COMPARATIVE BIOCHEMISTRY AND METABOLISM

RONALD C. SHANK, Ph.D.

THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
UNIVERSITY OF CALIFORNIA, IRVINE
IRVINE, CALIFORNIA 92717

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AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

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AMRL-TR-78-89

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 Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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Co-administration of an LD0.01 dose of hydrazine with ^{14}C-methylmethionine to rats or mice results in the methylation at the 7-position of guanine in liver DNA; in the mouse, but not in the rat, methylation at the O^6-position of guanine in liver DNA also occurs. Methylation of liver DNA in only the 7-position of guanine occurs in rats and mice treated with MMH and to a much lesser extent with UDMH. Carbon dioxide production from MMH and UDMH is most active in rat liver, less active in kidney and colon and only slightly active in lung; carbon dioxide production from MMH is 1.4 to 5.5 times greater than for UDMH.

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PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism 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 F33615-76-C5005, Work Unit #2312V117. This document describes the accomplishments of the Subprogram from June, 1977 through May, 1978.

R. C. Shank, Ph.D., served as coordinator for the Subprogram. Acknowledgement is made to J. A. Daggett, L. R. Barrows and D. C. Herron for their significant contributions and assistance in the preparation of this report. K. C. Back, Ph.D., Chief of the Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SECTION I
INTRODUCTION

This document constitutes the annual report of the Subprogram on Comparative Metabolism and Biochemistry, and describes the accomplishments of the laboratory from June, 1977 through May, 1978. The subprogram was established to characterize metabolic pathways of potential Air Force pollutants in selected animal and human tissues which will provide data for estimating whether man metabolizes the compounds at rates and by pathways similar to those for sensitive or resistant animals.

Hydrazine, monomethylhydrazine (MMH), and unsymmetrical dimethylhydrazine (UDMH) are used as rocket propellants and the United States Air Force is currently seeking the safe atmospheric concentrations for these compounds. Under conditions of high concentration and prolonged oral administration, these compounds have produced carcinomas in certain experimental animals.

The Toxic Hazards Research Unit (THRU), operated by the University of California, Irvine, at the Aerospace Medical Research Laboratories at Wright Patterson Air Force Base, Ohio, is currently conducting studies to determine quantitatively the carcinogenicity of hydrazine, MMH and UDMH under conditions appropriate to practical human exposure. These inhalation carcinogenicity studies are investigating the sensitivity of the mouse, rat, hamster and dog, recognizing the marked inter-species differences in response to these compounds.

Early studies on the metabolism of these hydrazines are incomplete in that the pathways were not fully defined and metabolism was not considered from the point of view of transformation of the hydrazines to proximate carcinogens. The fact that there are marked differences in the overall oxidation and excretion of hydrazines by various species strongly suggests that the current carcinogenicity studies will demonstrate a variety of responses in tumor production in the four species on test at THRU. The multi-species design provides a better basis for measuring carcinogenicity by reducing the possibility of performing the test in a species uniquely sensitive or resistant and therefore an inappropriate model for man. This design also raises an important question in interpreting the results of the cancer study: which species gives the most accurate prediction of the human response?

Extrapolation from animal data to man is difficult at best, but when carcinogenesis is the consideration, there are no proven measures upon which to base the extrapolation. If an environmental agent requires metabolic activation to generate a proximate carcinogen, which is thought to be the case with the hydrazines, then comparative metabolic studies on those agents in sensitive and resistant (to tumor formation) species could be useful in predicting which species is the best indicator of the human response, if the metabolism of the agent in man is known. The probable carcinogenicity of the agent precludes *in vivo* metabolism studies in man, but studies using fresh autopsy material may provide the information needed.

The ultimate objective of the Subprogram on Comparative Metabolism and Biochemistry is to measure the overall rate of metabolism of hydrazine, monomethylhydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine in liver, lung, kidney and large intestine preparations from the mouse, rat, hamster and human. The formation of several probable metabolites has been postulated for each agent, but these do not appear relevant to the potential carcinogenicity of these compounds; instead, attention will be focused on the ability of the hydrazines to bring about alkylation of deoxyribonucleic acids, thus identifying the compounds as indirect alkylating agents. The results of the comparative metabolic studies will be correlated with the results of comparative carcinogenicity studies currently in progress at THRU as an approach to judging human sensitivity to the tumor-producing capabilities of these rocket propellants.

SECTION II
RESEARCH PROGRAM

METHODS

Synthesis of ^{14}C-Labeled Methylated Hydrazines

The synthesis of ^{14}C-monomethylhydrazine and ^{14}C-1,2-dimethylhydrazine was carried out by our laboratory. Hydrazine (5.5 µl) was added slowly to 9.5 mg ^{14}C-dimethylsulfate (1mCi, 13.3 mCi/mmole); 50 µl of concentrated HCl were added to precipitate the hydrazines. The entire mixture was dissolved in 0.30 ml water and applied to thin layer chromatography plates (Avicel 250 microns, Analtech Labs) which were developed in isopropanol:water:HCl, 155: 25:20 (v:v). The hydrazines at one edge of each plate were visualized by

spraying with Folin-Ciocalteau phenol reagent followed by ammonia. The remainder of each plate was scraped to separate MMH and UDMH which were then eluted from the cellulose with water. The specific activity of the MMH was 6.6 μCi per μmole; the preparation contained about 20% UDMH. The specific activity of the UDMH was 13.3 mCi/mole. The aqueous extracts contained 102 μgMMH/ml and 72 μgUDMH/ml.

Liquid Chromatographic Fractionation of DNA Hydrolysates

The fractionation of DNA hydrolysates by high pressure liquid chromatography has undergone several refinements to improve resolution of pyrimidine bases and certain methylated purine bases and to permit direct counting of eluate fractions by liquid scintillation. Two types of hydrolysis are used routinely: when interest is limited only to purines, the DNA is dissolved in water and an equal volume of 0.2 N HCl is added to make a final concentration of 5 mg DNA/ml 0.1 N HCl; the hydrolysis is achieved by heating the mixture to 70°C for 30 minutes (this technique preserves labile bases such as O-methylated purines and pyrimidines). When interest includes labile pyrimidine derivatives, the DNA is hydrolyzed enzymatically; 5 mg DNA is dissolved in water containing 20 μg DNase (Sigma, DN-100) and incubated at 37°C for one hour; 0.22 unit venom phosphodiesterase (Sigma P-6877 Type II) and 32 μg alkaline phosphatase (Sigma P-4252) are added in 1 ml Tris buffer pH 8.9 and the incubation is continued an additional 18 hours.

Acid hydrolysates are fractionated on Partisil SCX, 10 micron columns (Whatman) with 0.045 M ammonium phosphate developed over 15 minutes at a flow rate of 2 ml/min. For larger samples, (more than 0.5 mg DNA) the preparative 50 cm column (Partisil SCX) is used; the gradient is linear from 0.05 to 0.1 M ammonium phosphate developed over 20 minutes at a flow rate of 4 ml/min. For both procedures the gradients are prepared automatically with distilled water as the weak solvent and 0.1 M ammonium phosphate pH 3.0 as the strong solvent. The eluate is monitored at 275 nm unless maximum sensitivity for O^6-methylguanine is desired, in which case the wavelength is 286 nm.

