide, which was expected to scavenge endogenous formaldehyde, did not decrease the amount of DNA methylation (Groups 2 vs 10), and that formate, which was expected to increase the concentration of endogenous formaldehyde, did not increase the amount of DNA methylation (Groups 2 vs 13). It appears that only formate administration may have been able to raise the level of formaldehyde in the livers of these animals under the conditions used.

The drug, aminopyrine, is known to be enzymatically demethylated to form two moles formaldehyde for every mole aminopyrine metabolized; this drug, then, was given to rats in an attempt to raise the level of formaldehyde in the liver. Six hooded Long Evans rats (350-440 g; gift of the Department of Pharmacology) were given 0, 45, 90, or 180 mg aminopyrine/kg body wt and killed 2 hr later; the animals had not been fasted. The livers were assayed for formaldehyde, and the results are presented in Table 3. The results of these experiments suggest that either the level of formaldehyde in the liver is closely regulated or that the analytical method is not measuring the true formaldehyde levels.

Table 1

Schedule for Rats Treated with Hydrazine and Modifiers of the 1-Carbon Pool

Group	No. Rats	Hydrazine	Disulfiram	Methanol	Semi-carbazide	Formate
1	6	-	-	-	-	-
2	3	+	-	-	-	-
3	6	-	+	-	-	-
4	6	-	-	+	-	-
5	3	+	+	-	-	-
6	3	+	-	+	-	-
7	6	-	+	+	-	-
8	3	+	+	+	-	-
9	3	-	-	-	+	-
10	3	+	-	-	+	-
11	3	+	+	+	+	-
12	6	-	-	-	-	+
13	3	+	-	-	-	+
14	3	-	-	-	+	+
15	3	+	-	-	+	+

Table 2

DNA Methylguanine and Formaldehyde Levels in Liver

Group	Methylguanines (μmol/mol guanine)		Formaldehyde (nmol/g liver)
	7-methylguanine	O^6-methylguanine	
1	ND	ND	76.9, 81.0
2	660, 760, 805	52, 56, 100	no analysis
3	ND	ND	81.4, 74.6*
4	ND	ND	89.9, 71.5
5	355	18	no analysis
6	animals died	animals died	animals died
7	ND	ND	84.9, 80.3
8	animals died	animals died	animals died
9	ND	ND	no analysis
10	260, 760	8, 46	no analysis
11	animals died	animals died	animals died
12	ND	ND	153.7, 97.1
13	430, 450	11, 14	no analysis
14	ND	ND	no analysis
15	100, 430, 730	21, 54, lost	no analysis

* also, elevated level of acetone (approx. double the control level)

Table 3

Liver Formaldehyde Levels in Rats Treated With Aminopyrine

Dose (mg/kg)	Formaldehyde (nmol/g liver)
Control	68
45	59
90	63
90	62
180	60
180	67

Alteration of Formaldehyde and Acetaldehyde Levels in Liver and Hydrazine-induced Methylation of DNA Guanine

The study on altering endogenous formaldehyde levels in liver continued with administration of methanol and ethanol to rats pretreated with buthionine sulfoximine to inhibit glutathione synthetase I; glutathione is a cofactor in the oxidation of aldehydes by aldehyde dehydrogenase, and most of the endogenous formaldehyde in liver is believed to exist in the bound form as S-formylglutathione (Heck et al., 1982). Depression of endogenous glutathione levels, therefore, might be expected to alter endogenous levels of formaldehyde in the rodent liver.

Buthionine sulfoximine (BSO) was synthesized according to Griffith and Meister (1979). Twenty-four male Sprague-Dawley rats (210 - 240 g body wt) were fasted 14 hr and then given 4 mmol buthionine sulfoximine/kg body wt in 0.1 M HCl orally; controls received vehicle only. Six hr after receiving buthionine sulfoximine the animals were either killed or given intraperitoneally 0.5 mL undiluted methanol or 4.12 mL undiluted ethanol per kg body wt, in which case they were killed 1 hr later. Three animals were treated per group. Livers were removed and frozen immediately after decapitation of the animals, and were subsequently assayed for reduced glutathione (by the method of Ellman, 1959) and formaldehyde and acetaldehyde levels according to our own HPLC method. The results are summarized in Table 4.

The reduced glutathione levels in liver did not change appreciably during the 1 hr period chosen for alcohol exposure (Groups 1 and 2); buthionine sulfoximine administration lowered glutathione levels to 26 - 31% of control for the same time period (Groups 3 and 4), but had no effect on hepatic formaldehyde levels in methanol-treated rats and little effect, if any, on acetaldehyde levels. Although formaldehyde levels seemed to be refractory, acetaldehyde levels did appear to be slightly higher in animals given ethanol alone and higher still in animals given buthionine sulfoximine before ethanol.

The rat liver appears to have a great capacity to regulate endogenous formaldehyde levels, as this pool was refractory to several attempts to expand it by administration of formaldehyde generators and inhibitors of formaldehyde dehydrogenase. Another attempt to increase endogenous formaldehyde levels was made in a second rodent species. Male Syrian golden hamsters (59-89 g, 3 animals/group except 2 controls) were given disulfiram (Aldrich Chemical Co.; 500 mg/kg body wt) ip to inhibit aldehyde dehydrogenase. Two hours later the animals were given ip 0, 1, 4, or 6 mL reagent grade

Table 4

Effect of Buthionine Sulfoximine (BSO)
on the Hepatic Production of Aldehydes from Methanol and Ethanol*

Treatment	Kill Time (hr)	Glutathione (μg/g liver)	Formaldehyde (nmol/g liver)	Acetaldehyde (nmol/g liver)
Control	0	1081 ± 132	82 ± 12	13 ± 1
Control	1	997 ± 207	93 ± 16	14 ± 1
BSO only	0	282 ± 81	89 ± 16	28 ± 0
BSO only	1	310 ± 63	95 ± 20	13 ± 3
MeOH only	1	973 ± 107	106 ± 18	14 ± 1
EtOH only	1	816 ± 71	104 ± 23	22 ± 3
BSO/MeOH	1	258 ± 63	101 ± 15	10 ± 4
BSO/EtOH	1	295 ± 62	97 ± 18	34 ± 5

* Values are means ± standard deviations; MeOH, methanol; EtOH, ethanol

methanol/kg body wt and decapitated 1 hr later. Controls were given neither disulfiram nor methanol. In a second experiment to test the effect of ethanol on acetaldehyde levels, five male Syrian golden hamsters (72–98 g body wt) were fasted overnight and then given 500 mg disulfiram/kg body wt orally in olive oil. Two hours later one animal received 2 mL absolute ethanol/kg body wt ip and two animals received either 4 or 6 mL ethanol/kg; there were no controls. The animals were decapitated 1 hr later. For both experiments hepatic aldehyde levels were determined by HPLC; the results are summarized in Table 5. Formaldehyde levels did not change appreciably by this treatment, except perhaps at the highest dose of methanol where formaldehyde levels were raised approximately 30% above those of the controls. Acetaldehyde levels increased in ethanol-treated hamsters as anticipated.

Table 5

Liver Aldehydes from Methanol and Ethanol in Disulfiram-treated Hamsters*

Treatment		Formaldehyde (nmol/g liver)	Acetaldehyde (nmol/g liver)
Control (MeOH expt)		82	tr
MeOH,	1 mL/kg	80	tr
	2 mL/kg	80	tr
	4 mL/kg	95, 81	tr
	6 mL/kg	99, 108	tr
EtOH,	2 mL/kg	85	118
	4 mL/kg	97, 100	182, 160
	6 mL/kg	111, 95	226, 185

* Each value represents the determination of aldehyde on a single animal liver

This procedure was successful in elevating not only acetaldehye levels but those of formaldehyde as well. Control liver usually contains no detectable or trace amounts of acetaldehyde; ethanol administration to hamsters pretreated with disulfiram resulted in a dose-related increase in acetaldehyde levels. Formaldehyde levels increased up to almost 30% above what is usually seen in control hamster (80 nmol/g liver). Perhaps acetaldehyde competes with formaldehyde in the catalase oxidation of the latter, resulting in this elevation of both aldehydes (acetaldehyde inhibition of formaldehyde dehydrogenase is less likely, because inhibition of this enzyme in the rodent has not been completely successful in raising endogenous levels of formaldehyde).

Since administration of ethanol to hamsters pretreated with disulfiram was successful in elevating the level of acetaldehyde in liver, a time-response study was done to determine how rapidly the aldehyde levels increased and how long the elevated levels persisted. At T = -2 hr, 12 male Syrian golden hamsters (85-107 g body wt) were given 500 mg disulfiram (Aldrich Chemical Co)/kg orally in olive oil to inhibit aldehyde dehydrogenase. At T = 0, two animals were decapitated, and the remaining animals received 2 mL absolute ethanol/kg ip and were decapitated 0.25, 0.5, 1, 1.5, or 2 hr later. Levels of formaldehyde and acetaldehyde in liver were determined and the results are summarized in Table 6 and Figure 1.

