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BIOASSAY OF MILITARY RELEVANT COMPOUNDS FOR CARCINOGENIC ACTIVITY BY THE STRAIN A MOUSE LUNG TUMOR BIOASSAY

FINAL REPORT

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2,4-Dinitrotoluene, 2,6-dinitrotoluene, a 2:1 mixture of 2,4-dinitrotoluene and 2,6-dinitrotoluene, 2,4-diaminotoluene, 2,6-diaminotoluene, benzo(a)pyrene, diethylnitrosamine, 1,2-dimethylhydrazine, ethylnitrosourea, 3-methylcholanthrene and urethan were evaluated for carcinogenic potential in the strain A mouse lung tumor (adenoma) bioassay. All compounds and the mixture were dissolved in either tricaprylin (glycerol trioctanoate) or water and administered intraperitoneally (i.p.) or orally (p.o.; by gavage) at three dose levels: the maximum tolerated dose (MTD), and 1:2 and 1:5 dilutions of the MTD. At either 24 or 30 weeks after the first treatments, the mice were harvested and the lung tumor response in chemically treated animals was compared to that in the appropriate vehicle controls.

2,4-Dinitrotoluene, 2,6-dinitrotoluene, the 2:1 mixture of 2,4-dinitrotoluene and 2,6-dinitrotoluene and 2,4-diaminotoluene did not induce lung tumors in A/J mice at any of the three dose levels following either route of administration. Therefore, at the total doses administered, these compounds were non-carcinogenic in the lung tumor bioassay. 2,6-Diaminotoluene induced a significant (albeit marginal) increase in the lung tumor response when given i.p. at a total dose of 375 mg/kg. After p.o. administration at the same dose, the compound produced an increase in the tumor response when compared to tricaprylin controls, but the difference was not significant.

Diethylnitrosamine, ethylnitrosourea, 3-methylcholanthrene and urethan produced significant and dose-related increases in the lung tumor response when given either i.p. or p.o. Benzo(a)pyrene was carcinogenic at all doses when given i.p. and at the highest dose (100 mg/kg) after p.o. administration. 1,2-Dimethylhydrazine was positive at the high (25 mg/kg) and middle (12.5 mg/kg) p.o. doses and at the highest dose (25 mg/kg) following i.p. administration.

The tumor responses to 3-methylcholanthrene, benzo(a)pyrene, the MTD of diethylnitrosamine, and the middle doses of both ethylnitrosourea and 1,2-dimethylhydrazine varied as a function of the route of administration. This finding was most evident for the polycyclic hydrocarbons. At all doses, 3-methylcholanthrene and benzo(a)pyrene induced significantly more lung tumors when given i.p. as compared to p.o. For example, the tumor response in animals receiving the middle dose of 3-methylcholanthrene or the highest dose of benzo(a)pyrene by the i.p. route exceeded that by the p.o. route by factors of 12 and 13, respectively. Diethylnitrosamine (200 mg/kg) and ethylnitrosourea (50 mg/kg) induced 1.45 and 2 times as many tumors, respectively, when given i.p. as compared to p.o. In contrast, 1,2-dimethylhydrazine, at the dose of 12.5 mg/kg, produced significantly more tumors per lung when administered p.o. The lung tumor response to urethan did not vary significantly by either route of administration.

2,3-Dinitrotoluene, 2,5-dinitrotoluene, 3,4-dinitrotoluene, 3,5-dinitrotoluene, and two dyes utilized in colored smoke grenades; i.e., C.I. Solvent Yellow 33 and a mixture of C.I. Solvent Green 3/C.I. Solvent Yellow 33 were also evaluated for their ability to induce lung adenomas in strain A/J mice. However, these compounds were administered only by the i.p. route. All five compounds and the mixture failed to produce an increase in the lung tumor response at the dose levels given. Therefore, these compounds were non-carcinogenic in the lung tumor bioassay. At necropsy, mice tested with C.I. Solvent Yellow 33 and the mixture of C.I. Solvent Green 3/C.I. Solvent Yellow 33 had significant quantities of accumulated dye in the peritoneum. This observation suggests that the dyes were either not metabolized or only partially metabolized by the animals. A final report on bioassays of the two dyes was submitted to the U.S. Army Medical Research and Development Command in January, 1985.

The distribution, elimination and metabolism of 2,4-dinitrotoluene (2,4-DNT) was examined in strain A/J mice. The urine was found to be the major route of elimination of [3-^{3}H]-labeled 2,4-DNT with 52.5, 60.1, and 70.0% of i.p. doses of 1, 10, and 100 mg/kg, respectively, excreted within 4 hours after administration. The distribution of 2,4-DNT in various tissues (blood, liver, kidneys, lungs, adipose tissue, small and large intestine) showed no evidence for preferential uptake or retention at any of the doses tested. At 0.25 to 4 hours after administration, the lungs contained 0.05 to 0.2% of the dose. Terminal half-lives of radioactive material in liver (1.1 to 1.7 hour) and kidney (0.9 to 1.4 hour) were not related to dose. At all doses (1, 10, and 100 mg/kg), rapid and extensive metabolism of 2,4-DNT by liver and small intestine was observed, as judged by the low amounts of 2,4-DNT (less than 13% of the total ^{3}H/tissue) that could be re-isolated from these tissues. Blood and lungs contained much higher levels of unchanged 2,4-DNT and, in most cases, the extent of 2,4-DNT metabolism was similar in these two tissues, suggesting that the lung is not an active site of 2,4-DNT metabolism. Therefore, the liver and the small intestine are major sites of 2,4-DNT metabolism, while the lungs are relatively inactive.