The retention volumes for the various DNA fractions are summarized in Table 1 and Figures 1 and 2. Limits of detection for the DNA fractions using ultraviolet absorbance are approximately 1 ng applied to the column, depending

TABLE 1

CHROMATOGRAPHIC (HPLC) FRACTIONATION

OF DNA HYDROLYSATES

	Retention Volumes, milliliters*	
Acid Hydrolysate	Analytical column	Preparative column
Pyrimidine oligonucleotides-I	2.8	16.0
-II	4.4	24.8
-III	6.2	35.2
Guanine	8.8	49.6
Adenine	14.3	73.6
3-Methylguanine	about 15	about 75
7-Methylguanine	18.2	78.4
1-Methyladenine	27.0	-
7-Methyladenine	31.3	-
O^6-Methylguanine	36.4	145.6
3-Methyladenine	44.8	-
Enzyme Hydrolysate		
X	7.6	22.4
Thymidine	9.2	30.4
Deoxyguanosine	11.2	35.2
X'	18.4	46.4
X''	20.4	51.2
Deoxycytidine	24.0	54.4
Deoxyadenosine	27.2	65.6
X'''	33.6	78.4

*Elution systems: acid hydrolysates - 0.045 M ammonium phosphate pH 2.3; analytical column run at 2 ml/min, prep. column at 4 ml/min., enzyme hydrolysates - analytical column uses convex gradient (N=2.5) of water to 0.025 M ammonium phosphate made from 0.1 M ammonium phosphate pH 3, developed over 15 minutes at 2 ml/min; prep. column uses linear gradient of 0.05 to 0.1 M ammonium phosphate made from 0.1 M ammonium phosphate pH 3, developed over 20 minutes at 4 ml/min.

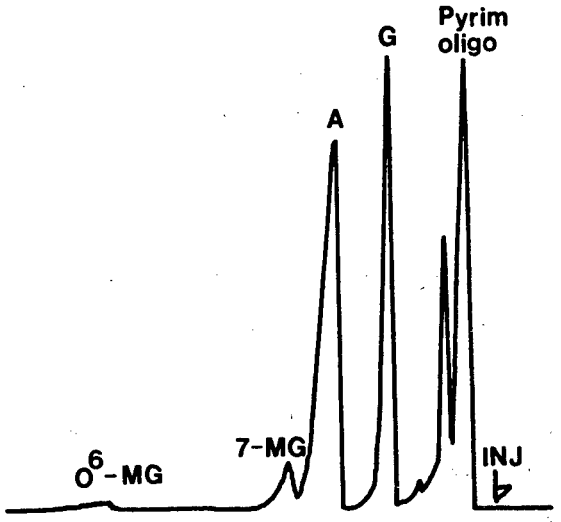


Figure 1. Chromatographic separation of purines and pyrimidine oligonucleotides in DNA hydrolysate (dilute acid).

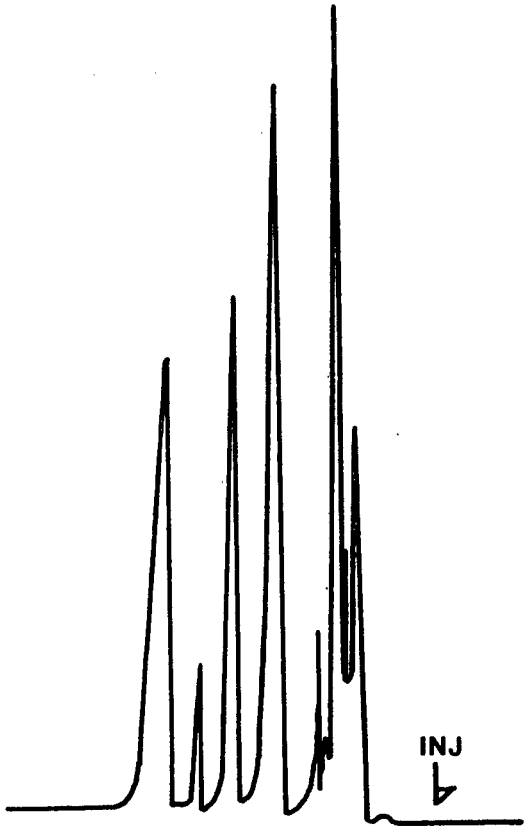


Figure 2. Chromatographic separation of nucleosides from DNA enzyme hydrolysate.

upon the individual fraction. Using a fluorescence detector, the limits of detection are brought down to approximately 100 picograms (0^6-methylguanine) applied to the column.

Gas Chromatography - Mass Spectroscopy

A Model 5992A Hewlett Packard GC/MS has been used to 1) examine the stock supplies of propellant hydrazines for contaminants and 2) identify abnormal pyrimidine bases in liver DNA from rats treated with hydrazine.

A method for a gas chromatographic separation of UDMH and its contaminants, suggested by the Toxic Hazards Research Unit, was slightly modified for the GC/MS analysis of hydrazine, MMH, and UDMH for impurities. The method involves injecting 10 µg of propellant hydrazine in 1 µl methanol into a 10 foot, 2mm ID glass column packed with Tenax GC, 60/80 mesh; the injector temperature was 117°C and the helium flow rate was 25 ml/min.; the column oven temperature was held at 90°C for 4 minutes after injection and then raised 5°C per minute to 140°C. A mass spectrum was obtained on a contaminant common to hydrazine and UDMH but not MMH; its probable molecular weight is 93.1 and is present at concentrations less than one percent. No further attempts have been made to identify the contaminant. Other contaminants at lower concentrations are present in the UDMH.

Administration of hydrazine to rats and mice subsequently treated with ^{14}C-methyl-methionine results in the labeling of pyrimidine oligonucleotides in liver DNA. Individual labeled pyrimidine nucleosides from animals so treated have been isolated but not yet identified. The DNA is hydrolyzed enzymatically to free the individual purine and pyrimidine nucleosides. Commercial sources for methylated nucleosides, except 5-methyldeoxyribocytosine, are not available and it is not feasible to synthesize these compounds in the laboratory to use as authentic standards. The nucleosides, isolated from the DNA, have been silylated for GC/MS analysis but the silylation has not been reproducible to permit identification of the compounds. Much of the problem lies with the presence of ammonium phosphate with the nucleoside (the salt comes from the buffer used to elute the fractions from the column). Attempts are in progress to substitute dilute HCl for the buffer to elute the nucleosides.

^{15}N-hydrazine was purchased from a local supplier to study the fate of the nitrogen atoms in the metabolism of the hydrazine compounds. These studies will receive a high priority in the next year.

HYDRAZINE METABOLISM

Several experiments have been done on the methylation of liver DNA in rats treated with hydrazine and a paper describing early results was presented at the March, 1978 Society of Toxicology Annual Meetings in San Francisco.

Rats are fasted overnight to reduce the hepatic stores of glycogen which interfere with the DNA isolation. Between 8 and 9 a.m., the rats are given orally a solution of hydrazine in 0.1 N HCl (intubation volume approximately 0.1 ml); controls are given 0.1 N HCl only. At the same time and every hour thereafter for four hours, all rats are given ^{14}C-methylmethionine (approximately 20 μCi) intraperitoneally, and are killed by decapitation one hour after the last methionine injection. Liver DNA is isolated, acid hydrolyzed and chromatographically separated into pyrimidine oligonucleotides and free purine bases. At a dose of 60 mg hydrazine/kg body weight (LD0.01), but not at a dose of 30 mg/kg, the presence of 7-methylguanine in the DNA hydrolysate can be detected. In early experiments 7-methylguanine could also be detected, albeit at much lower concentrations, in liver DNA from control rats; however, as intubation technique improved and became less stressful, this abnormal base was not found in control liver DNA.