Table 6

Time Course of Hepatic Aldehyde Levels
in Hamsters Treated with Disulfiram and Ethanol*

Time after ethanol administration, hr	Formaldehyde (nmol/g liver)	Acetaldehyde (nmol/g liver)
0	78	46
	74	40
0.25	77	140
	78	144
0.5	93	122
	89	131
1	97	127
	87	124
1.5	98	138
	92	112
2	91	117
	95	116

* Each value represents the determination of aldehyde on a single animal liver

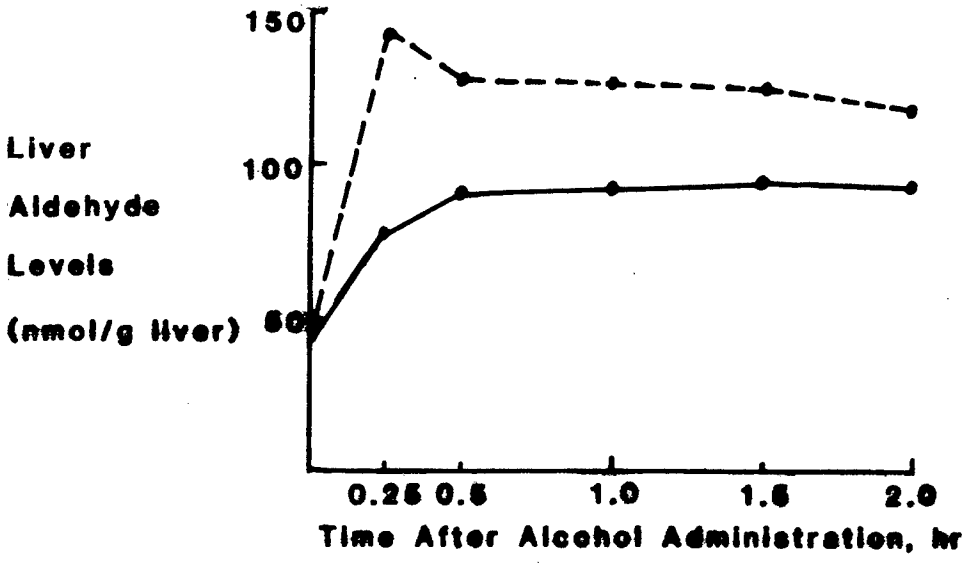


Fig. 1. Persistence of formaldehyde and acetaldehyde in liver after administration of methanol to disulfiram-treated rats. Formaldehyde (solid line) and acetaldehyde (dashed line) concentrations in noml/g liver.

Hepatic acetaldehyde levels rose rapidly and remained elevated over the course of the 2-hr experiment. Formaldehyde levels rose slightly and more slowly than did acetaldehyde levels. The conditions for substantial alteration of hepatic aldehyde levels in vivo appeared to have been established, and the first experiment with hydrazine in this system was conducted.

Hamsters could not be obtained, so young adult male Sprague Dawley rats (253-313 g) were pretreated with disulfiram (500 mg/kg, po; Aldrich Chemical Co), then given absolute ethanol (2 mL/kg) ip, hydrazine (90 mg/kg, po), and decapitated according to the protocol in Table 7.

Table 7

Protocol for Pretreatment of Rats
With Disulfiram and Administration of Ethanol and Hydrazine

Time, hr	Treatment	Group 1	Group 2	Group 3	Group 4	Group 5
-3	disulfiram	+	-	+	-	+
-1	ethanol	-	+	+	-	+
0	hydrazine	-	-	-	+	+
1	decapitation	+	+	+	+	+

The livers from individual animals were divided into two portions, one for aldehyde analysis and one for DNA isolation and alkylguanine determination. The results are summarized in Table 8.

Table 8

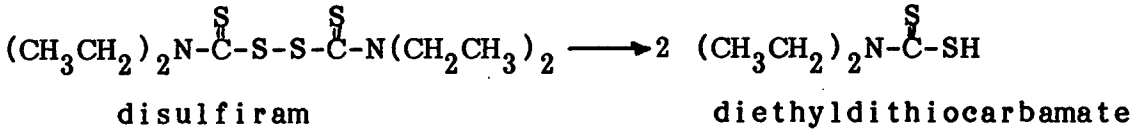
Levels of Hepatic Aldehydes and Alkylguanines in DNA
After Hydrazine Administration to Rats Pretreated with Disulfiram and Ethanol

	Aldehydes (nmol/g liver)		Methylguanines (μmol/mol guanine)*	
Treatment	Formaldehyde	Acetaldehyde	7-Methylguanine	O^6-Methylguanine
Disulfiram only	76	-	ND	ND
	66	85	ND	ND
	69	86	ND	ND
Ethanol only	76	-	ND	ND
	68	110	ND	ND
	93	123	ND	ND
Disulfiram, ethanol	64	-	ND	ND
	89	96	ND	ND
	81	67	ND	ND
Hydrazine only	106	-	239	12
	105	79	176	9
	122	73	314	22
Disulfiram, ethanol, hydrazine	117	-	ND	ND
	131	215	ND	ND
	80	-	13	ND

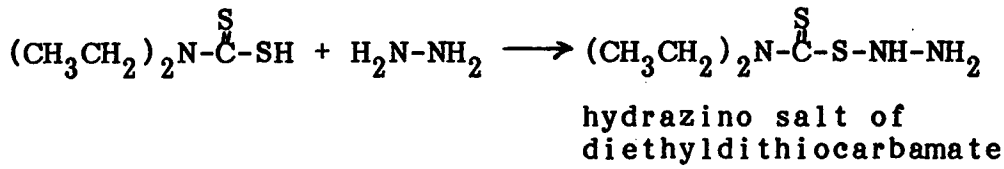
* No ethylguanines could be detected in any DNA samples

Several interesting findings were obtained: 1) rats treated with disulfiram only (no ethanol) had as much acetaldehyde as formaldehyde in their livers; 2) administration of ethanol to disulfiram-treated rats raised the acetaldehyde levels only slightly, but administration of ethanol to naive rats raised acetaldehyde levels by 34%. The methylation of liver DNA guanine in rats treated with hydrazine only was at expected levels based on earlier studies (Becker et al., 1981), but little alkylation of DNA guanine could be detected in disulfiram-pretreated rats given methanol or ethanol, in spite of elevated formaldehyde and acetaldehyde levels. It is known that pretreatment of rats with disulfiram protects the liver from the carcinogenic action of dimethylnitrosamine (Schmahl et al., 1976) and decreases the methylation of DNA by dimethylnitrosamine (Schmahl et al., 1971). It is also possible that the nucleophilic metabolite of disulfiram, diethyldithiocarbamate, may react with the electrophilic metabolite of dimethylnitrosamine, thus reducing the level of methylating agent available to interact with DNA. If such a mechanism occurs, it may explain why disulfiram appears to inhibit the alkylation of DNA in hydrazine-treated rats.

It is also possible that diethyldithiocarbamate, the metabolic product from disulfiram, may react directly with hydrazine to form the hydrazino salt and thus remove hydrazine from the biochemical pathway(s) responsible for methylation of DNA.

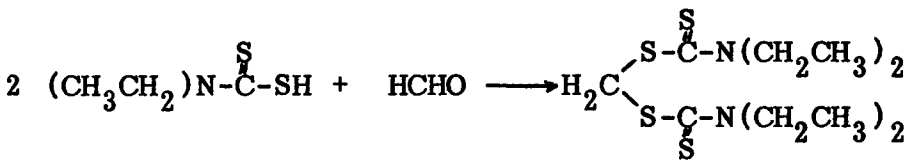


disulfiram — diethyldithiocarbamate



hydrazino salt of diethyldithiocarbamate

Also, diethyldithiocarbamate may react directly with formaldehyde to form the thioacetal, thus removing formaldehyde from the biochemical pathway(s) responsible for methylation of DNA.



Therefore, the metabolic product of disulfiram may scavenge formaldehyde, acetaldehyde, and hydrazine, thereby blocking the DNA alkylation reaction. The method used for aldehyde determinations measures both free aldehydes and those complexed with reduced glutathione (the major form of cellular aldehydes); it may also measure aldehydes bound to diethyldithiocarbamate.

The above experiment was repeated in the hamster, but disulfiram was not used as the inhibitor of aldehyde dehydrogenase because of the demonstrated inhibition of aberrant methylation in hydrazine-poisoned rats. A pilot study was run first to determine whether cyanamide, $H_2NC{\equiv}N$, a known inhibitor of aldehyde dehydrogenase (Marchner and Tottmar, 1978), would be compatible with the hydrazine/aldehyde system.

Young adult male Syrian golden hamsters (38-61 g) were pretreated with cyanamide (Sigma Chemical Co., 25 mg/kg body wt, 1% neutral solution in saline, ip), then given absolute ethanol (2 mL/kg, ip) and/or hydrazine (90 mg/kg, ip instead of the usual po), and decapitated, according to the protocol in Table 9.

Each group contained six hamsters. After decapitation, the livers were cut in half; two halves from different animals in the same group were pooled for DNA analysis. Aldehyde analyses were done on one individual half from each animal. Thus, there were 3 DNA analyses and 6 aldehyde analyses per group. The results obtained are summarized in Table 10.