Metabolic studies revealed that from 0.5 to 4 hours after intraperitoneal administration of [3-^{3}H] 2,4-DNT, 3.6 to 8.8% of the urinary metabolites was unconjugated while 2.4 to 8.8% was present in the glucuronide fraction. After oral administration these amounts were 5.5 to 6.8% and 20.5 to 28.2% respectively. After both intraperitoneal and oral administration, no unchanged 2,4-DNT could be detected in the urine, and 2,4-dinitrobenzyl alcohol (2,4-DNBAlc) represented the most abundant identifiable neutral metabolite. Small amounts of 2,4-diaminotoluene, 2-amino-4-nitrobenzyl alcohol, 2-(N-acetyl)amino-4-nitrotoluene, 4-amino-2-nitrotoluene (4A2NT), and 2-amino4-nitrotoluene (2A4NT) were also present. In almost all cases the largest proportion of metabolites represented unknowns, some of which exhibited the chromatographic properties of carboxylic acid metabolites. Metabolism of 2,4-DNT by liver and lung microsomes yielded mainly 2,4-DNBAlc with lower amounts of 4A2NT and 2A4NT, and their formation was dependent on the presence of oxygen and NADPH. Pretreatment of the animals with 2,3,7,8-tetrachlorodibenzo-p-dioxin resulted in increased yields of all three metabolites. Aerobic metabolism of 2,4-DNT by explants of the small intestine, large intestine, or by cecal contents yielded 2,4-DNBAlc, 2A4NT, 4A2NT and 4-(N-acetyl)amino2nitrotoluene (4Ac2NT). The proportion of reduced metabolites (2A4NT, 4A2NT, and 4Ac2NT) was much higher in these systems than with liver or lung microsomes and their formation by small intestine and cecal contents was enhanced severalfold under anaerobic conditions, while that of 2,4-DNBAlc was abolished. In summary, 2,4-DNT metabolism in the A/J mouse is rapid and complete and the major neutral urinary metabolite is 2,4-DNBAlc. Minor amounts of reduced or partially reduced products appear to be formed mainly in the intestine, with a major role by its microflora.

The distribution and elimination of 2,6-dinitrotoluene (2,6-DNT) was also examined in strain A/J mice. The urine was the major route of elimination of both i.p. and p.o administered ^{3}H-labeled 2,6-DNT, with 87.6, 55.2, and 49.1% of i.p. doses of 1, 10 and 100 mg/kg, respectively, excreted within 4 hours. The corresponding amounts excreted after p.o. administration were 33.6, 25.2, and 24.3%, which increased to 53.7, 53.5, and 48.6% after 8 hours. The distribution of 2,6-DNT in various tissues (blood, liver, kidneys, lungs, small and large intestine) showed no evidence for preferential uptake or retention at any of the i.p. or p.o. doses. Terminal half-lives of radioactive material in the liver were 1.11, 0.95, and 1.16 hours after i.p. doses of 1, 10, and 100 mg/kg, respectively. At all i.p. doses (1, 10, and 100 mg/kg), rapid and extensive metabolism of [3-^{3}H]-labeled 2,6-DNT was observed, as judged by the

low amounts (<2% of the total [^{3}H]/tissue) of unchanged [3-^{3}H]-labeled 2,6-DNT that could be reisolated from blood, liver, small intestine, or lungs at 2 hours after administration. The extent of ^{3}H-labeled 2,6-DNT metabolism by these tissues after p.o. administration was similar, except for the relatively high amounts of unchanged ^{3}H-labeled 2,6-DNT (approximately 50% of the total ^{3}H/tissue between 1 and 8 hours after 100 mg/kg) present in the small intestine. At these times, negligible (<2%) unchanged ^{3}H-labeled 2,6-DNT was present in the large intestine. Thus, excretion in the urine is the major mode of elimination after either i.p. or p.o. administration, but is slower after p.o. than after i.p. dosing. It is likely that the liver and small intestine are major sites of 2,6-DNT metabolism.

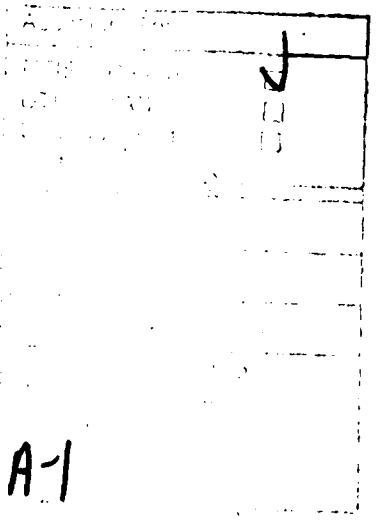
Metabolic studies of 2,6-dinitrotoluene were conducted in both the strain A/J mouse (in which the compound is non-carcinogenic) and Fischer-344 rats (in which the compound is carcinogenic). The in vitro metabolism of 2,6-DNT by rat and mouse hepatocytes was similar and resulted mainly in the formation of 2,6-dinitrobenzyl alcohol, either unconjugated or as a glucuronide (57.5-85.5% of the total per fraction), with smaller amounts of polar, acidic metabolites (8.4-38.7%) and minor amounts (1.2-5.3%) of 2-amino-6-nitrotoluene. Anaerobic metabolism of 2,6-DNT by rat or mouse cecal enzymes resulted mainly in the formation of 2-amino-6-nitrotoluene and 2-(N-acetylamino)-6-nitrotoluene and minor amounts of 2,6-diaminotoluene. I.p. administration of 2,6-DNT (150 mg/kg) to Fischer-344 rats resulted, after 24 hours, in covalent binding to DNA of the liver, (131.1-259.9 pmol 2,6-DNT/mg DNA) and lower binding to DNA of the lungs and the intestine (14.9-22.7 pmol 2,6-DNT/mg DNA, respectively). Similar treatment of A/J mice resulted in lower binding of 2,6-DNT in the liver (25.9-31.9 pmol 2,6-DNT/mg DNA) and no detectable binding in extrahepatic tissues. In vitro binding of 2,6-DNT to DNA of cultured hepatocytes from both A/J mice and Fischer-344 rats required prior metabolism of 2,6-DNT by the respective cecal enzymes. DNA binding was nondetectable in hepatocytes incubated with 2,6-DNT only. Binding of 2,6-DNT to liver DNA requires its prior reductive metabolism, probably by intestinal microorganisms. The higher levels of binding of 2,6-DNT in the Fischer-344 rat than in the A/J mouse may, in part, be responsible for the higher susceptibility of the Fischer-344 rat to 2,6-DNT carcinogenesis.