The 7-methylguanine was radioactive indicating the ^{14}C-methyl-methionine as the source of the methyl group. The most likely pathways for such a methylation of DNA to occur are 1) via methylation of hydrazine to monomethylhydrazine which is then metabolically converted to a reactive methonium ion (CH_3^+), or 2) via an enzymatic methylation of DNA by DNA methylase, the enzyme which normally methylates cytosine in the 5-position using methionine as the source of the methyl moiety. Rats given 15 mg MMH/kg body weight (^{3}H-MMH) were found to have 20.6 μmoles 7-methyl-guanine per mole guanine in their liver DNA: this is compared to 150-196 μmoles 7-methylguanine per mole guanine in rats given an equitoxic dose of hydrazine (60 mg/kg body weight). Thus, on an equitoxic basis, hydrazine administration results in 7 to 10 times more 7-methylguanine in rat liver DNA than does ^{3}H-MMH administration. This

experiment will have to be repeated using equal molar doses of the two hydrazines; in the above experiments the hydrazine dose was 1.88 mmoles/kg body weight while the MMH dose was only 0.33 mmoles, a ratio of 5.7 to 1. Dividing 150-196 μmoles 7-methylguanine/mole guanine (observed in hydrazine-treated rats) by 5.7 yields 26.3 - 34.4 μmoles 7-methylguanine/mole guanine expected for MMH-treated rats if the pathway for DNA methylation is methylation of hydrazine to MMH and then to methonium ion.

These experiments have been repeated in the mouse. Groups of 10 female C57BL6 mice were given orally 10 mg hydrazine/kg body weight (LD0.01) or 14.4 mg ^{3}H-MMH/kg body weight (equimolar doses of hydrazine and MMH). The oral LD50 for hydrazine in the C57BL6 mouse, measured in the laboratory, was 100 mg/kg body weight for four hours using the Litchfield Wilcoxon (1949) method; the LD0.01 was 11.3 mg/kg body weight. Hydrazine-treated mice were given five injections ip of 4 μCi ^{14}C-methyl-methionine every hour for five hours. All mice were killed five hours after hydrazine or MMH administration, and liver DNA was isolated and hydrolyzed in dilute acid. The hydrolysates were fractionated by liquid chromatography to detect methylated purines. The pyrimidine oligonucleotide peaks in DNA from both treated groups contained the most activity. Liver DNA from hydrazine-methionine mice contained 7-methylguanine and O^{6}-methylguanine, while that from MMH-treated mice contained only 7-methylguanine, as shown in Table 2.

TABLE 2

METHYLATION OF LIVER DNA IN MICE TREATED WITH HYDRAZINE AND ^{14}C-METHYL-METHIONINE OR ^{3}H-METHYL-MONOMETHYLHYDRAZINE

Toxin	Methylated purine, μmoles/mole guanine	
(equimolar doses)	7-methylguanine	O^{6}-methylguanine
Hydrazine	220	65
MMH	338	none detected

Thus, in this preliminary experiment, the mouse seems to differ considerably from the rat: administration of hydrazine at doses equitoxic to the rat and mouse results in methylation of DNA guanine at the 7-position

to about the same extent in both species but results in methylation at the O-6 position only in the mouse; also, approximate equimolar (but not necessarily equitoxic) doses of MMH to both species labels mouse liver DNA at the 7-position of guanine to a much greater extent than rat liver DNA, implying a greater rate of metabolic conversion of MMH to methonium ion in the mouse (Table 3). These experiments need confirmation and expansion.

TABLE 3

COMPARISON OF LIVER DNA METHYLATION IN RATS AND MICE TREATED WITH HYDRAZINE AND ^{14}C-METHYL-METHIONINE OR ^{3}H-METHYL-MONOMETHYLHYDRAZINE

		Methylated purine, µmoles/mole guanine					
		Hydrazine + ^{14}C-Me-Meth.			^{3}H-Me-MMH		
Species	Doses	Dose, mg/kg	7MG	O^6MG	Dose, mg/kg	7MG	O^6MG*
Rat (Fischer 344)	equitoxic	60	150-196	nd	15	20.6	nd
Mouse (C57BL6)	equimolar	10	220	65	14.4	338	nd

*nd = none detected

One possible mechanism for the methylation of DNA in hydrazine-treated animals is the stimulation of aberrant behavior in DNA methylase, which normally transfers the methyl group from methionine to the 5-position of cytosine. A Russian study (Kudryashova and Vanyushin, 1976) has shown that administration of hydrocortisone to mimic stress results in almost twice as much 5-methylcytosine in rat liver DNA compared to untreated rats. It is possible that toxic levels of hydrazine act non-specifically by inducing stress. A preliminary study was done to determine whether stress conditions alone could result in 7-methylguanine production in rat liver DNA. Rats were given 20 mg hydrocortisone hemisuccinate per kg body weight and 12 µCi ^{14}C-methyl-methionine ip at time t = 0, at t = 1, 2, 3 and 4 hours the methionine injections

were repeated; at t = 5 hours the rats were killed and liver DNA was isolated; control animals were given saline in place of the hormone. DNA was hydrolyzed in dilute acid and analyzed by liquid chromatography and scintillation spectrometry. Control DNA containing 3.24 μmoles guanine had no detectable 7-methylguanine; DNA from hydrocortisone-treated rats containing 2.66 μmoles guanine had 14.9 μmoles 7-methylguanine per mole guanine, about one-tenth that seen in hydrazine-treated rats. These results suggest the action of hydrazine may be related to toxic stress; the studies will be expanded.

The original proposal for this work suggested that hydrazine might be acetylated in liver, and in 1976 Nelson and coworkers suggested that acetylhydrazine might acetylate macromolecules such as DNA. A single experiment in our laboratory was conducted to see if any acetylation of DNA could be detected in rats given orally 60 mg hydrazine/kg body weight and intraperitoneally ^{14}C-acetate. Three young male Fischer 344 rats were fasted overnight and at t = 0 hour given 60 mg hydrazine per kg body weight (approx. LD 0.01) per os and 291 μg (30 μCi) ^{14}C-sodium acetate (8.45 mCi/mmole) ip. The acetate injections were repeated hourly for an additional 4 hours and the animals were killed at t = 5 hours. Control animals were treated similarly but received solvent (0.1 N HCl) only instead of hydrazine. DNA was isolated from each individual liver and from pooled kidneys, brains, lungs and colons. Between 2 to 5 mg of DNA were hydrolyzed from each tissue and little radioactivity was detected indicating no incorporation of acetate-derived carbon into DNA of hydrazine-treated animals. One liver sample from a control animal contained 2400 cpm (2.7 mg DNA); the hydrolysate did not fractionate well on the column but 1132 cpm (about 2300 dpm) were recovered from the column (about 2700 dpm applied). Figures 3 and 4 illustrate the chromatograms for control colon DNA and hydrazine colon DNA respectively.