Table 9

Protocol for Pretreatment of Hamsters
with Cyanamide and Administration of Ethanol and Hydrazine

Time, hr	Treatment	Group Number 1	2	3	4	5	6	7	8	9	10
-1.5	cyanamide	-	-	+	+	-	-	+	+	-	+
-1	ethanol	-	-	-	-	+	+	+	+	-	+
0	decapitation	+	-	+	-	+	-	+	-	-	-
0	hydrazine		-		-		-		-	+	+
1	decapitation		+		+		+		+	+	+

Table 10

Levels of Hepatic Aldehydes and Methylguanines in DNA After Hydrazine
Administration to Hamsters Pretreated with Cyanamide and Ethanol

Treatment		Aldehydes (nmol/g liver) (mean ± s.d.) Formaldehyde	Acetaldehyde	Methylguanines (μmol/mol guanine) 7-methylguanine	O^6-methylguanin
1.	None Zero-hr kill	119 ± 21	ND	ND	ND
2.	None 1-hr kill	118 ± 13	ND	ND	ND
3.	Cyanamide Zero-hr kill	124 ± 16	ND	ND	ND
4.	Cyanamide 1-hr kill	131 ± 12	ND	ND	ND
5.	Ethanol Zero-hr kill	104 ± 15	49 ± 2	ND	ND
6.	Ethanol 1-hr kill	124 ± 24	tr	ND	ND
7.	Cyan., ethanol Zero-hr kill	142 ± 28	63 ± 6	ND	ND
8.	Cyan., ethanol 1-hr kill	135 ± 19	67 ± 5	ND	ND
9.	Hydrazine 1-hr kill	175 ± 14	ND	296 ± 63	77 ± 11
10.	Cyan., ethanol hydrz., 1-hr kill	175 ± 21	572 ± 94	43 ± 4	ND

ND = none detected, less than 10 μmol 7-methylguanine and 1 μmol O^6-methylguanine/mol guanine

tr = trace (approx. 20 - 40 nmol/g liver)

Cyanamide treatment alone appeared to increase the level of endogenous formaldehyde in the liver to a small extent. Cyanamide pretreatment increased acetaldehyde levels in animals given ethanol. More interestingly, administration of hydrazine alone appeared to increase the size of the endogenous formaldehyde pool in the liver by 50% (compare Groups 2 and 9). The level of 7-methylguanine and O^6-methylguanine in the liver DNA of these animals was the same as seen in earlier experiments. When hydrazine was given to hamsters pretreated with cyanamide and ethanol, two surprising changes occurred: a) the level of acetaldehyde increased by an order of magnitude over that seen in animals given only cyanamide and ethanol; b) the level of methylguanines in the liver DNA of these animals decreased by an order of magnitude, and, in spite of the extraordinarily high levels of acetaldehyde in the liver, no ethylguanines could be detected. It may be that cyanamide inhibited the DNA alkylation process.

The experimental protocol was modified to eliminate the cyanamide pretreatment to determine whether the same elevated levels of acetaldehyde would be found. It was also decided to administer methanol as well as ethanol in combination with hydrazine to determine whether elevations in formaldehyde levels could be detected. Liver DNA was also isolated for determination of alkylated purines. Additionally, kidney, lung, and brain were saved for aldehyde analysis; in a separate experiment (reported in the another section of this report - see Table 28) the methylation of DNA in liver, kidney, lung, and brain in response to hydrazine administration was determined.

Young adult male Syrian golden hamsters (57-72 g) were pretreated with 50% methanol (500 µL/kg body wt, ip), or absolute ethanol (2 mL/kg, ip). An hour later the animals were given hydrazine (90 mg/kg in 0.1 M HCl, po), and decapitated 60 min later. The protocol is summarized in Table 11. Each group contained six hamsters. After decapitation, the livers were cut in half; two halves from different animals in the same group were pooled for DNA analysis. Aldehyde analyses were done on one individual half from each animal. Thus, there were 3 DNA analyses and 6 aldehyde analyses per group. The results are summarized in Table 12. In addition, determinations for formaldehyde and acetaldehyde were done on kidney, lung, and brain, and those results are summarized in Table 13.

Table 11

Protocol for Pretreatment of Hamsters
with Methanol or Ethanol Before Administration of Hydrazine

		Group Number					
Time, hr	Treatment	1	2	3	4	5	6
-1	methanol	-	+	-	+	-	-
-1	ethanol	-	-	+	-	+	-
0	hydrazine	-	-	-	+	+	+
1	decapitation	+	+	+	+	+	+

Little change in formaldehyde levels in kidney, lung, or brain could be seen in the various groups; the hepatic formaldehyde levels determined in this experiment were about half of the levels seen in an earlier experiment (Table 10); even so, hydrazine administration increased formaldehyde levels 48% in both experiments, but ethanol had

no effect on formaldehyde or acetaldehyde levels. The results for the two experiments are compared in Table 14. In the first experiment (Table 10) hydrazine had been administered by intraperitoneal injection,; in the second (Table 12), hydrazine had been administered by stomach tube, as usual. To determine whether this difference in route of administration for hydrazine had a significant effect on hepatic aldehyde and DNA methylation levels, two hamsters (71 and 74 g) were treated with absolute ethanol at 0 hr and hydrazine at 1 hr, as above; both ethanol and hydrazine were give ip. The results of the liver aldehyde and DNA analyses are summarized in Table 15.

Table 12

Levels of Hepatic Aldehydes and Methylguanines in DNA After Hydrazine Administration to Hamsters Pretreated with Methanol or Ethanol

Treatment		Aldehydes (nmol/g liver) (mean ± s.d.)		Methylguanines (μmol/mol guanine)	
		Formaldehyde	Acetaldehyde	7-Methylguanine	O^6-Methylguanine
1.	None	58 ± 31	ND	ND	ND
2.	Methanol	70 ± 35	ND	ND	ND
3.	Ethanol	68 ± 14	ND	ND	ND
4.	Methanol, hydrazine	90 ± 28	ND	298 ± 89	16 ± 1
5.	Ethanol, hydrazine	59 ± 26	ND	ND	ND
6.	Hydrazine	106 ± 53	ND	509 ± 164	53 ± 18

ND = none detected
No ethylguanines were detected in any DNA sample

Table 13

Aldehyde Levels in Kidney, Lung and Brain After Hydrazine Administration to Hamsters Pretreated With Ethanol

Treatment		Formaldehyde (nmol/g tissue)*		
		Kidney	Lung	Brain
1.	No treatment	38 (41, 35)	67 ± 17	37 ± 3
2.	Methanol	35 (27, 43)	70 ± 31	37 ± 3
3.	Ethanol	37 (32, 42)	85 ± 37	36 ± 4
4.	Methanol, hydrazine	56 (65, 43)	59 ± 25	34 ± 4
5.	Ethanol, hydrazine	49 (56, 41)	69 ± 19	40 ± 7
6.	Hydrazine	43 (39, 47)	90 ± 33	46 ± 6

* No acetaldehydes detected; values are means ± s.d. except for kidney where they are means of 2 analyses with actual concentrations in parentheses; kidneys and brains from 2 animals pooled for analysis; kidneys from 2 of 6 animals per group were lost

Table 14

Comparison of Aldehyde Levels in Livers of Hamsters
Given Ethanol and Hydrazine in Two Experiments

	Aldehydes (nmol/g liver)			
	Formaldehyde		Acetaldehyde	
Treatment	Expt 1*	Expt 2**	Expt 1*	Expt 2**
None	118 ± 13	58 ± 31	ND	ND
Ethanol only	124 ± 24	68 ± 14	trace	ND
Hydrazine only	175 ± 14	106 ± 53	ND	ND
Ethanol, then hydrazine	-	59 ± 26	-	ND

* see Table 10
** see Table 12

Table 15

Liver Aldehydes and DNA Methylguanines
in Hamsters Given Hydrazine Intraperitoneally

	Aldehydes (nmol/g liver)		Methylguanines (μmol/mol guanine)	
Treatment	Formaldehyde	Acetaldehyde	7-methylguanine	O^6-methylguanine
Ethanol, then hydrazine	51, 42	47, 123	149	6

Intraperitoneal administration of hydrazine seems to allow detectable levels of acetaldehyde to accumulate in liver after giving ethanol to hamsters without the need for disulfiram as in earlier experiments (Table 5). Also, the presence of ethanol, but not methanol, appears to greatly decrease the amount of 7-methylguanine and O^6-methylguanine formed in liver DNA in response to either oral or intraperitoneal administration of hydrazine. Perhaps acetaldehyde from ethanol combines with the hydrazine to form the corresponding hydrazone which may be slowly oxidized to diazoethane, or the diazoethane is a much slower/poorer alkylating agent for DNA.

Effect of the 1-Carbon Pool on DNA Methylation in Hydrazine Toxicity: In Vitro

In vitro studies were begun in the investigation of the role of the 1-carbon pool in the methylation of DNA guanine in hydrazine toxicity as they offered greater capability to control the concentrations of biochemical intermediates in proximity to cell components.

In the first experiment the incubation system included rat liver S9 fraction (supernatant produced by centrifuging liver homogenate - 3 g liver/mL 20mM Tris in 154 mM KCl pH 7.4 - at 9000 x g for 20 min at 4°C; 32 mg protein/mL) to provide liver enzymes for metabolic conversion of hydrazine-formaldehyde intermediates such as the

proposed aldehyde hydrazone. S9 fraction was prepared from fasted untreated Sprague Dawley rats. Three milliliters of incubation medium contained 0.47 mL S9 fraction (15 mg protein), 7.5 mg calf thymus DNA (Sigma Chemical Co., St. Louis, MO), and toxicant, in Tris buffer. The toxicants were added to give the following final concentrations: dimethylnitrosamine (DMN, 0.01 mM, positive control for required metabolic activation), hydrazine (0.1 or 1.0 mM), formaldehyde (0.1 or 1 mM), N-nitrosopyrrolidine (NP, 1 or 100 mM, a second positive control for required metabolic activation), methylnitrosourea (MNU, 0.1 mM, positive control not requiring metabolic activation), and monomethylhydrazine (MMH, 1 mM).