Attempts were made to develop a transplacental carcinogenesis bioassay in the strain A/J mouse. Pregnant A/J mice were treated on the sixteenth day of gestation with a single i.p. injection of ethylnitrosourea at either 10 or 2 mg/kg. Of 114 A/J females that were bred, only 70 had litters. Due to cannabalism, only 3 of the 70 litters survived. This survival rate was considered to be too low to justify continuation of the experiment.

The strain A/J mouse lung tumor bioassay has a limited ability to detect many liver carcinogens, some bladder carcinogens, and certain of the unstable, direct-acting alkylating agents. Therefore, a preliminary experiment was initiated to develop a mouse strain with the potential for a broader range of carcinogenic susceptibility. A/J mice (high lung tumor susceptibility) were bred with C_3HeB/FeJ mice (high liver tumor susceptibility) to develop hybrids that might be susceptible to both "lung" and "liver" carcinogens. Fifteen day-old A/J mice, C_3HeB/FeJ mice and the two hybrid crosses; A/J male X C_3HeB/FeJ female and A/J female X C_3HeB/FeJ male, each received a single i.p. dose of diethylnitrosamine (DENA) (50 mg/kg). Three, six and twelve months after dosing, animals from all groups were sacrificed and their surface lung and liver tumors enumerated. At three months, tumors were not observed on the livers of any of the DENA-treated parental and hybrid mice. Lung tumors were observed in all strains at 3 months, with the highest tumor response in A/J mice. Six months after DENA treatment, all parental and hybrid strains exhibited liver tumors, and the liver tumor response was significantly

higher in males than in females. The relative frequency of liver tumors in the various mouse strains was C_3HeB/FeJ > hybrids > A/J. Lung tumors occurred in all strains at 6 months, and the relative frequency was A/J $\geq$ hybrids > C_3HeB/FeJ. Twelve month data was not available at the time of termination of this contract.

In summary, the hybrid crosses each develop both lung and liver tumors within six months after exposure to DENA. The lung tumor frequency in the hybrid crosses was similar to the parental A/J strain and significantly higher than the C_3HeB/FeJ parent. The liver tumor frequency in the hybrid crosses was lower than in the C_3HeB/FeJ parent, but significantly higher than in the A/J parent. Therefore, the hybrid crosses appear promising for the detection of both "lung" and "liver" carcinogens. Additional studies indicated that 15 day-old hybrids were more susceptible to DENA-induced lung and liver tumors than six to eight week-old hybrids.



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EXECUTIVE SUMMARY

Under this contract from the U.S. Army Medical Research and Development Command, the Medical College of Ohio conducted studies to determine the carcinogenic potential of a series of dinitrotoluene compounds, diaminotoluenes, dyes, and other selected compounds for carcinogenic activity in the strain A mouse lung tumor bioassay. In most cases, a comparison was made of the carcinogenic potential of the chemicals following either intraperitoneal (i.p.) or oral (p.o., by gavage) administration.

2,4-Dinitrotoluene, 2,6-dinitrotoluene, a 2:1 mixture of 2,4-dinitrotoluene and 2,6-dinitrotoluene did not induce lung tumors in A/J mice at any of the three dose levels given, or by either route of administration. 2,3-Dinitrotoluene, 2,5-dinitrotoluene, 3,4-dinitrotoluene, 3,5-dinitrotoluene, and two dyes used in colored smoke grenades; i.e., C.I. Solvent Yellow 33 and a mixture of C.I. Solvent Green 3/C.I. Solvent Yellow 33 were also negative for lung tumor induction following i.p. administration. 2,6-Diaminotoluene, a metabolite of 2,6-dinitrotoluene, induced a significant (albeit marginal) increase in the lung tumor response when given i.p. at a total dose of 375 mg/kg. After p.o. administration at the same dose, the compound produced an increase in the tumor response, but the increase was not significant. 2,4-Diaminotoluene was negative for lung tumor induction in strain A mice.

In certain instances, our data on the carcinogenic potential of the dinitrotoluenes and diaminotoluenes are in disagreement with the results of other bioassays. 2,6-Dinitrotoluene appears to be a potent carcinogen in the rat liver and 2,4-diaminotoluene is carcinogenic in both rats and mice. The published results on the carcinogenic potential of 2,4-dinitrotoluene in other systems are equivocal. The probable reason for the inability of the lung tumor bioassay to detect the carcinogenic potential of these compounds is its relative insensitivity to detect liver carcinogens. At the present time, we do not understand the basis of this insensitivity; however, it may be related to an inability of the strain A mouse to metabolize liver carcinogens to a sufficient extent to induce tumors.

There is very little published data on the ability of compounds to induce lung tumors in strain A mice when given by different routes of exposure. Moreover, there are no data on the carcinogenic potential of compounds in strain A mice following oral administration. Since the oral route is a major route of human exposure to carcinogenic agents, we compared the ability of several chemicals to induce lung tumors in A mice following both i.p. and p.o. administration. The data indicated that the polycyclic hydrocarbons, benzo(a)pyrene and methylcholanthrene were significantly more active when given i.p. as compared to p.o. Similarly, diethylnitrosamine and ethylnitrosourea were more active when given i.p. In contrast, 1,2-dimethylhydrazine induced more lung tumors when given p.o. These results suggest that the i.p. route is more appropriate when testing polycyclic hydrocarbons or other fat soluble compounds. Pharmacokinetic studies with methylcholanthrene indicated that these compounds are retained in the body cavity for longer periods following i.p. administration; therefore, the carcinogenic stimulus is likely to persist for a longer period of time. However, the observation that at least one carcinogen, 1,2-dimethylhydrazine, was more active after p.o.

administration suggests that both the i.p. and p.o. routes should be used routinely in the lung tumor bioassay.