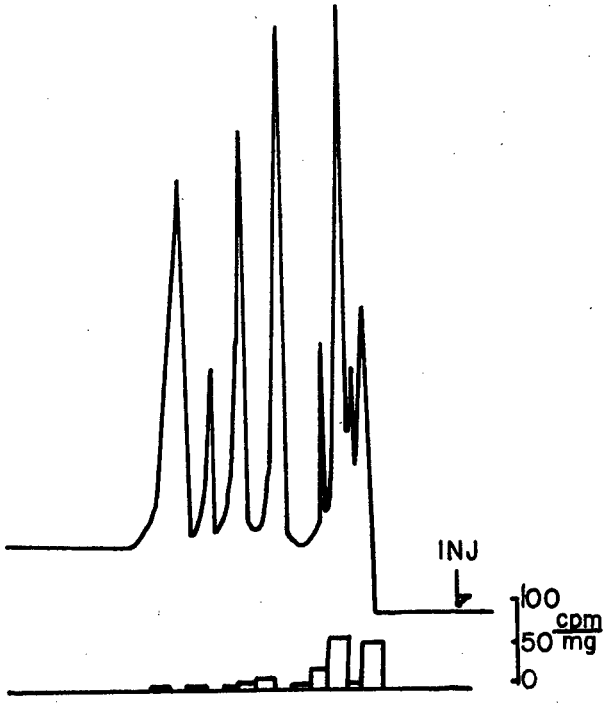


Figure 3. Chromatographic separation of colon DNA nucleosides and radioactivity from control rats given ^{14}C-acetate.

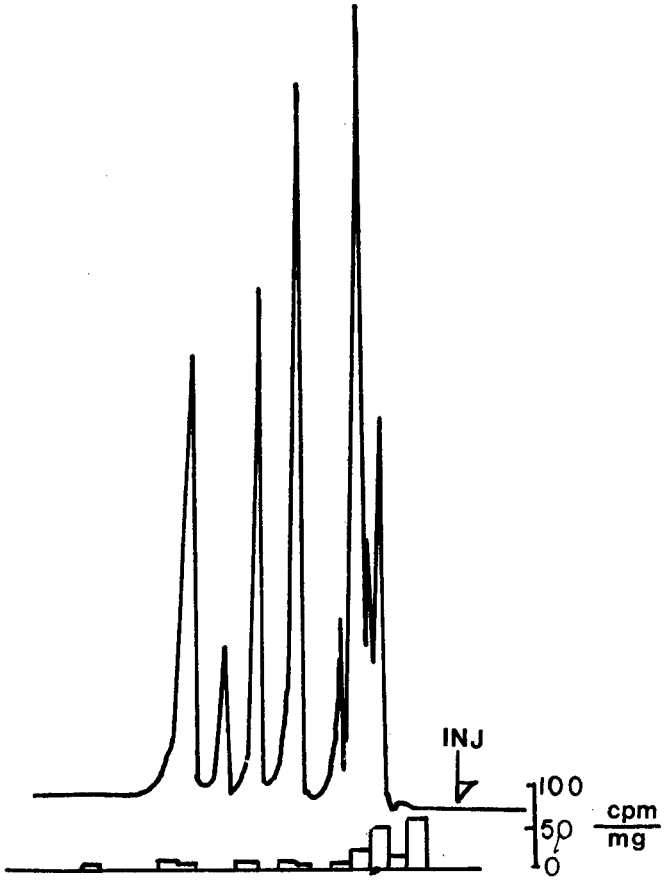


Figure 4. Chromatograph separation of colon DNA nucleosides and radioactivity from rats treated with hydrazine and ^{14}C-acetate.

There was no significant difference between the distribution of radioactivity in the two samples. It was noted that these DNA specimens did not hydrolyze completely (enzymatic method) and that approximately 80% of the label in each crude hydrolysate was filtered out before application to the column. These experiments will be repeated using more tissue DNA and weak acid hydrolysis of the DNA.

MONOMETHYLHYDRAZINE (MMH) METABOLISM

Several experimental approaches have been made to the study of MMH metabolism. The earliest studies examined the rate of disappearance of MMH from incubation mixtures containing tissue slices or tissue homogenates; in these systems the rate of disappearance with live tissue was no different from that for boiled tissue, suggesting that spontaneous decomposition was quantitatively more important than metabolism for MMH disappearance.

The emphasis of the research was changed to the study of interactions of MMH with DNA, on the rationale that focusing on such possible interactions would be more pertinent to mechanisms in carcinogenesis. Tritiated MMH was prepared in the laboratory; rat liver DNA (10.8 mg) was dissolved in 5% sodium acetate and incubated with 25 µCi ^{3}H-MMH (4.6 mCi/mmole) for 3 hours at 37^{o}C. The DNA was recovered by repeated precipitations and washings (37% recovery), hydrolyzed in 0.1 N HCl and fractionated by high pressure liquid chromatography. The results indicated that most of the radioactivity eluted from the column (50% of the radioactivity did not elute) was associated with the slowest migrating pyrimidine oligonucleotides, and a minor radioactive product chromatographed with a retention volume similar to that of 7-methylguanine. A second experiment used DNA isolated from rats which had been treated with ^{14}C-formate in infancy to incorporate ^{14}C into rings of the normal bases. Four milligrams of this DNA were dissolved in 0.1 M phosphate buffer (pH 7.4) and incubated with 1.0 mg MMH (not labeled) for 3 hours at 37^{o}C. The DNA was recovered and hydrolyzed to nucleosides; the nucleosides were separated chromatographically; no abnormal nucleosides were detected, indicating that MMH does not vigorously interact covalently with rat liver DNA *in vitro*. A small amount of abnormal nucleoside may have eluded detection due to the low specific activity of the ring labeled nucleosides. The net result of the two experiments suggests that MMH itself or a spontaneous breakdown product may bind covalently to naked DNA.

It has long been known that at high concentrations, hydrazine reacts with pyrimidines in nucleic acids to form 5,6-dihydrothymine and 4-methylpyrazole from thymine and 5,6-dihydrocytosine, N^4-aminocytosine, and 3-aminopyrazole from cytosine (Bacon and Brown, 1955; Lingens and Schneider-Bernlohr, 1965; Brown, McNaught and Schell, 1966). It may be, then, that the tritiated material eluting with the slow pyrimidine oligonucleotides represents a MMH-DNA adduct: the putative presence of 7-methylguanine would argue for reaction of DNA with a breakdown product (methonium ion?) of MMH.

The next step was to determine whether MMH would alter tissue DNA under in vivo conditions. A non-fatal but convulsive dose of ^{3}H-MMH (300 µCi, 15 mg/kg body weight) was given ip to each of 4 adult male rats. The rats were killed 4 hours later and DNA from liver, kidney, lungs, brain and colon (pooled tissues) was isolated and hydrolyzed in dilute acid. The hydrolysates contained ^{3}H-labeled material in the following order (decreasing, cpm/mg DNA): liver, lung, kidney, brain, colon. None of the radioactivity eluted with known purine or pyrimidine bases during chromatographic fractionation; in fact, only in one case, lung DNA hydrolysate, did any radioactive material elute at all and that material could not be identified.