Vials were capped (air atmosphere) and incubated in a shaking water bath at 37°C for 30 min. Reactions were stopped by plunging the vials into ice, adding 1 mL 2% naphthalenediaminesulfonic acid sodium salt and 4 mL phenol reagent (phenol, m-cresol, and 8-hydroxyquinoline), and DNA was isolated and purified; methylguanine levels were determined, but not quantitatively in this pilot experiment as DNA yields were low. The results are given in Table 16.

The DMN positive control failed to produce methylated guanines in the DNA, but the N-nitrosopyrrolidine positive control did yield NP-DNA adduct (Hunt and Shank, 1982) when the lower concentration of carcinogen was used. Thus the metabolic activation system in the S9 fraction appeared to be intact. The high concentration of NP did not produce any DNA adduct, but then it is already known that at high concentrations NP inhibits its own metabolism (Herron and Shank, 1981).

The combination of hydrazine and formaldehyde gave the response expected from the working hypothesis that the putative hydrazone produced from the reaction of formaldehyde and hydrazine appeared to require enzymic activity in its conversion to a methylating agent. These results suggest the following pathway:

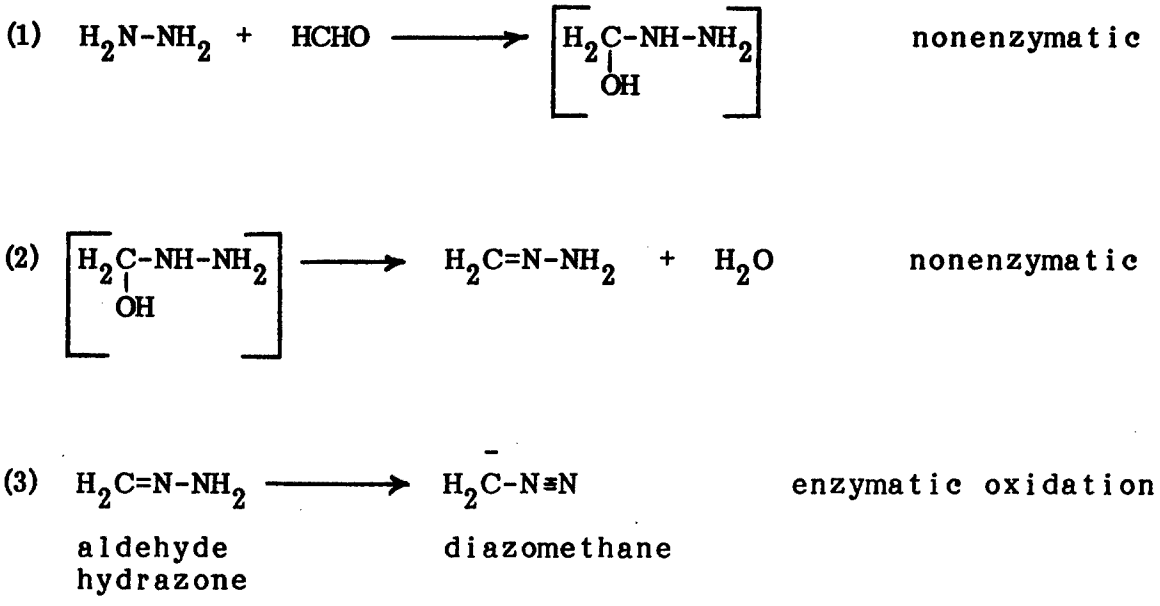


Table 16

In Vitro Methylation of DNA by Rat Liver S9 and Various Toxicants

Treatment*	S9 Fraction	Methylguanines in DNA (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine	
none	fresh	ND	ND	
	boiled	ND	ND	
DMN	fresh	ND	ND	
(0.1 mM)	boiled	ND	ND	
Hz + HCHO	fresh	lost	lost	
(1 mM ea)	boiled	ND	ND	
Hz + HCHO	fresh	++	+++	
(0.1 mM ea)	boiled	ND	ND	
Hz	fresh	lost	lost	
(1 mM)	boiled	ND	ND	
Hz	fresh	(t?)	(t?)	
(0.1 mM)	boiled	(t?)	ND	
HCHO	fresh	(t?)		
(1 mM)	boiled	(t?)		
none	none	ND	ND	
NP	fresh	(t?)	ND	NPa +**
(1 mM)	boiled	ND	ND	NPa -
NP	fresh	ND	ND	NPa -
(100 mM)	boiled	ND	ND	NPa -
MNU	fresh	+	+	
(0.1 mM)	boiled	++++	++++	
MMH	fresh	+	(t?)	
(1 mM)	boiled	+++	++++	

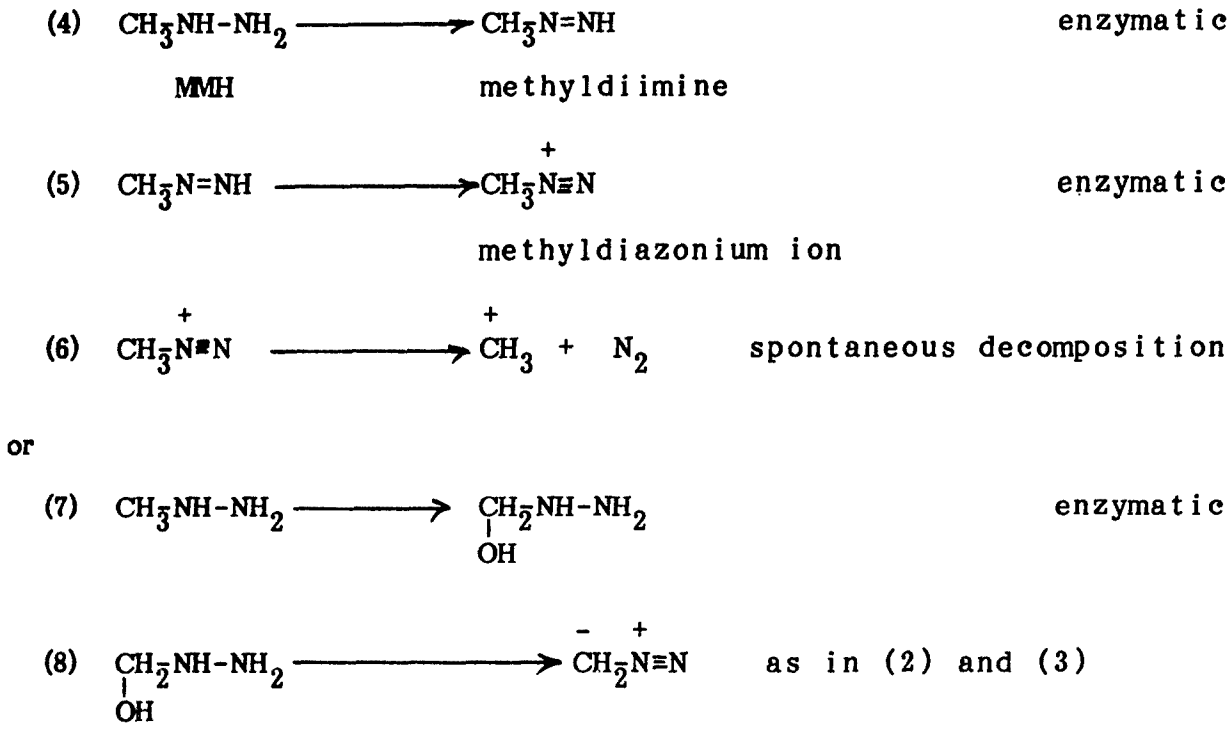
* DMN, dimethylnitrosamine; Hz, hydrazine; HCHO, formaldehyde; NP, nitrosopyrrolidine; MNU, methylnitrosourea; MMH, monomethylhydrazine

**NPa: N-nitrosopyrrolidine-derived DNA adduct, identity still unknown

ND: none detected

(t?): possible trace amount, peak barely distinguishable from noise in baseline

The methylnitrosourea control (not requiring metabolic activation) produced the anticipated response; the methylnitrosourea apparently broke down spontaneously at pH 7.4 to a methylating agent. Boiling the S9 fraction may have made nucleophilic sites on the S9 macromolecules less available and thereby less competitive with the DNA for the methylating agent, because there was more methylation of the calf thymus DNA when boiled S9 was used compared to fresh S9. The same was true for monomethylhydrazine, a result which was not expected, for it has been widely held that monomethylhydrazine is enzymatically converted to a methylating agent, presumably as follows:



A second in vitro experiment was done to determine whether monomethylhydrazine could methylate DNA non-enzymatically. Calf thymus DNA was incubated at 37°C for 30 min in 10 mM sodium cacodylate, pH 7.4 with 1) no additive, 2) methylnitrosourea (0.1 mM), or 3) monomethylhydrazine (1 mM). The only methylation of DNA occurred in the methylnitrosourea treatment (as expected) and none was seen with monomethylhydrazine. The experiment was repeated using a greater concentration of monomethylhydrazine (5mM), and 7-methylguanine was detectable in the DNA.