The distribution, metabolism and elimination of both 2,4-dinitrotoluene and 2,6-dinitrotoluene were examined in strain A mice. The data indicated that, for both compounds, the urine was the major route of elimination. The distribution of both 2,4-dinitrotoluene and 2,6-dinitrotoluene in various tissues (blood, liver, kidneys, lungs, small intestine, large intestine, adipose tissue) showed no evidence of preferential uptake or retention at any of the doses tested. The liver and small intestine appeared to be major sites of metabolism of both compounds whereas the lungs (the target organ in the bioassay) were relatively inactive. Metabolic studies showed that the most abundant identifiable metabolite of 2,4-dinitrotoluene was 2,4-dinitrobenzyl alcohol. Small amounts of 2,4-diaminotoluene, 2-amino-4-nitrobenzyl alcohol, 2-(N-acetyl)amino-4-nitrotoluene, 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene were also present. The largest proportion of the metabolites were unidentified, and some of these exhibited the chromatographic properties of carboxylic acid intermediates. The microflora of the intestine seemed to play a major role in the production of reduced or partially reduced metabolites of 2,4-dinitrotoluene.

Metabolic studies of 2,6-dinitrotoluene were conducted in strain A/J mice (in which the compound is non-carcinogenic) and Fischer-344 rats (in which the compound is carcinogenic) to investigate the basis for the difference in carcinogenic susceptibility between the two species. *In vitro* studies with rat and mouse hepatocytes showed that the metabolism of 2,6-dinitrotoluene in the two species is similar. The principal identifiable metabolite was 2,6-dinitrobenzyl alcohol, either unconjugated or as a glucuronide, with smaller amounts of polar, acidic metabolites and minor amounts of 2-amino-6-nitrotoluene. The *in vivo* binding of 2,6-dinitrotoluene metabolites to DNA was significantly higher in the rat than in the mouse which may, in part, be responsible for the higher susceptibility of the rat to 2,6-dinitrotoluene carcinogenesis.

In utero exposure of strain A mice to carcinogens generally results in a higher lung tumor response than treatment of newborn or adult mice. In addition, the latency period for tumor development is shorter in animals exposed *in utero*. Therefore, we attempted to develop a rapid and sensitive transplacental bioassay for both initiators and promoters of carcinogenesis in strain A mice. Unfortunately, we observed a high rate of cannabalism of newborn mice from mothers that had been exposed to ethylnitrosourea, the initiator in the bioassay. This problem was not solved by attempts to protect the newborn mice and the experiments were terminated. Subsequently, we learned that other investigators had similar problems in conducting transplacental carcinogenesis bioassays with strain A mice.

A hybrid mouse model is under development which, in our opinion, is likely to have a broader range of carcinogenic susceptibility than that of the strain A mouse. Strain C_3HeB/FeJ mice (high liver tumor susceptibility) are bred with A/J mice (high lung tumor susceptibility) to develop hybrids that might be susceptible to both "lung" and "liver" carcinogens. Preliminary results from treatment of 15 day-old offspring with diethylnitrosamine indicate that the hybrids develop both lung and liver tumors in six months whereas the offspring of parental crosses develop predominately lung or liver tumors. The frequency

of lung tumors in the offspring of carcinogen-treated hybrid mice is similar to that of the A/J parent. However, the liver tumor occurrence in the hybrids is lower than in the A/J parent. Further studies are required to fully evaluate this model for potential use in carcinogenesis bioassays.

FOREWORD

All animal facilities used in conducting the research described in this report have been accredited by the American Association for the Accreditation of Laboratory Animal Care. Maintenance and research practices in the use of laboratory animals were conducted according to the principals and standards enumerated in the Guide for Care and Uses of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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This work was conducted in the Carcinogenesis Research Laboratory under the direction of Dr. Gary D. Stoner, Director of Experimental Pathology, Department of Pathology.

Bioassays of nitrotoluene compounds for their ability to induce lung tumors in strain A/J mice, and all transplacental carcinogenesis studies, were directed by Dr. Stoner, and supervised by Elizabeth Greisiger. Technical assistance for the bioassays was provided by Diane Ash, Tamara Gmitter, Dawn Crawford and Mary Morgan.

Pharmacokinetic and metabolism studies of 2,4-dinitrotoluene and 2,6-dinitrotoluene in strain A/J mice were directed by Dr. Herman A.J. Schut. Dr. Schut was assisted by Drs. Timothy Loeb and Rakesh Dixit. Technical assistance on a part-time basis was provided by Laura Grimes. Medical student volunteers involved in these studies were Cindy Butcher and Terri Sackl.

Dr. Philip B. Conran, a Board-Certified Veterinary Pathologist, supervised the autopsies and monitored the health status of the breeding colony and mice used for the bioassays. Histological examination of liver tissues from nitrotoluene-treated A/J mice, and from parental and hybrid mice treated with diethylnitrosamine was provided by Dr. James E. Klaunig.

Drs. Ronald Krol and Daniel Nordlung of the Department of Family Medicine performed statistical analyses on the biassay data.