The experiment was repeated twice in the rat under the same conditions except the animals were killed 5 hours after oral ^{3}H-MMH administration (instead of 4 hours after ip administration). In one experiment acid hydrolysis of liver DNA yielded 1.73 nmoles of 7-methylguanine per mole of guanine, and enzymatic hydrolysis of the same DNA yielded 1.55 nmoles of 7-methyldeoxyguanosine per mole deoxyguanosine. In the second repeat experiment, 20.6 µmoles of 7-methylguanine per mole of guanine were found in the liver DNA from ^{3}H-MMH-treated rats. In all three experiments between 248 and 378 µCi ^{3}H-MMH were given to each rat, although the dose of 15 mg/kg body weight did not change; in the experiment with the largest yield of 7-methylguanine, the amount of tritium given to each rat was 248 µCi.

The same experiment has been repeated in the C57BL6 mouse and the results were reported under the section on Hydrazine Metabolism. Ten female mice were given 14.4 mg ^{3}H-MMH/kg body weight (28.3 µCi/mouse) orally and killed 5 hours later. Liver DNA was isolated and hydrolyzed in dilute acid. The hydrolysate contained 338 µmoles 7-methylguanine per mole guanine; the pyrimidine oligonucleotides also contained radioactivity but this has not been characterized.

The third approach to MMH metabolism went back to incubation of tissue preparations with the hydrazine, but, rather than measuring rates of disappearance, the rates of oxidation to carbon dioxide were measured; this was made possible by the synthesis of ^{14}C-MMH in the laboratory. Note: the MMH in this work contained 20% UDMH as a contaminant. Fresh slices of rat liver, kidney, colon, and lung were incubated with ^{14}C-MMH (20.1 µg per 200 mg tissue) at 37^{o} for 15, 30, 60, 120 and 240 minutes. Fresh tissue was sliced (0.25 mm thickness) and washed in cold saline; 200 mg of tissue slices were added to a metabolism flask containing 3 ml Krebs-Ringer phosphate buffer, pH 7.4; other slices were first boiled at 100^{o}C for one minute to serve as controls. Preincubation at 37^{o} was carried out for 10 minutes, and then 0.1 µg ^{14}C-MMH (14.6 µCi/ml, 6.6 mCi/mmole) and 2 µl containing 20 µg MMH in 0.1 N HCl were added; for UDMH 0.00135 µg ^{14}C-UDMH (18 µCi/ml, 13.3 mCi/mmole) and 2 µl containing 100 µg UDMH in 0.1 N Cl were added. Carbon dioxide was trapped in the center well in NaOH. After the appropriate incubation time, the slices were inactivated by the addition of 6 ml cold absolute ethanol. Sodium carbonate was added to the NaOH as carrier and the carbonate was precipitated as the barium salt. The barium carbonate was washed, boiled in water, and counted for ^{14}C; in this way the ^{14}C represented only CO_2 and not entrapped MMH or UDMH. The filtered incubation medium was also assayed for radioactivity as a check on how much ^{14}C was added to the flask initially.

The percentage of administered radioactivity recovered as CO_2 was determined for each time interval, and regression lines were calculated for rates of MMH oxidation for each tissue. The equation for these lines are given in Table 4 where y equals the percentage of administered radioactivity recovered as carbon dioxide, x equals the incubation time in minutes and R is the correlation coefficient.

TABLE 4

REGRESSION LINES FOR THE CONVERSION OF ^{14}C-MMH TO $^{14}CO_2$ BY RAT TISSUES IN VITRO

Liver	$y = 0.03x - 0.36$	$R = 0.95$
Kidney	$y = 0.124x + 0.207$	$R = 0.95$
Colon	$y = 0.011x + 0.14$	$R = 0.87$
Lung	$y = 0.0044x + 0.01$	$R = 0.99$

Liver has the highest activity for producing carbon dioxide from MMH; expressing the activity of the other tissues in terms of liver, the kidney has 41% of the activity, colon 37% and lung has 15% of the activity on an equal weight basis.

Although in each case, a single regression line is given for the overall 240-minute incubation period, a biphasic response was observed with each tissue, showing a greater rate of oxidation occurring in the first 60 minutes of incubation. No experimental attempt has yet been made to explain the biphasic response. In light of the earlier experiments on incubating ^{3}H-MMH with naked DNA which suggested direct interaction of the macromolecule with MMH and a probable decomposition product, it may be possible that the biphasic oxidation pattern represents first a rapid metabolic oxidation of a spontaneous decomposition product of MMH followed by a slower oxidation of MMH itself. It may also represent first a rapid oxidation of unbound MMH followed by a slower oxidation of MMH bound to tissue protein.

1,1-DIMETHYLHYDRAZINE (UDMH) METABOLISM

As was true in the MMH studies, measuring the rate of disappearance of UDMH in various tissue preparations failed to distinguish between spontaneous decomposition and metabolism of the hydrazine, and it was decided to study the interactions between UDMH and DNA. In an in vitro test, 10 mg of rat liver DNA were dissolved in 5% sodium acetate and 36 µg (10 µCi, 16.6 mCi/mmole) ^{3}H-methyl-UDMH were added to the mixture which was then incubated for 3 hours at 37^{o}C. The DNA was recovered (7.4 mg) by repeated precipitations and washings and hydrolyzed in dilute acid. The hydrolysate was fractionated by liquid chromatography, and each fraction was assayed for radioactivity. The detailed results are summarized in Table 5.

Not only was there considerable tritium activity associated with the pyrimidine oligonucleotides and free purine bases, but activity also co-chromatographed with authentic carrier 7-methylquanine and O^{6}-methylguanine (although it is possible the activity in the 7-methylguanine peak could be due to "spillover" from the adenine in the preceding fraction). Since hydrazine in high concentration is known to react directly with nucleic acid pyrimidines (as described under MMH metabolism), this experiment was extended to in vivo studies.

TABLE 5

DISTRIBUTION OF ^{3}H IN HYDROLYSATE FRACTIONS OF LIVER DNA INCUBATED WITH ^{3}H-UDMH

	Fraction	DPM
1.		0
2.	early pyrim. oligo.	2533
3.	late pyrim. oligo.	1390
4.		142
5.	guanine	2861
6.	adenine	4619
7.	7-methylguanine	825
8.		175
9.	O^{6}-methylguanine	116
10.	O^{6}-methylguanine	175
11.	O^{6}-methylguanine	253
12.	3-methyladenine	49
13.	3-methyladenine	22
14.	3-methyladenine	15
15.		34
16.		24
17.		6

Male Fischer 344 rats (150 g) were given ^{3}H-UDMH (6 mg/kg body weight, 250 µCi/rat; approximately one-twentieth the published LD50) by intraperitoneal injection and killed 4 hours later. DNA from liver, kidney, lung and colon was analyzed for aberrant ^{3}H-labeled purines and pyrimidine oligonucleotides. Liver DNA was unlabeled except for a trace of material co-chromatographing with carrier 7-methylguanine. DNA from lung, kidney and colon contained tritium in the pyrimidine oligonucleotide peaks and in the carrier 7-methylguanine peak; material eluting with carrier O^{6}-methylguanine was also labeled to a small extent. The experiment was repeated using ^{14}C-UDMH (4.5 mg/kg body weight, 40 µCi/rat) but the dose was too small to yield detectable levels of alkylation. Of the DNA isolated from liver, kidney, lung, brain and colon, lung DNA contained the most radioactivity, as seen in Table 6. Only 5.6 mg of lung DNA was available (800 cpm), precluding analysis of individual DNA fractions. This experiment has not yet been repeated using a higher dose of UDMH and more radioactivity.