In another in vitro study hydrazine, formaldehyde, monomethylhydrazine, methylnitrosourea, and diethylnitrosamine were incubated with naked calf thymus DNA and S9 fraction of rat liver as described above; the protocol for and results from that experiment are summarized in Table 17. DNA yields were poor, and quantitative results could not be obtained.

Table 17

In Vitro Methylation of DNA by Various Hydrazine Analogs

Treatment*	S9 Fraction	Methylguanines in DNA (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine
blank (DNA only)	fresh	-	-
	boiled	-	-
Hz (1 mM)	fresh	-	-
	boiled	-	-
HCHO (1 mM)	fresh	-	-
	boiled	-	-
Hz + HCHO (1 mM)	fresh	++	+
	boiled	-	-
Hz + HCHO (0.1 mM)	fresh	+	-
	boiled	-	-
MMH (1 mM)	fresh	-	-
	boiled	-	-
MNU (0.1 mM)	fresh	+++	+
	boiled	+++	+
DEN (1 mM)	fresh	+++ (**)	+++(**)
	boiled	-	-

* Hz, hydrazine; HCHO, formaldehyde; MMH, monomethylhydrazine; MNU, methylnitrosourea; DEN, diethylnitrosamine; concentrations are expressed as millimoles per liter incubation medium.

** Ethylguanines, rather than methylguanines, were determined

DNA incubated in buffer alone contained no detectable alkylguanines. Incubation of DNA with either hydrazine or formaldehyde alone, with or without active S9 fraction, did not produce detectable levels of alkylguanines; however, when the two reactants, hydrazine and formaldehyde, were added together to the incubation medium containing fresh S9 fraction, both 7-methylguanine and O^6-methylguanine were readily detectable in the DNA; boiling the S9 fraction before adding it to this incubation mixture prevented this formation of alkylated purines, suggesting that the formaldehyde hydrazone formed in the reaction mixture requires metabolic oxidation to the alkylating agent, presumably diazomethane. Addition of monomethylhydrazine (1 mM) did not result in formation of methylguanines whether fresh or boiled S9 fraction was present; apparently higher molar concentrations of monomethylhydrazine are need to achieve detectable levels of 7-methylguanine in the DNA. Addition of methylnitrosourea (a positive control which does not require metabolic activation to a methylating agent) to the incubation system

resulted in the formation of 7-methylguanine and O^6-methylguanine with either fresh or boiled S9 fraction, as expected. DNA incubated with diethylnitrosamine (a positive control; this compound requires metabolic activation to an ethylating agent) and fresh, but not boiled, S9 fraction contained 7-ethylguanine and O^6-ethylguanine.

The in vitro experiments were expanded to determine the location within the liver cell of the enzyme(s) necessary for formation of the methylating agent produced during hydrazine exposure. Also, hamster liver replaced rat liver as the source of the metabolic activation system.

Livers (av wt 2.6 g) from adult male Syrian golden hamsters were homogenized in 3 volumes of cold 0.02 M Tris in 0.15 M KCl pH 7.4. The homogenate was centrifuged in the cold at 9000 x g for 20 min and the supernatant was used as the S9 fraction. Microsomes were prepared by centrifuging the S9 fraction at 100,000 x g for 1 hr; the supernatant was used as the cytosol and the pellet was resuspended in cold buffer and centrifuged again in the cold at 100,000 x g for 1 hr. Microsomes and cytosol were stored at -70°C until used.

Individual cell fractions were incubated in closed vials containing 10 mg calf thymus DNA (Sigma), 5 mM substrate (hydrazine, formaldehyde, or dimethylnitrosamine), 50 mg protein as S9 fraction, 10 mg protein as microsomes, or 20 mg protein as cytosol. Microsome and cytosol cell fractions were supplemented with an NADPH-generating system. The incubations were carried out at 37°C for 20 min for S9 fraction systems and 15 min for microsome and cytosol systems. The reactions were stopped by quick freezing the vials with liquid nitrogen; the vials were stored at -70°C until DNA isolation.

An improved method for the isolation of the DNA from the reaction medium was used; the method was a modification of that described by Lai et al. (1979). Five milliliters of phenol reagent was added to each vial and the mixture shaken for 30 min at room temperature. After centrifugation at 12,000 x g for 10 min, the upper layer was mixed with an equal volume of 2-ethoxyethanol to precipitate the DNA. The DNA was washed twice with absolute ethanol, once with 1:1 ethanol:ether, and twice with anhydrous diethylether. After rapid drying, the DNA was dissolved in 5 mL 5% sodium acetate and incubated at 37°C with ribonuclease A for 30 min; after the addition of pronase, the incubation was continued for another 60 min. The DNA was isolated again by one phenol extraction and washed with ethanol, ethanol:ether, and ether, as above. This method improved the yield and purity of the DNA.

The DNA was analyzed for the presence of 7-methylguanine and O^6-methylguanine, and the results are reported in Tables 18 and 19. As seen in earlier experiments, neither hydrazine nor formaldehyde alone produced methylguanines in the calf thymus DNA, but when the two were incubated together and in the presence of fresh hamster liver S-9 fraction, both 7-methylguanine and O^6-methylguanine could be detected in the ratio of 0.19 (O^6-methylguanine:7-methylguanine) in the DNA. Boiling the S-9 fraction before incubation eliminated the DNA methylation response. The enzymatic activity of the S-9 fraction seemed to be concentrated in the cytosol, and to a lesser extent, in the smooth endoplasmic reticulum (microsomes). This is in contrast to the metabolic activation of dimethylnitrosamine, which is carried out principally in the microsomal fraction of rat liver cells. It was noted that the variability in the levels of DNA methylation in the in vitro experiments was greatly reduced from that experienced in the in vivo studies, yet the average alkylation levels were similar.

Table 18

In Vitro Formation of Methylguanines in Calf Thymus DNA
Incubated with Hydrazine and Formaldehyde and Hamster Liver S9 Fraction

Treatment	Methylguanines (μmol/mol guanine)	
	7-Methylguanine	O^6-Methylguanine
Hydrazine (5 mM)	tr, tr, tr	ND, ND, ND
Formaldehyde (5 mM)	ND, ND, ND	ND, ND, ND
Hz + HCHO (5 mM ea)	220 ± 19	41 ± 5
Control (buffer)	ND, ND, ND	ND, ND, ND
Dimethylnitrosamine (5 mM)	33 ± 5	7 ± 2

All treatments were tested with boiled hamster liver S-9 fraction; these preparations showed no detectable levels of methylguanines in the DNA
Trace: less than 10 μmol 7-methylguanine or 1 μmol O^6-methylguanine per mol DNA guanine
ND: none detected; below the limits of detection given above

The experiment summarized in Table 18 was repeated but added acetaldehyde among the variables; the results for the S9 fraction system in the repeat experiment are summarized in Table 20. Addition of hydrazine alone to the incubation system leads to little methylation of DNA guanine; what does occur may be assisted by endogenous formaldehyde. Addition of both hydrazine and formaldehyde produces readily detectable levels of both 7-methylguanine and O^6-methylguanine. Addition of formaldehyde alone does not appear to result in methylation of DNA guanine.

Liver fractions were analyzed for aldehyde levels to determine whether there is sufficient endogenous formaldehyde in these preparations to explain the 7-methylguanine levels seen in DNA from incubations with hydrazine but not added formaldehyde. Formaldehyde levels (in nmol/g liver equivalents) were: fresh whole liver, 75 ± 2; fresh S-9 fraction, 65 ± 1; frozen cytosol, 102; frozen microsomes, 39. The added time to produce the cytosol and microsome fractions may explain why together these fractions contained more formaldehyde than fresh S-9 or whole liver. In any case there appears to be enough endogenous formaldehyde in the liver fractions to account for the small amount of 7-methylguanine seen in incubation systems to which hydrazine but not formaldehyde was added. Acetaldehyde was not detected in any of these liver fractions.

It was proposed that the mechanism of action of DNA methylation in hydrazine-treated animals involved first reaction of hydrazine with endogenous formaldehyde to form formaldehyde hydrazone; this intermediate would have to be oxidized to an alkylating agent, presumably diazomethane. Apparently the hydrazone requires metabolic activation for the methylation reaction to take place in the presence of fresh, but not boiled, S-9 liver fraction. It was also proposed that if the hydrazone was an

Table 19

In Vitro Formation of Methylguanines in Calf Thymus DNA
Incubated with Hydrazine and Formaldehyde and Hamster Liver Cell Fractions

Treatment	Methylguanines (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine
Microsome only		
Hydrazine	ND, ND	ND, ND
Hydrazine and formaldehyde	53, 49 (mean = 51)	11, 12 (mean = 12)
Dimethylnitrosamine	318, 359 (mean = 339)	59, 60 (mean = 60)
Microsomes with cytosol		
Hydrazine	trace, 17	ND, trace
Hydrazine and formaldehye	121, 102 (mean = 112)	24, 26 (mean = 25)
Dimethylnitrosamine	679, 885 (mean = 782)	110, 119 (mean = 115)
Boiled microsomes with fresh cytosol		
Hydrazine	ND, ND	ND, ND
Hydrazine and formaldehye	100, 87 (mean = 94)	23, 24 (mean = 24)
Dimethylnitrosamine	26, 70 (mean = 48)	6, 6 (mean = 6)

Concentrations of hydrazine, formaldehyde, and dimethylnitrosamine in the incubation medium were 5mM
ND and trace: as in Table 18
All treatments incubated with boiled microsomes without cytosol failed to produce detectable levels of methylguanines in the DNA