CONTENTS

TABLES

INTRODUCTION

This report is divided into six sections in accordance with the objectives of our research investigations. These sections are:

1. Bioassay of a series of dinitrotoluene compounds for carcinogenic activity in the strain A mouse lung tumor bioassay.

2. Pharmacokinetics and metabolism of dinitrotoluenes.

3. Lung tumor response in A/J mice to chemicals administered intraperitoneally and orally.

4. Bioassay of dye compounds for carcinogenic activity in strain A/J mice.

5. Development and evaluation of a transplacental carcinogenesis bioassay in strain A/J mice.

6. Development of a hybrid mouse lung/liver model for carcinogenesis bioassays.

The first year of these investigations (August, 1980 - September, 1981) was supported by Army Contract No. DAMD 17-80-C-0135, and the final three years (September, 1981 - September, 1984) by Army Contract No. DAMD 17-81-C-1178. Bioassays of the two dye compounds; C.I. Solvent Yellow 33 and a mixture of C.I. Solvent Green 3 and C.I. Solvent Yellow 33, were supported by a subcontract under DAMD 17-81-C-1178, and a separate final report for these bioassays was submitted to the U.S. Army Medical Research and Development Command in January, 1985.

SECTION 1 - BIOASSAY OF DINITROTOLUENE COMPOUNDS IN THE STRAIN A MOUSE

In Section 1, we describe the results on bioassays of 2,3-dinitrotoluene (2,3-DNT), 2,4-dinitrotoluene (2,4-DNT), 2,5-dinitrotoluene (2,5-DNT), 2,6-dinitrotoluene (2,6-DNT), 3,4-dinitrotoluene (3,4-DNT), 3,5-dinitrotoluene (3,5-DNT) and a 2:1 mixture of 2,4-dinitrotoluene and 2,6-dinitrotoluene (2,4,6-DNT) for lung tumor induction in strain A/J mice following intraperitoneal (i.p.) administration.[1,2]

PROCEDURES

Animals and Housing

Six to eight week-old male and female mice were used in the bioassays. They were obtained from our breeding colony, derived from A/J mice purchased from the Jackson Laboratories, Bar Harbor, ME. The mice were kept on corn-cob bedding in polycarbonate cages, covered with stainless steel lids and nonwoven polyester fiber filter tops. There were four animals housed per cage, in accordance with National Cancer Institute weight-space specifications. Hygienic conditions were maintained by twice-weekly changes of the animal cages and water bottles, and routine sterilization of the cages. An adequate supply of food (NIH-07 Certified Rodent Diet, Ziegler Brothers, Gardner, PA) and water was provided ad libitum. The temperature in the rooms was maintained at 22 ± 2°C, with a relative humidity of 30-60%. An automatic timing device provided an alternating 12 hour cycle of light and darkness.

The health status of the breeding colony and mice used in the bioassays was periodically examined by:

(a) complete gross necropsy, tabulating gross lesions, general condition and body weight of each animal;

(b) histopathological evaluation of formalin-fixed tissues involving all major organs and;

(c) serological tests for the presence of murine virus infections.

Chemicals

All dinitrotoluene compounds were coded by numbers and obtained through Dr. Mary C. Henry, Health Effects Research Division, U.S. Army Medical Bioengineering Research and Development Laboratory. They were stored at 4°C in an explosion-proof refrigerator, and in an area designated for the storage of carcinogens. This area is at a distance from the animal quarters.

The purity of 2,4-DNT was 92-95%, with the major impurity being 2,6-DNT. The purity of the other chemicals was as follows: 2,3-DNT, 98%; 2,5-DNT, >99%; 2,6-DNT, 98%; 3,4-DNT, >98%; and 3,5-DNT, >98%.

The dinitrotoluenes were tested for solubility in deionized doubly-distilled water or in glycerol trioctanoate (tricaprylin; Eastman Kodak, Rochester, NY). All compounds were found to be insoluble in water and soluble in tricaprylin; therefore, tricaprylin was used as the vehicle in all bioassays of dinitrotoluene compounds.

Dinitrotoluene solutions were prepared fresh each day before the injections. The compounds were weighed in a chemical fume hood under yellow fluorescent lights, and dissolved in tricaprylin to the appropriate concentration. Amber bottles were used to protect the chemicals from fluorescent light. The dinitrotoluenes were administered i.p. in tricaprylin as 0.1 ml per dose.

Preliminary Toxicology

A maximum tolerated dose (MTD) was determined for each dinitrotoluene compound under test. Serial two-fold dilutions of each chemical were injected i.p. (0.1 ml per dose) into groups of 4 mice. The MTD for each chemical was the maximum single dose that all 4 mice tolerated (survived) after receiving six injections over a two-week period. For evidence of delayed toxicity, animals receiving six doses of the MTD were held for four weeks before experimental groups were initiated.

Using the preliminary toxicology as a guide, experimental groups were started for each dinitrotoluene compound. Three dose levels were used: the MTD, and 1:2 and 1:5 dilutions of the MTD. In most cases, there were 52 mice per dose level; 26 males and 26 females. All chemicals were injected i.p., three times weekly for a total of 24 injections. However, fewer injections of the highest doses of 3,4-DNT and 3,5-DNT were administered since these doses were found to cause mortality during the course of the injections. The mice were weighed every two weeks during the injection period and at monthly intervals thereafter.

Untreated and vehicle controls were maintained in parallel with the dinitrotoluene-treated groups. The untreated mice (96 animals; 48 males and 48 females) were killed along with the treated mice to determine the incidence of "spontaneous" lung adenomas. Vehicle controls (100 animals; 50 males and 50 females) received 24 injections of tricaprylin (0.1 ml per dose). Positive controls consisted of animals treated with three dose levels of urethan (1000, 500 or 200 mg/kg; 12 mice per dose) to determine whether the adenoma response was dose-related and comparable to that observed in previous studies with strain A mice.[3-5]

Thirty weeks after the initial injections, treated and control animals were killed by cervical dislocation and their lungs removed and fixed in Tellyesniczky's fluid (a mixture of glacial acetic acid and formalin). In Tellyesniczky's fluid, the tumors appear as pearly-white nodules that can easily be observed on the surface of the lung 24 hours after fixation. The tumors on the lung surface were counted and a few taken for histological examination to confirm the appearance of adenoma. The lungs were examined grossly and microscopically for the presence of other abnormalities such as inflammatory reactions and adenomatosis. Other organs examined at autopsy for

the presence of neoplasms were the kidneys, liver, spleen, thymus, salivary and endocrine glands, and the stomach and intestines. Grossly abnormal tissues were excised and examined histologically.