TABLE 6

INCORPORATION OF ^{14}C INTO DNA FROM VARIOUS TISSUES OF RATS TREATED WITH ^{14}C-UDMH

Tissue DNA	Specific Activity, cpm/mg DNA
liver	8
colon	29
brain	54
kidney	67
lung	143

One experiment was done in which a relatively large dose of isotopically-stable UDMH was given to rats whose DNA was radiolabeled. Newborn rats were given ^{14}C-formate (10 μCi) ip each day for the first 6 days of life to label the rings of guanine and adenine, and the methyl group of thymidine. When the rats reached adulthood (2 females 160-170 g, one male 284 g) they were given 55 mg UDMH/kg body weight ip and killed 4 hours later. DNA isolated from pooled liver, kidney, lung, brain, and colon was hydrolyzed and fractionated into component bases. No abnormal bases were found in the DNA sample from any of the tissues, as seen in Table 7. Although this experiment gives no evidence for interaction between UDMH and DNA, it must be kept in mind that the specific activities of the individual bases are low, perhaps too low to permit detection of minor abnormal bases; for example, an abnormal derivative of guanine in liver DNA which occurs to the extent of 0.1% of the original guanine would contain 0.1% of the radioactivity found in guanine, or about 4 dpm per mg liver DNA. A study was made to determine the limits of detection of abnormal bases using these formate-labeled animals and an authentic carcinogen known to produce abnormal DNA bases, 1,2-dimethylhydrazine (SDMH); those experiments are reviewed in the next section of this report.

The availability of ^{14}C-labeled UDMH permitted the return to simple metabolism systems to determine the extent to which each tissue can metabolize this propellent hydrazine. As was done with MMH, rat tissue slices were incubated with ^{14}C-UDMH at 37°C for 15, 30, 60, 120 and 240 minutes and the rate of $^{14}CO_2$ production was measured. Regression lines were calculated for the percentage of administered radioactivity recovered as carbon

TABLE 7

DISTRIBUTION OF RADIOACTIVITY AMONG DNA FRACTIONS PREPARED FROM PRELABELED RATS TREATED WITH UDMH

Fraction	Specific Activity, dpm/mg DNA				
	Liver	Kidney	Lung	Brain	Colon
1.	0	0	0	44	0
2.	32	130	0	250	43
3. pyrim. oligonucl.	1800	7840	9190	7080	1465
4. " "	670	3010	4700	2480	560
5. " "	290	1410	2820	1015	240
6.	62	175	200	135	50
7. guanine	4280	20,400	22,130	9070	3050
8.	130	650	990	267	80
9. adenine	4600	25,210	26,570	11,730	4445
10. "	1605	8180	16,075	2835	920
11.	105	615	1140	210	57
12.	20	105	180	45	18
13.	0	50	70	27	0
14.	0	31	38	18	0
15.	0	18	27	0	0
16.	0	20	21	0	0
17.	0	17	13	0	0
18.	0	15	20	0	0
19.	0	15	0	0	0
20.	0	20	0	0	0
21.	0	0	0	0	0
22.	0	0	0	0	0
23.	0	0	0	0	0

dioxide over the 240-minute interval; the equations for these lines are given in Table 8 where y equals the percentage of administered radioactivity recovered as carbon dioxide, x equals the incubation time in minutes, and R is the correlation coefficient. Liver has the highest

TABLE 8

REGRESSION LINES FOR THE CONVERSION OF ^{14}C-UDMH TO $^{14}CO_2$ BY RAT TISSUES IN VITRO

Liver	$y = 0.01x - 0.02$	$R = 0.96$
Kidney	$y = 0.0072x + 0.085$	$R = 0.90$
Colon	$y = 0.008x + 0.17$	$R = 0.84$
Lung	$y = 0.0008x + 0.0006$	$R = 0.91$

activity for producing carbon dioxide from UDMH; expressing the activity of other tissues in terms of liver, the colon is 80% as active, kidney 72% and lung has only 8% of the activity of liver on an equal weight basis. Comparing the activities of each tissue to oxidize MMH and UDMH, monomethylhydrazine is oxidized faster by all four tissues tested (Table 9).

TABLE 9

COMPARISON OF RATES OF CO_2 PRODUCTION FROM MMH AND UDMH BY VARIOUS RAT TISSUES (IN VITRO)

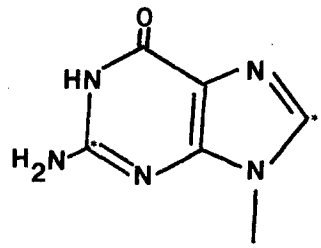
	Slope of Regression Line		Ratio of Slopes
Tissue	MMH	UDMH	MMH; UDMH
Liver	0.03	0.01	3.0
Kidney	0.0124	0.0072	1.7
Colon	0.011	0.008	1.4
Lung	0.0044	0.0008	5.5

A biphasic response in the $^{14}CO_2$ production curve for UDMH was also seen in liver, kidney and colon but not lung incubations. The rate of $^{14}CO_2$ production was greater in the first 60 minutes incubation for all tissues but the lung. As stated in the case of MMH, the biphasic oxidation pattern may represent first a rapid metabolic oxidation of a spontaneous decomposition product of UDMH followed by a slower oxidation of UDMH itself. It may also represent first a rapid oxidation of unbound UDMH followed by a slower oxidation of UDMH bound to tissue protein.

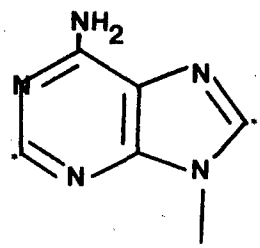
1,2-DIMETHYLHYDRAZINE (SDMH) METABOLISM

Earlier studies on the in vivo methylation of DNA by MMH and UDMH indicated little or no labeling of the nucleic acid following administration of the toxic agents, and we questioned whether our methods lacked sufficient sensitivity to detect the methylation if it had occurred. Several experiments have been done on SDMH, which serves as a positive control, to determine how sensitive our methods are for detecting methylation of DNA.

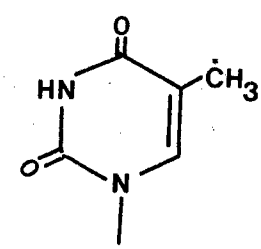
A group of Fischer 344 rats was given 10 µCi ^{14}C-formate ip daily for the first six days of life. This results in the labeling of the purine bases of DNA in the 2 and 8 positions and of thymine in the 5-methyl position as shown below (indicated carbons derived from formate).



guanine



adenine



thymine

DNA has a relatively long half-life in most mammalian tissues and when the animals reach adulthood, the DNA is still radioactive while other macromolecules have little or no carbon-14. Such animals constitute "pre-labeled" rats.

Three groups of animals were given a single ip dose of 150 mg SDMH/kg body weight. One group of animals was "prelabeled" with formate as above and given isotopically-stable SDMH. The second group of animals had not been treated with formate but was given ^{14}C-SDMH (133 μCi/rat). The third group of animals was not "pre-labeled" and was given isotopically-stable SDMH, that is, no radioactivity at all. The object of the experiment was to determine to what extent the amount of radioactivity in the DNA influenced the amount of methylation detected.