Table 20

In Vitro Formation of Methylguanines in Calf Thymus DNA Incubated with Hydrazine, Formaldehyde or Acetaldehyde and Hamster Liver S9 Fraction

	Methylguanines (μmol/mol guanine)*	
Treatment**	7-Methylguanine	O^6-Methylguanine
Hydrazine	12 ± 8	ND
Hydrazine and formaldehyde	209 ± 17	29 ± 5
Formaldehyde	ND	ND
Hydrazine and acetaldehyde***	ND	ND
Acetaldehyde***	ND	ND
Monomethyl-hydrazine	ND	ND
Dimethyl-nitrosamine	225 ± 76	38, 40, tr
Control (buffer)	ND	ND

* The mean and standard deviation are given; all treatments were tested with boiled hamster liver S9 fraction; no methylguanines were detected for any treatment expect for monomethylhydrazine, in which case 2 of the 3 incubation systems contained 7-methylguanine in the DNA (113 and 115 μmol/mol guanine) and all 3 contained O^6-methylguanine (18 μmol/mol guanine in each instance)

** Concentration of hydrazines, aldehydes, and dimethylnitrosamine in incubation medium was 5.0 mM

*** Neither methylguanines nor ethylguanines could be detected

important intermediate, that hydrazine could react with acetaldehyde, if present, to form acetaldehyde hydrazone, which could yield diazoethane and hence lead to ethylation of the DNA. No evidence, however, has been obtained so far to support the in vitro ethylation of DNA in the presence of hydrazine and acetaldehyde. It may be that 1) acetaldehyde inhibits the alkylation reaction, 2) that the rate of metabolic activation of the putative acetaldehyde hydrazone is considerably slower than that for the formaldehyde analog, or 3) diazoethane is not as effective as diazomethane in alkylating the DNA under the experimental conditions used.

The results with monomethylhydrazine at a concentration of 5mM in the incubation medium were as expected. Monomethylhydrazine added to the incubation system with fresh S-9 fraction didn't result in the formation of methylguanines in the naked calf thymus DNA; however, if the S-9 fraction was boiled before addition to the incubation system, then the presence of monomethylhydrazine in high concentration resulted in the formation of both 7-methylguanine and O^6-methylguanine in the DNA.

A reanalysis of the monomethylhydrazine used in these studies was done by gas chromatography - mass spectrometry (GC/MS) to determine if a contaminant is present in the material. Most monomethylhydrazine produced commercially is made by reacting chloramine with methylamine:

$$NH_2Cl + CH_3NH_2 \longrightarrow CH_3\text{-}NH\text{-}NH_2$$

but it may be that the material used in these studies (Aldrich Chemical Co.) may have been prepared by reacting hydrazine with dimethylsulfate:

$$H_2N\text{-}NH_2 + (CH_3O)_2SO_2 + NaOH \longrightarrow CH_3\text{-}NH\text{-}NH_2 + CH_3OSO_3Na + H_2O$$

Dimethylsulfate is a direct methylating agent and known to react with DNA to produce 7-methylguanine. GC/MS analysis was unable to detect any impurities in the monomethylhydrazine, and specifically, no dimethylsulfate.

Hepatotoxicity and Liver Formaldehyde Levels

A pilot experiment was conducted to determine whether the amount of endogenous formaldehyde in liver could be altered significantly and nonspecifically by inducing hepatotoxicity. It was proposed earlier that hydrazine may react with endogenous formaldehyde in liver to form the corresponding hydrazone which could be oxidized to the methylating agent, diazomethane; hydrazine-induced hepatotoxicity might cause an elevation in the amount of free formaldehyde in the liver, thereby providing more subtrate with which the hydrazine could react.

Two Fischer 344 rats (male, 280-300 g) were fasted overnight and given 1.5 g carbon tetrachloride or 7.73 mL ethanol per kg body wt, ip, undiluted; an additional rat was given 2.8 mg yellow phosphorus/kg body wt, in olive oil, ip. One ethanol-treated rat died an hour after treatment; its liver was removed in less than 10 min after death and frozen immediately. The remaining animals were decapitated 24 hr after treatment. Livers of rats treated with carbon tetrachloride or phosphorus were pale; the liver from the surviving ethanol-treated rat appeared normal. The livers from the rats given carbon tetrachloride were cut in half, and one half was rapidly frozen while the other half was used immediately for formaldehyde determination. The hepatic level of formaldehyde was determined by HPLC; freezing the liver before chemical analysis did not alter the formaldehyde level in the tissue, according to the assay. The results are summarized in Table 21.

Table 21

Hepatic Formaldehyde Levels in Poisoned Rats

Treatment	Formaldehyde (nmol/g liver)
Control	88.6
Carbon tetrachloride	75.9
Carbon tetrachloride	74.1
Ethanol (died in 1 hr)	100.0*
Ethanol	65.0
Phosphorus	62.0**

* Also 189 nmol acetaldehyde/g liver in animal that died 1 hr after ethanol administration but none detectable in the animal that was killed 24 hr after treatment
** Also elevated level of acetone in liver.

Effect of Pyrazole Pretreatment of Hamsters on Hydrazine-induced Methylation of DNA Guanine

The several studies designed to test the hypothesis that hydrazine reacts with endogenous formaldehyde in the liver to form formaldehyde hydrazone which in turn could be oxidized to the potent methylating agent, diazomethane, have not strongly supported the hypothesis. Some, but not all, of the results of the in vivo and in vitro studies have been consistent with this process. The studies have suggested rather strongly, however, that 1) hydrazine and formaldehyde react to yield an intermediate which requires metabolic activation, and 2) that metabolic activation seems to be sensitive to inhibitors of the soluble enzyme, aldehyde dehydrogenase; disulfiram and cyanamide inhibit aldehyde dehydrogenase and inhibit the hydrazine-induced methylation of DNA. Methanol and ethanol are substrates for alcohol dehydrogenase, and their oxidation products in turn are substrates for aldehyde dehydrogenase; methanol and ethanol could compete with the putative product of the hydrazine-formaldehyde reaction for the dehydrogenase, blocking the activation of that product and thus inhibiting the hydrazine-induced methylation of DNA. A preliminary experiment was carried out to determine whether inhibition of alcohol dehydrogenase by pyrazole could effect the methylation of DNA guanine in hydrazine-poisoned animals.

Twelve adult male Syrian golden hamsters (109-145 g body wt.) were fasted overnight and divided into 4 groups: Group 1 received no treatment (control); Group 2 was given 360 mg pyrazole/kg body wt. in saline at T = -2 hours; Group 3 was given 90 mg hydrazine/kg body wt. in 0.1 M HCl p.o. at T = 0; Group 4 was given pyrazole at T = -2 hours and hydrazine at T = 0. All animals were decapitated at T = 5 hours and liver DNA was isolated and analyzed for 7-methylguanine and O^6-methylguanine; the results are summarized in Table 22.

Table 22

Methylguanines in Liver DNA of Hamsters
Pretreated with Pyrazole Before Hydrazine Administration

Treatment	Methylguanines (μmol/mol guanine)	
	7-Methylguanine	O^6-Methylguanine
Control	ND, ND, ND	ND, ND, ND
Pyrazole	ND, ND, ND	ND, ND, ND
Hydrazine	74, 127, 354	trace, 3, 45
Pyrazole, Hydrazine	113, 31, ND	4, ND, ND

Values represent average of duplicate determinations from individual livers
ND: none detected

Pyrazole pretreatment inhibited the methylation of liver DNA guanine in hydrazine-poisoned hamsters, a finding which is consistent with the hypothesis that hydrazine may react with endogenous formaldehyde to form an intermediate which is metabolically activated by alcohol dehydrogenase and aldehyde dehydrogenase. A new hypothesis for the mechanism of action of hydrazine-induced methylation of DNA, taking into account the new findings, is presented in the Summary and Conclusions section of this report.

Methylation of DNA Guanine in Hamsters Chronically Exposed to Hydrazine in the Drinking Water

It was shown earlier that daily administration of hydrazine to hamsters in their drinking water for nine weeks resulted in the accumulation of 7-methylguanine and O^6-methylguanine in liver DNA (Bosan and Shank, 1983). Such genotoxicity is generally regarded as important in the mechanism of action in the initiation of liver cancer by methylating agents. However, in four independent chronic studies, all at lower doses than used in the nine-week study, hydrazine administration to hamsters failed to detect any liver cancer (Biancifiori, 1970; Toth, 1972; MacEwen et al., 1981; Dr. E. Weisburger, personal communication). Since the results of the DNA methylation study appear to conflict with the results of the bioassays, a study was begun in which liver DNA methylation will be measured over a two-year hydrazine exposure period to test the relationship between DNA methylation and carcinogenesis, if any.

Hydrazine sulfate and dimethylnitrosamine, the positive control, are being administered in the drinking water to groups of male Syrian golden hamsters (male, initial body wt 50-60 g, for two years; control animals are receiving distilled water only. The hydrazine sulfate dose levels are 170, 340, and 510 mg/liter (equivalent to 5.5, 11, and 17 mg hydrazine/kg body wt); dimethylnitrosamine is administered at 10 mg/liter. Solutions are prepared fresh twice weekly and kept in brown glass water bottles to protect the carcinogens from light. Water consumption was measured twice weekly during the first month

of exposure, and 4 additional times for 2-week periods at randomly-selected dates during the study. All animals were weighed weekly during the first month of exposure and monthly thereafter. The animals are maintained in laminar flow Porta-room enclosures that have been modified to exhaust through an external stack to prevent exhausting hydrazine or dimethylnitrosamine into the atmosphere. Animals are being fed Purina Formulab ad libitum.