The lung tumor responses in dinitrotoluene-treated mice were compared to those in the appropriate control group by analysis of variance of the number of tumors developed per mouse, and a Chi-square analysis of the number of mice developing at least one tumor.[6] The analysis proceeded in a stepwise manner using programs from the BMDP statistical package.[7]

First, two-way analyses of variance, sex by dose, were conducted using the P7D program from the BMDP series of programs. The dose variable included the appropriate control group. Of primary concern were significant main effects of dose. However, whenever sex interacted with dose, the intepretation of dose effects was appropriately qualified. Second, whenever the Levine test for heterogeneity of variance was significant, both the Brown-Forsythe and the Welch tests were reported. These are one-way analysis of variance statistics which do not assume homogeneity of within group variances. In the event that these tests were not significant, the original analysis was interpreted cautiously. Third, where there were significant main effects of dose, mean differences between dose levels were tested using a separate variance t-test which is appropriate when within group variances are not assumed to be equal.[6]

Finally, Chi-square analyses of the number of mice developing at least one tumor were conducted for each compound using the P4F program from the BMDP series of programs.

RESULTS

Lung Tumor Response in Controls

Table 1 presents results on the occurrence of lung tumors in untreated and vehicle-treated A/J mice. The tumor responses in males were not significantly different from those in females ($p = > 0.05$); therefore, results from the two sexes were combined. Data from untreated mice represent the "spontaneous" occurrence of lung tumors in A/J mice and agree with earlier results on mice of equivalent age.[3-5] The tumor responses in mice administered tricaprylin were not significantly different from those in the untreated mice, indicating that the occurrence of lung tumors was not affected by the vehicle. The tumor responses to urethan, the positive carcinogen control, were dose-related and similar to those observed with urethan in previous studies involving the strain A mouse.[3-5]

Lung Tumor Responses in Dinitrotoluene-Treated Mice

Table 2 presents data from bioassays of the dinitrotoluene compounds following i.p. administration. As for the controls, the tumor responses in male mice were not significantly different from those in the females; therefore, data from the two sexes were combined. The MTD of 3,4-DNT was toxic (25 animals died) and treatment was discontinued after 13 injections. Similarly, the MTD of 3,5-DNT was toxic (11 animals died) and treatment was discontinued after 18 injections. When compared to tricaprylin controls and untreated controls, all 6 dinitrotoluene compounds and the mixture (2,4,6-DNT) were negative for lung tumor induction in strain A mice. Therefore, these compounds were non-carcinogenic in the strain A mouse lung tumor bioassay.

Table 1. LUNG TUMOR RESPONSE IN UNTREATED, VEHICLE-TREATED AND URETHAN-TREATED A/J MICE[a]

Treatment	Route	Number of Treatments	Duration of Experiment (weeks)	Survivors/ Initial[b]	Mice With Lung Tumors (%)	Average Number Lung Tumors/ Mouse
Untreated	-	-	30	94/96	30	0.38 ± 0.10[c]
Tricaprylin	i.p.	24	30	98/100	23	0.31 ± 0.06
Urethan						
1000 mg/kg	i.p.	1	30	12/12	100	27.0 ± 1.10
500 mg/kg	i.p.	1	30	12/12	100	10.7 ± 1.50
200 mg/kg	i.p.	1	30	12/12	100	4.2 ± 0.50

[a] Table contains control data for bioassays of the dinitrotoluene compounds (Section 1; see Table 2).
[b] Data are combined males and females.
[c] Values are mean ± standard error.

Table 2. LUNG TUMOR RESPONSE IN A/J MICE TO SEVEN DINITROTOLUENES ADMINISTERED INTRAPERITONEALLY

Compound	Vehicle	Number of Treatments	Duration of Experiment (weeks)	Total dose[a] (mg/kg)	Survivors/ Initial[b]	Mice with Lung Tumors (%)	Average Number Lung Tumors/Mouse
2,3-Dinitrotoluene	T[c]	24	30	3000	46/50	22	0.22 ± 0.06[d]
		24		1500	47/50	35	0.49 ± 0.07
		24		600	49/50	22	0.33 ± 0.10
2,4-Dinitrotoluene	T	24	30	3000	50/52	26	0.28 ± 0.07
		24		1500	52/52	19	0.19 ± 0.06
		24		600	52/53	44	0.46 ± 0.08
2,5-Dinitrotoluene	T	24	30	3000	48/52	42	0.50 ± 0.09
		24		1500	52/52	25	0.37 ± 0.10
		24		600	51/52	33	0.41 ± 0.09
2,6-Dinitrotoluene	T	24	30	3000	47/52	30	0.40 ± 0.10
		24		1500	51/52	45	0.53 ± 0.09
		24		600	50/52	34	0.40 ± 0.09
2,4,6-Dinitrotoluene	T	24	30	4800	40/52	23	0.25 ± 0.08
		24		2400	50/52	28	0.32 ± 0.08
		24		960	48/52	33	0.39 ± 0.08
3,4-Dinitrotoluene	T	13	30	3250	27/52	22	0.22 ± 0.08
		24		3000	51/52	33	0.35 ± 0.07
		24		1200	49/52	20	0.29 ± 0.08

TABLE 2 (cont.)