The animals were killed 6 hours after SDMH administration to permit comparison of results with those of other investigations. DNA was isolated from liver, kidney, and colon and hydrolyzed in dilute acid. The hydrolysates from "pre-labeled" animals and from ^{14}C-SDMH-treated animals contained carrier authentic 7-methylguanine and O^6-methylguanine, but carrier was not added to hydrolysates from the animals given no radioactivity. The detailed analysis of these latter hydrolysates (no radioactivity) awaits installation of the fluorescence detector on the liquid chromatograph for optical detection of methylated bases. Table 10 summarizes the data available regarding the different amounts of methylated bases obtained with SDMH depending upon whether the DNA or the carcinogen was radioactive.

TABLE 10

IN VIVO METHYLATION OF DNA IN RATS TREATED WITH 1,2-DIMETHYLHYDRAZINE (SDMH)

	moles 7-methylguanine/10^5 moles guanine		
	UCI-THRU		Literature*
Tissue	^{14}C-DNA**	^{14}C-SDMH***	^{14}C-SDMH
Liver	145	516	669
Kidney	35	81	74
Colon	100	168	94

	moles O^6-methylguanine/10^5 moles guanine		
	UCI-THRU		Literature*
	^{14}C-DNA**	^{14}C-SDMH***	^{14}C-SDMH
Liver	n.d.	73	78
Kidney	trace	9	8
Colon	trace	24	9

* Rogers and Pegg, Cancer Research 37:2082-2087 (1977). 200 mg SDMH/kg body weight, 0.208 mCi/mmole; 6 hours before cervical dislocation.

** ^{14}Cformate daily first 6 days of life; as adults, 150 mg SDMH/kg body weight, 6 hours before decapitation.

*** ^{14}C-SDMH, 150 mg/kg body weight, 0.47 mCi/mmole; 6 hours before decapitation.

n.d. - none detected.

When comparing the amounts of methylated guanines seen using "prelabeled" rats (^{14}C-DNA) versus normal rats treated with ^{14}C-labeled carcinogen, the use of a "pre-labeled" rat model apparently severely underestimates the levels of alkylation. The degree of underestimation is greater for O^6-methylguanine than for 7-methylguanine; the former base is enzymatically removed from DNA by normal repair mechanisms while most of the 7-methylguanine in DNA is removed by spontaneous (non-enzymatic) depurination. The possibility then arises that the lower levels of alkylation seen in DNA from "prelabeled" animals may be due to higher rates of enzymatic repair in these animals; that is, the presence of the carbon-14 in the bases of normal DNA may induce DNA repair, perhaps by inducing dimerization and other abnormalities in DNA as the carbon-14 atoms decay. Whatever the reasons for this

underestimation of the extent of methylation of DNA by SDMH, it is clear that one is not able to continue use of the "pre-labeled" animal in studies on DNA alkylation by the propellant hydrazines and that it is necessary to rely on ^{14}C-MMH, ^{14}C-UDMH, and ^{15}N-hydrazine as investigations are continued on metabolic activation of these compounds.

BIOCHEMICAL ASSESSMENT OF TOXIC INJURY TO THE LUNG

There is a need to develop biochemical means to assess toxic injury to the lung, means which promise to be more sensitive and more directed to mechanism of action than physiological parameters now in use. As a preliminary step in developing such means, a small pilot experiment was conducted to determine whether toxic insult to the lung could readily be detected by monitoring two major parameters of lung biochemical function. Preliminary to the use of hydrazines, the model lung carcinogen, N-nitrosoheptamethyleneimine (NHMI), was used to provide the toxic insult.

Infant Fischer 344 rats were given 40 mg NHMI/kg body weight sc in saline: ethanol (1:1) weekly for 10 weeks; this routine is reported to induce only alveolar adenomas and adenocarcinomas in Fischer rats in approximately 16 weeks (Taylor and Nettesheim, 1975). This treatment killed approximately 20% of the animals, and the body weights of surviving male and female rats were 20% and 16% below control values, respectively.

One week after the last NHMI injection, the female rats were used to measure the uptake and oxidation of 5-hydroxytryptamine by perfused lung and male rats were used to measure the rate of synthesis of pulmonary surfactant and phospholipid. The uptake of 5-hydroxytryptamine and metabolic oxidation to 5-hydroxyindoleacetic acid by the isolated perfused lung was measured by a slight modification of the methods of Junod (1972). Animals were infused with 32 μmoles of 5-hydroxytryptamine in 3 minutes. The uptake of the amine varied considerably between animals in the same group, and average values did not indicate a significant difference between control and NHMI-treated animals (Figure 5). The oxidation of 5-hydroxytryptamine to 5-hydroxyindoleacetic acid did appear to be less in 4 of the 7 NHMI-treated animals compared to controls (Figure 6); unfortunately a histopathologic examination of the individual lungs could not be made to determine whether the lungs with low metabolic activity had neoplastic or preneoplastic lesions.

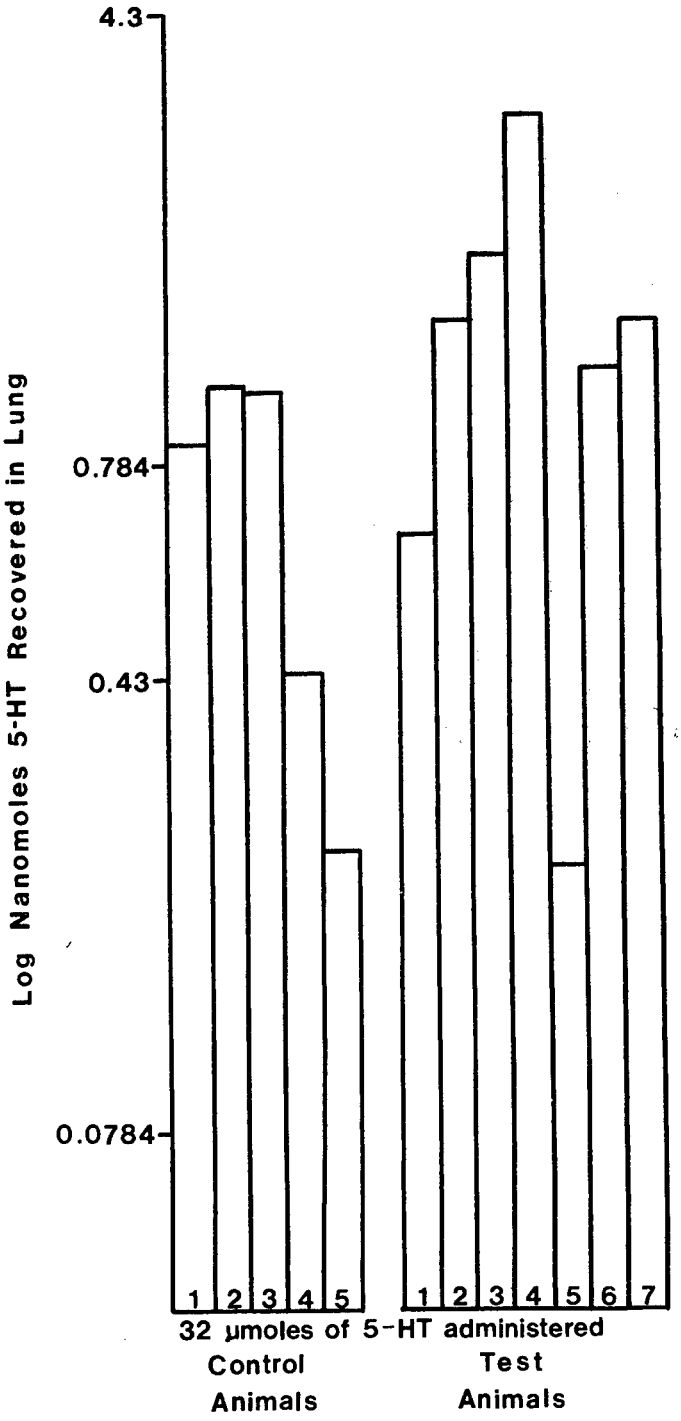


Figure 5. Effect of chronic administration of N-nitrosoheptamethyleneimine on 5-hydroxytryptamine uptake by rat lung.