Moribund hamsters are killed to reduce loss of tissues due to autolysis. Livers from all control and test animals that die during the study are being saved for histopathologic examination. Three animals from each group were killed by halothane overdose at 6 months, and similarly 3 animals will be killed at 12, 18, and 24 months for determination of liver DNA methylation levels (7-methylguanine, O^6-methylguanine, and 5-methylcytosine) and histologic examination.

Three animals from each group were killed in April 1984, six months after the experiment began. Some animals given dimethylnitrosamine had died earlier. DNA from liver, kidney, and lung were analyzed for 7-methylguanine and O^6-methylguanine, and the results are reported in Tables 23-25 and summarized in Table 26. Results of the 5-methylcytosine HPLC analyses on liver DNA are summarized in Table 27.

Table 23
Methylation of Liver DNA Guanine in Hamsters
Given Hydrazine or Dimethylnitrosamine in the Drinking Water for Six Months

	Methylguanines (μmol/mol guanine; mean ± S.D.)		
Treatment	7-Methylguanine	O^6-Methylguanine	Ratio: O^6:7
Control	ND, ND, ND	ND, ND, ND	
Hydrazine 170 mg/L	153 ± 76	4 ± 3	0.03 ± 0.03
Hydrazine 340 mg/L	118 ± 26	13 ± 4	0.12 ± 0.06
Hydrazine 510 mg/L	124 ± 17	105*	0.78*
Dimethyl-nitrosamine**	340*	603*	1.79*

* mean of two values
* low yields of DNA

Table 24

Methylation of Kidney DNA Guanine in Hamsters
Given Hydrazine or Dimethylnitrosamine in the Drinking Water for Six Months

Treatment	Methylguanines (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine	Ratio: O^6-MeG:7-MeG
Control	ND, ND, ND	ND, ND, ND	
Hydrazine 170 mg/L	ND, ND, ND	ND, ND, 2	
Hydrazine 340 mg/L	ND, ND, ND	5, ND, 8	
Hydrazine 510 mg/L	tr, tr, ND	6, 2, tr	
Dimethyl-nitrosamine	140, 92, 85 (106)	248, 85, 193 (175)	1.77, 0.92, 2.27) (1.65)

Table 25

Methylation of Lung DNA Guanine in Hamsters
Given Hydrazine or Dimethylnitrosamine in the Drinking Water for Six Months

Treatment	Methylguanines (μmol/mol guanine) 7-Methylguanine	O^6-Methylguanine	Ratio: O^6-MeG:7-MeG
Control	ND, ND, ND	ND, -, ND	
Hydrazine 170 mg/L	ND, -, ND	2, -, ND	
Hydrazine 340 mg/L	ND, ND, ND	5, 4, 5 (4)	
Hydrazine 510 mg/L	ND, ND, ND	6, 5, 5 (5)	
Dimethyl-nitrosamine	46, -, 46 (46)	133, -, 117 (125)	2.90, -, 2.52) (2.71)

Table 26

Comparison of Methylguanine Levels in Liver, Kidney and Lung DNA from Hamsters Given Hydrazine or Dimethylnitrosamine in the Drinking Water for Six Months

Treatment	Methylguanines (μmol/mol guanine)					
	7-Methylguanine			O^6-Methylguanine		
	Liver	Kidney	Lung	Liver	Kidney	Lung
Control	ND	ND	ND	ND	ND	ND
Hydrazine 170 mg/L	153	ND	ND	3.6	tr	tr
Hydrazine 340 mg/L	118	ND	ND	12.7	4.3	4.2
Hydrazine 510 mg/L	124	tr	ND	104.6	2.7	5.2
Dimethyl-nitrosamine	340	106	46	602.6	175.2	124.9

Table 27

5-Methylcytosine Content of Liver DNA from Hamsters Given Hydrazine or Dimethylnitrosamine in Drinking Water for Six Months

Treatment	5-Methylcytosine (% total cytosine)
Control	2.8 ± 1.6
Hydrazine (170 mg/L)	2.7 ± 1.2
Hydrazine (340 mg/L)	3.0 ± 1.2
Hydrazine (510 mg/L)	2.5 ± 1.0
Dimethylnitrosamine	2.4

Values are means of duplicate determinations on three liver samples except for dimethylnitrosamine where DNA could be isolated from only two livers.

The positive control, dimethylnitrosamine, provided liver DNA with twice as much 7-methylguanine as seen in the animals treated with hydrazine and six times more O^6-methylguanine than seen in the hamsters exposed to the highest concentration of hydrazine; in the dimethylnitrosamine-treated animals the ratio of O^6-methylguanine to 7-methylguanine is almost 2, the highest ratio ever seen for any methylating carcinogen (no one has reported alkylation levels in liver DNA after 6-months exposure to dimethylnitrosamine). The variability in the 5-methylcytosine results (Table 27) was so great that no difference between groups, if they existed, could be identified.

Histopathology has not yet been done on sections taken from the livers analyzed above; in gross appearance the livers from the hydrazine animals were unremarkable, but those from the dimethylnitrosamine-treated animals were small and nodular, and cut with the texture of wet sand. Because of the severe liver injury in the dimethylnitrosamine-treated animals and the increasing rate of mortality in this group, dimethylnitrosamine exposure was terminated and the surviving animals given distilled water; the hydrazine exposures are continuing.

Effect of Route of Administration of Hydrazine on Methylguanine Formation in Hamster Liver, Kidney, Lung, and Brain DNA

All the studies on the formation of 7-methylguanine and O^6-methylguanine in DNA in response to hydrazine administration have focussed on liver DNA after oral administration of the toxicant. This experiment was designed to expand the observations to include additional routes of administration and organs.

Twenty-four young adult male Syrian golden hamsters (34-64 g) were given 90 mg hydrazine/kg body wt in saline pH 7.4, or saline only by gavage or by intraperitoneal or subcutaneous (nape) injection. Of the 24 fasted hamsters treated with hydrazine by each of the 3 routes of administration, no animals died within the 24-hr observation period following oral or intraperitoneal administration, but 4/24 died following subcutaneous injection; death was preceded by running in circles and intermittent tonic/clonic convulsions.

The animals were decapitated 24 hr later, and DNA was isolated from 3 pooled livers, 8 pooled kidneys, 8 pooled lungs, and 8 pooled brains from each group; 3 DNA analyses were done for each group. The results are summarized in Table 28; no methylguanines were found in any tissues from control animals or in brain (in spite of the obvious central neurotoxicity) from hydrazine-treated animals. Both 7-methylguanine and O^6-methylguanine were detected in DNA from liver, kidney, and lung, and route of administration had little effect on the extent of this alkylation.

Hydrazine-derivative Drugs and Methylation of Liver DNA Guanine

Hydralazine and isoniazid (structures shown) are two chemotherapeutic agents which can induce hepatotoxicity. Both of these compounds contain free hydrazino groups and are thought to be metabolized to free hydrazine. Although it has been proposed that acetylhydrazine, a further metabolite of these drugs, is responsible for the hepatotoxicity (McKennis et al., 1959; Nelson and Gordon, 1980), it is also plausible that hydrazine itself could be the active agent. If this is the case, then administration of these drugs might be expected to result in methylation of DNA guanine, as is the case in hydrazine administration; that is, administration of these drugs might be viewed as another means of administering hydrazine. It has been suggested that these drugs could be weak carcinogens. The mechanism of their carcinogenicity, then, could be due to hydrazine formation and resultant aberrant methylation of DNA.

Male Sprague Dawley rats and C57Bl/6 mice were fasted overnight and then given the published LD_{50} of hydralazine or isoniazid (Sigma Chemical Co.): rats, 155-190 g body wt, 50 mg hydralazine or 325 mg isoniazid per kg body wt, orally; mice, 18-19 g

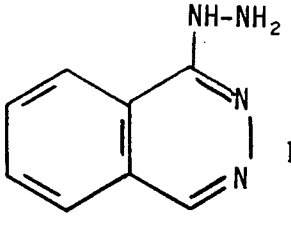
Table 28

Effect of Route of Administration of Hydrazine
on Levels of Methylguanines in Hamster Liver, Kidney, Lung, and Brain DNA

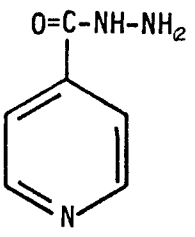
Tissue	Methylguanines (μmol/mol guanine)		Ratio
	7-Methylguanine	O^6-Methylguanine	O^6-MeG:7-MeG
Oral			
Liver	672 ± 100	139 ± 27	0.21
Kidney	67 ± 8	12 ± 4	0.18
Lung	10 ± 1	trace	-
Brain	ND	ND	-
Intraperitoneal			
Liver	724 ± 287	118 ± 22	0.16
Kidney	39 ± 19	12 ± 5	0.31
Lung	7 ± 1	trace	-
Brain	ND	ND	-
Subcutaneous			
Liver	516 ± 95	112 ± 19	0.22
Kidney	53 ± 3	12 ± 2	0.24
Lung	trace	trace	-
Brain	ND	ND	-