LUNG TUMOR RESPONSE IN A/J MICE TO SEVEN DINITROTOLUENES ADMINISTERED INTRAPERITONEALLY

Compound	Vehicle	Number of Treatments	Duration of Experiment (weeks)	Total dose[a] (mg/kg)	Survivors/ Initial[b]	Mice with Lung Tumors (%)	Average Number Lung Tumors/Mouse
3,5-Dinitrotoluene	T	18	30	2250	39/50	49	0.64 ± 0.12
		24		1500	50/50	16	0.16 ± 0.05
		24		600	48/50	38	0.46 ± 0.09

[a] Total cumulative dose per animal.
[b] Data are combined males and females.
[c] T = tricaprylin
[d] Values are mean ± standard error.

DISCUSSION

Dinitrotoluene (DNT) compounds have been tested for carcinogenic potential when administered in the diet. Technical grade DNT (75.8% 2,4-DNT, 19.5% 2,6-DNT, and 4.7% other isomers), when fed to F-344 rats at 3.5 to 35 mg/kg/day, produced a dose-dependent increase in hepatocellular carcinoma with a higher incidence among males than among females.[8] 2,6-DNT was classified as a "weak" hepatocarcinogen when fed to F-344 rats and exhibited promoting activity in rats initiated with a single dose of DENA.[9] A recent preliminary report showed that the feeding of pure 2,6-DNT (14 and 7 mg/kg diet) to 20 male Fischer-344 rats for 1 year resulted in a 100% (14 mg/kg diet) and 85% (7 mg/kg diet) incidence of hepatocellular carcinoma.[10] 2,4-DNT (>98% pure) was also a hepatocarcinogen when fed to CD-1 rats at 34 and 45 mg/kg/day, and induced both preneoplastic and neoplastic renal tumors in male CD-1 mice when fed at 13.5 and 95 mg/kg/day.[11] A study, conducted by the National Cancer Institute, showed no evidence for carcinogenicity of 2,4-DNT in either rats or mice.[12]

In our study, 2,4-DNT and 2,6-DNT were negative for lung tumor induction when administered i.p. at total doses of 3000, 1500 and 600 mg/kg, and the 2:1 mixture of 2,4-DNT and 2,6-DNT was inactive after i.p. doses of 4800, 2400 and 960 mg/kg. The inability of these dinitrotoluenes to induce lung tumors in A/J mice is not too surprising since it is known that the lung tumor bioassay is relatively insensitive to moderately and/or weakly active liver carcinogens.[3-5] The assay is, however, quite sensitive to strong liver carcinogens such as aflatoxin B_1[13], 2-acetylaminofluorene[3], dimethylnitrosamine[5], and diethylnitrosamine.[14]

The other dinitrotoluene isomers; i.e., 2,3-DNT, 2,5-DNT, 3,4-DNT and 3,5-DNT have not been reported to be carcinogenic in any other model system and were negative for lung tumor induction in A/J mice.

SECTION 2 - PHARMACOKINETICS AND METABOLISM OF DINITROTOLUENES

INTRODUCTION

The principal components of technical grade dinitrotoluene are 2,4-DNT and 2,6-DNT. The metabolism, distribution and elimination of these two isomers in the strain A mouse have hitherto not been investigated. As is known for other compounds, these parameters may affect the tumor response. This section contains a report on the pharmacokinetics and metabolism of these compounds, including data on the effects of the route of administration, effects of pretreatment and different doses. In addition, because the covalent interaction of a carcinogen with DNA is believed to be related to the initiation of the carcinogenic process, the covalent binding of 2,4-DNT and 2,6-DNT to DNA of the lungs, the target organ in the bioassay (see Section 1), as well as that to DNA in nontarget organs has been studied and these results are also included in this section.

A. 2,4-DINITROTOLUENE

1. Purity of 2,4-Dinitrotoluene and [3-^{3}H]2,4-Dinitrotoluene

The purity of 2,4-dinitrotoluene (2,4-DNT) was checked by high-pressure liquid chromatography (HPLC) using a Waters (Milford, MA) system (model 6000 A pump, model M45 pump, model U6K injector, model 720 system controller, model 730 data module). An ISCO (Lincoln, NE) model UA-5 absorbance detector with peak separator was used to monitor the absorbance (254 nm) of the column effluent. A 10 µl aliquot of a solution of 2,4-DNT in methanol (500 ppm) was chromatographed on a C_{18}-µBondapak (Waters) column. The column was eluted isocratically using water:methanol (1:1) as the solvent. Two U.V.-absorbing peaks were eluted, a major peak with a retention time of 11.54 min and a minor peak with a retention time of 13.70 min (Fig. 1). The minor peak comprised 0.6%, while the major peak comprised 99.4% of the material injected, as concluded from integration of the areas under the respective peaks. It was therefore concluded that 2,4-DNT was at least 99.4% pure. This material was used without further purification.

[3-^{3}H]2,4-DNT (specific activity 1.4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). For the verification of the radiochemical purity of [3-^{3}H]2,4-DNT, approximately 15,000 cpm of this material was cochromatographed with 2,4-DNT as described above. The column effluent was collected in 0.5 ml fractions using an ISCO model 328 fraction collector which was interfaced with the UA-5 detector and was operated with an ISCO flow interruptor valve. Determination of the radioactivity in eluted fractions of the entire chromatogram revealed that 99.5% of the total amount of radioactive material injected cochromatographed with the major peak, representing 2,4-DNT. Therefore, the [3-^{3}H]2,4-DNT was greater than 99.5% radiochemically pure. It was used in all of the pharmacokinetic and metabolism studies.