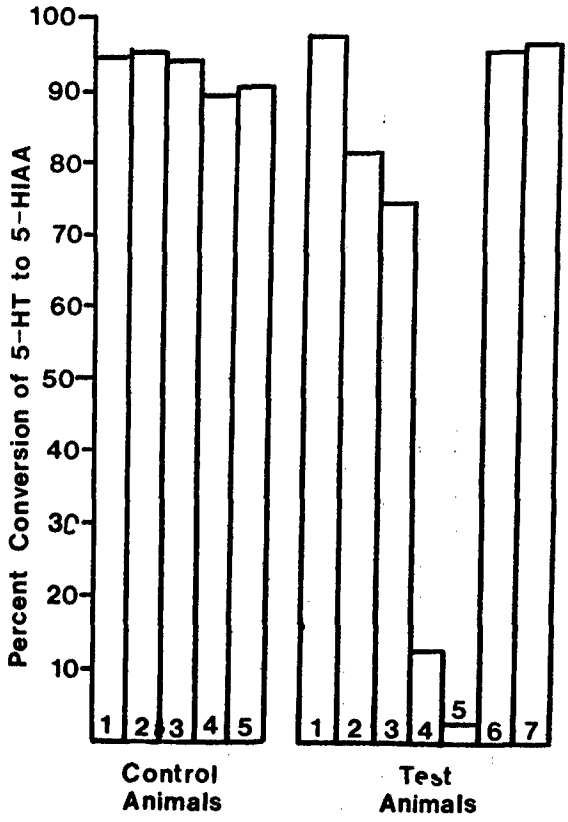


Figure 6. Effect of chronic administration of N-nitroso-heptamethyleneimine on metabolic oxidation of 5-hydroxytryptamine to 5-hydroxyindoleacetic acid in rat lung (Results for individual animals).

Phospholipid synthesis and turnover in lung was measured according to Spitzer and Norman (1970). Rats were given 103 μCi ^{3}H-choline (13 Ci/mmole) and 12.5 μCi ^{14}C-methyl-methionine (56 mCi/mmole) ip, and 4, 24, and 48 hours later incorporation of radioactivity into pulmonary phospholipid and surfactant was measured. Between animal variation in these measurements was also large. No difference between control and treated animals was seen in the incorporation of label from choline or methionine into lung phospholipid. Table 11 summarizes the results on the incorporation of the label from choline and methionine into pulmonary surfactant.

TABLE 11

EFFECT OF NHMI PRETREATMENT ON
INCORPORATION OF LABEL FROM ^{3}H-CHOLINE AND
^{14}C-METHYL-METHIONINE INTO PULMONARY SURFACTANT IN RATS

Time after precursor administration (hours)	Picomoles precursor incorporated into surfactant, mean (range)			
	^{3}H-choline		^{14}C-methyl-methionine	
	Control	NHMI	Control	NHMI
4	4.5(1.9-7.2)	9.1(8.4-10.1)	0.1(0.1-0.2)	0.2(0.2-0.3)
24	26.2(15.8-35.4)	33.6(22.8-43.8)	5.5(5.0-6.1)	7.1(4.2-11.7)
48	11.9*	52.2(22.6-101.5)	0.4*	1.0(0.9-1.5)

* one animal only

Peak incorporation of radioactivity in both cases occurred nearly 24 hours after administration of the precursors with no difference between NHMI and control rats; however, pulmonary surfactant from NHMI-treated rats appeared to retain the radioactivity longer than that from control rats, perhaps reflecting a decreased rate of degradation or an accumulation of surfactant in these animals.

These experiments, conducted in animals after chronic exposure to a lung carcinogen, have failed to demonstrate striking differences in two major parameters in lung biochemistry. It is unlikely that these parameters can be used as sensitive indices to toxic insult produced by low levels of propellant hydrazines, unless considerably more time is spent in reducing the between-animal variation.

SUMMARY AND CONCLUSIONS

Hydrazine administration at the LD0.01, but not at half that level, results in the production of 7-methylguanine in liver DNA in the rat; little if any 7-methylguanine is seen in liver DNA from control animals. This methylation of DNA may be enzymatically mediated and related to stress resulting from the toxic insult. Administration of hydrocortisone in place of hydrazine also results in formation of 7-methylguanine in rat liver DNA but to a

lesser extent than with hydrazine. At an equitoxic dose, but one-sixth the molar dose, in the mouse, hydrazine administration results in the same amount of 7-methylguanine formation in liver DNA; however, in the mouse, O^6-methylguanine is also formed and this site of alkylation is considered by many to be important in mutagenesis and carcinogenesis; O^6-methylguanine is not seen in rat liver DNA under these conditions.

Administration of ^{3}H-methyl-monomethylhydrazine (MMH) to rats at a dose equitoxic to the hydrazine dose results in some 7-methylguanine production but less than that when hydrazine is administered; doses of MMH equimolar to those of hydrazine have not yet been studied in the rat. In the mouse, however, a dose of MMH equimolar to one of hydrazine results in more 7-methylguanine production than when hydrazine and methionine are administered, but O^6-methylguanine is not seen. If hydrazine stimulated the methylation of DNA via conversion first to MMH by methionine in the mouse, then one would expect to see both 7-methylguanine and O^6-methylguanine in MMH-treated mice as is seen in hydrazine-treated mice; since this is not the case, it may be more likely that the hepatotoxic hydrazine does not share the same mechanism of toxic action as the neurotoxic MMH. A preliminary study has not provided evidence that co-administration of hydrazine and ^{14}C-acetate results in the acetylation of DNA in various rat tissues as had been suggested in the literature.

Although the formation of formaldehyde from MMH cannot be measured in rat tissue incubation systems, the oxidation of ^{14}C-methyl-MMH to $^{14}CO_2$ has been measured quantitatively in tissue slice incubation systems prepared with rat liver, kidney, lung and colon. Liver oxidizes MMH faster than the other tissues, kidney and colon have approximately equal activity one-third that of liver, and lung has the least activity, approximately 7 times less than liver.

Some evidence is now available supporting the methylation of DNA guanine in the 7-position after the administration of UDMH to rats but this observation needs confirmation.

Carbon-14 carbon dioxide can be produced from ^{14}C-methyl-UDMH by rat tissues but at a slower rate than seen for $^{14}CO_2$ production from MMH. Rat liver slices produce CO_2 from UDMH at a rate three times slower than when MMH is used as the substrate. Kidney and colon slices have approximately

the same activity, about 70-80% of the activity seen in liver slices; lung slices have only 8% of the activity measured in liver.

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