Means ± s.d.
Trace: between 1 and 4 μmol/mol guanine
ND: none detected

body wt, 50 mg hydralazine/kg po or 50 mg isoniazid/kg ip. Five rats and seven mice were used per group. The animals were decapitated 24 hr after treatment, and liver DNA was analyzed for methylguanine content. Hepatic DNA 7-methylguanine contents were 9, 13, 28, 30 μmol/mol guanine for four of the five rats treated with isoniazid; the fifth animal had only a trace of 7-methylguanine in its liver DNA. No methylguanines were found in liver DNA of rats treated with hydralazine. Liver DNA from mice was pooled for the 7-methylguanine determination. More of this base was observed in the DNA from mice treated with hydralazine (35 μmol 7-methylguanine/mol guanine) compared to DNA from mice treated with isoniazid (3 μmol/mol guanine). No O^6-methylguanine was detected in any of the DNA samples. These results are consistent with the proposal that the drugs are metabolized to free hydrazine, and also suggest that they may be carcinogenic via the mechanism of aberrant methylation of DNA.



hydralazine



isoniazid

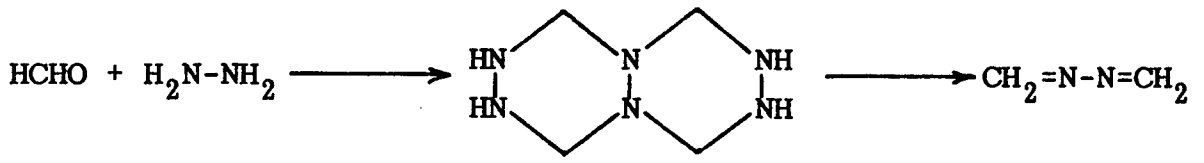
SUMMARY AND CONCLUSIONS

In vivo studies on the role of the 1-carbon pool in the methylation of DNA guanine following hydrazine exposure have not provided strong support for the hypothesis that hydrazine reacts with endogenous formaldehyde to form formaldehyde hydrazone, and subsequently, diazomethane. Administration of hydrazine appeared to have little effect on formaldehyde levels in liver. Large acetaldehyde levels in liver did not result in formation of ethylguanines in DNA of hamsters given hydrazine. On the other hand, several in vitro studies demonstrated that addition of hydrazine and formaldehyde to incubation medium containing mammalian enzymes and naked DNA resulted in the expected formation of 7-methylguanine and O^6-methylguanine in the DNA. It may be that formaldehyde is complexed with reduced glutathione in the liver cell in vivo and is not available to react with hydrazine to form the hydrazone, but in the in vitro system, to which glutathione is not added, the reaction between added hydrazine and added formaldehyde is favored. Acetaldehyde in the in vitro system may not form the corresponding hydrazone and diazoethane as rapidly as does formaldehyde, and alkylation of DNA by diazoethane may occur at a rate considerably less than that for diazomethane.

Evidence has been obtained to suggest that monomethylhydrazine may spontaneously methylate DNA, an observation which may be important in understanding the mechanism of DNA methylation in response to hydrazine administration. Until now, all efforts to support the hypothesis that monomethylhydrazine is an important intermediate in the hydrazine response have failed. Specifically, in the rat, mouse, and hamster hydrazine treatment was more efficient in producing methylguanines in DNA than was monomethylhydrazine treatment on an equimolar basis. The literature claims that monomethylhydrazine is distributed with the body water, as is hydrazine, and is sufficiently stable in body fluids and tissues to be measured hours after administration. If those reports are incorrect and monomethylhydrazine breaks down spontaneously and rapidly in blood, the monomethylhydrazine administered intraperitoneally or by stomach tube may never reach the liver; but hydrazine, administered similarly, may reach the liver and there in the liver cell become methylated by S-adenosylmethionine and a N-methyltransferase to monomethylhydrazine, where it immediately decomposes to a methylating agent. Such a mechanism would suggest that monomethylhydrazine would have carcinogenicity properties similar to those of MNU, which is known to rapidly decompose spontaneously at physiological pH to a methylating agent and produce tumors at the site of application. However, monomethylhydrazine does not have such carcinogenicity properties. Therefore, the role of monomethylhydrazine in hydrazine-induced DNA methylation remains confused.

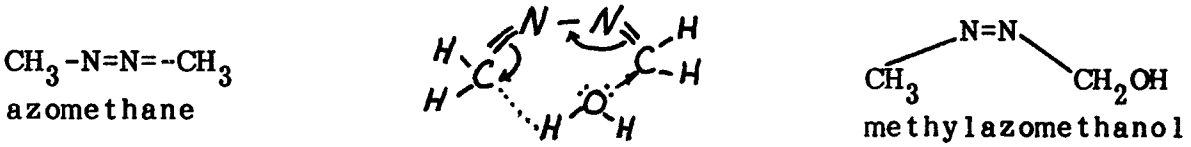
The several studies carried out to test the hypothesis that hydrazine might react with endogenous formaldehyde to form a methylating intermediate did indicate that inhibitors of alcohol and aldehyde dehydrogenases, used in an attempt to raise aldehyde levels by blocking their metabolism, resulted in a depressed DNA methylation response to hydrazine. Methanol and ethanol themselves appeared to interfere with the hydrazine-induced methylation of DNA, perhaps by competing with the putative hydrazine-formaldehyde reaction product for the dehydrogenases.

If the dehydrogenases are important in the metabolic activation of hydrazine to a methylating agent, then the proposed intermediate of diazomethane may be incorrect. Neureiter (1959) reported that the major reaction product of aqueous formaldehyde with hydrazine is tetraformyltrisazine (Kirk-Othmer, 1980) which depolymerizes to formaldazine (formaldehyde azine; methanal azine, Chem. Abs. Ser. No. 503-27-5):

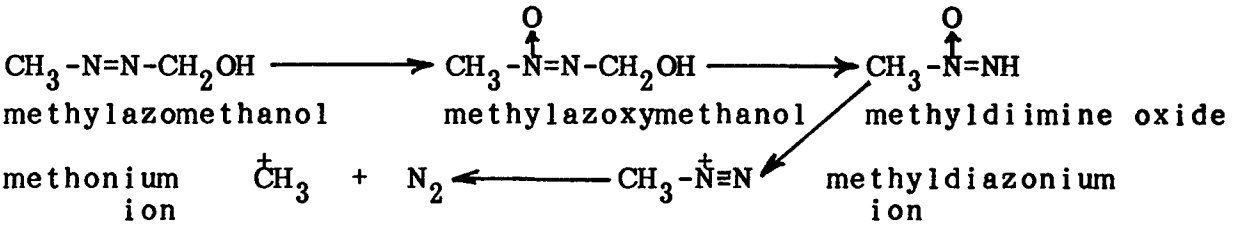


tetraformyltriazine formaldazine

If this reaction could take place in the liver cell, the formaldazine might be reduced to azomethane, a known methylating intermediate (Fiala, 1977), or, more likely, the formaldazine might undergo a nucleophilic attack by water to yield methylazomethanol:



A subsequent N-oxidation could yield methylazoxymethanol, a methylating intermediate more proximal than azomethane (Fiala, 1977):



The N-oxidation might be catalyzed by cytochrome P-450 as Fiala (1977) suggested for the oxidation of methylazomethanol, or it may be catalyzed by a tertiary amine oxidase (Ziegler and Mitchell, 1972).

Such a proposed mechanism could explain why hydrazine and acetaldehyde fail to produce ethylguanines in DNA; the initial reaction depends on formation of the tetraformyltrisazine polymer and the ethyl analog of the polymer apparently is not favored. Also this modified hypothesis could explain why in earlier studies disulfiram and cyanamide inhibited the methylation of DNA guanine in hydrazine-treated animals. These agents were tested in vitro and in vivo because they are known to inhibit aldehyde dehydrogenase; such inhibition was desired to increase the levels of endogenous aldehydes, by preventing their metabolism, to provide more substrate with which hydrazine could react to form the corresponding hydrazones. The modified hypothesis should indicate a need for aldehyde dehydrogenase in the putative activation of the methylating agent:

This oxidation is consistent with Schoental's suggestion that methylazoxymethanol is metabolically activated to the aldehydic derivative (Schoental, 1973).

Hamsters exposed for six months to hydrazine in the drinking water accumulated 7-methylguanine and O^6-methylguanine in their liver DNA, as predicted from the nine-week study reported last year; the levels of methylguanines accumulated were of the same order of magnitude as those for hamsters exposed to dimethylnitrosamine in the drinking water for the same period. The livers of the animals exposed to hydrazine appeared unremarkable, but those from the dimethylnitrosamine-treated hamsters were grossly damaged; some of the livers from the dimethylnitrosamine group had macroscopic nodules and were severely discolored. This chronic study is in its first quarter, and conclusions made at this time would be premature.

Methylation of DNA guanine in hydrazine-treated hamsters is not limited to only the liver; similar findings were obtained in kidney and lung, but not brain even at doses which were clearly toxic to the brain. Consistent with the distribution of hydrazine with the body water, route of administration (oral, intraperitoneal, subcutaneous) had little effect on the amount of 7-methylguanine and O^6-methylguanine formation in the DNA of those tissues.

Administration of drugs which are thought to yield hydrazine as an intermediary metabolite also results in the methylation of liver DNA guanine; the two drugs tested were isoniazid and hydralazine.

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