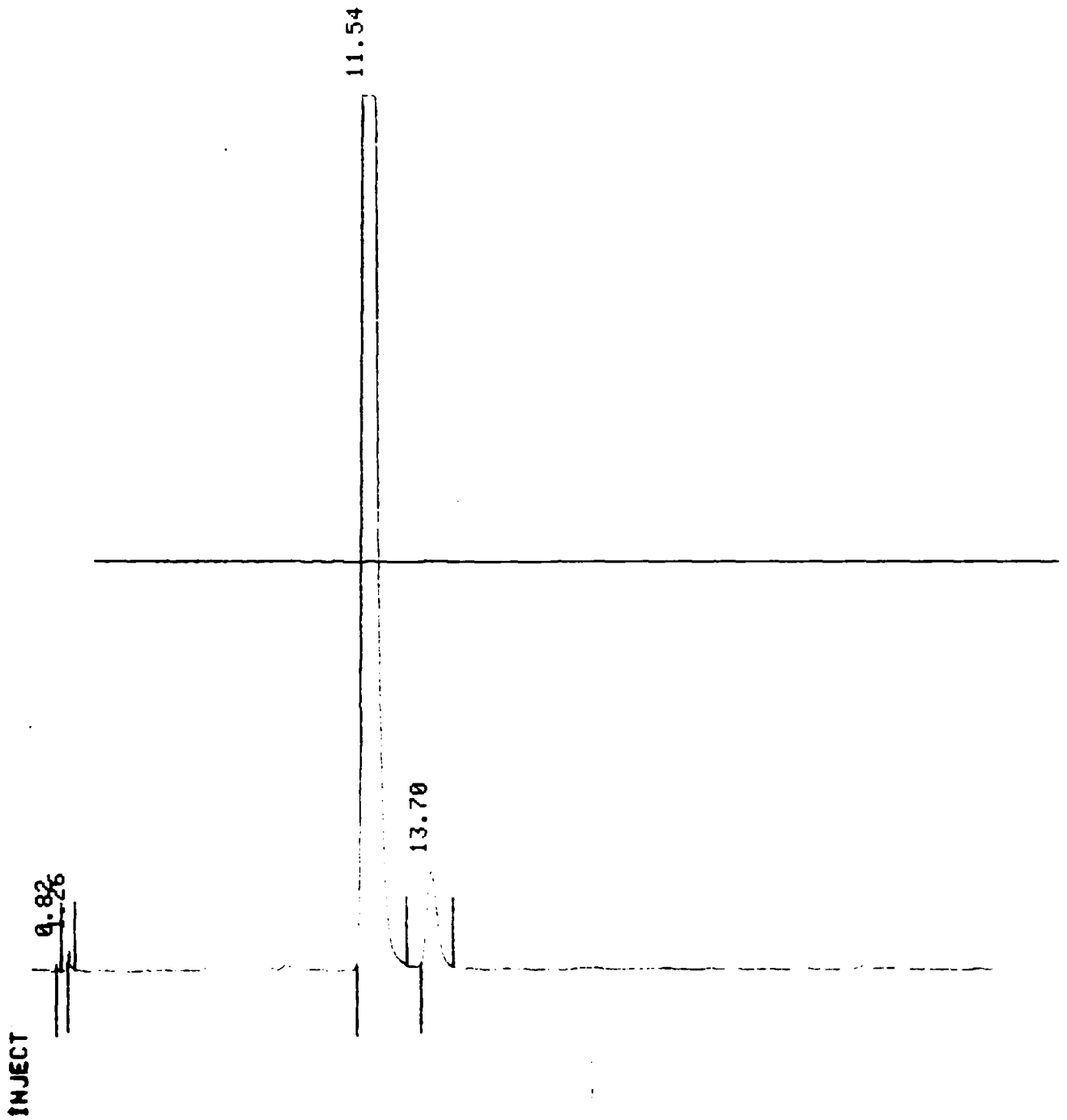

Fig. 1 Check of purity of 2,4-DNT. HPLC profile of 2,4-DNT, chromatographed as described in the text.

In certain experiments (see Section A.2.d. below) [U-ring-^{14}C]2,4-DNT was used as a recovery marker. The ^{14}C-label in this material was distributed randomly in the ring system. Approximately 200 µCi of [U-ring-^{14}C]2,4-DNT (s.a. 52.5 mCi/mmol) was received as a gift from Dr. John Dent (Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina). Its purity was verified by both high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC). For HPLC, approximately 44,000 dpm of the material was mixed with 5 µg of 2,4-DNT and the mixture was chromatographed on a µBondapak C_{18} column (Waters), as described above for 2,4-DNT. A total of 97.3% of the radioactivity injected onto the column eluted with the standard 2,4-DNT, which had a retention time of 14.6 min under these conditions. The majority of the remainder of the radioactivity (2.7%) eluted with a retention time of 5.4 min.

For TLC, a total of 12,000 dpm of the [U-ring-^{14}C]2,4-DNT was mixed with 100 µg of 2,4-DNT and the mixture was chromatographed on an Eastman Kodak sheet (silica gel, 100 µm), using n-hexane: ethyl acetate (4:1) as the solvent system. The R_f of 2,4-DNT under these conditions was 0.8; the spot of 2,4-DNT was localized under U.V. light (silica gel with fluorescent indicator was used) and eluted with 2 ml of methanol. The remainder of the TLC-plate was divided into 6 segments of 1 inch each and each segment was eluted as above. All eluates were counted using standard liquid scintillation techniques. A total of 98.2% of the applied radioactivity eluted with the 2,4-DNT standard, while the remainder (1.8%) eluted with an R_f of 0.4. This material was used as a recovery marker in all subsequent reisolation experiments.

2. Tissue Distribution and Elimination Studies

a) Effect of vehicle and route of administration

A set of preliminary experiments were performed using corn oil as the vehicle. Adult male A/J mice were injected intraperitoneally with 0.25 ml of a solution of [3-^{3}H]2,4-DNT in corn oil (10 µCi/ml). After various time periods the mice were anesthetized with diethyl ether, the animal was placed in a piece of aluminum foil, and blood was collected from the jugular veins. Urine was collected from the aluminum foil using a Pasteur pipet when the animal urinated during this procedure. Immediately thereafter, the organs listed in Table 3 were removed. Except for the liver, small and large intestine, the entire organ was placed in a counting vial, wetted with 0.2 ml of distilled water and digested at 50°C in 3.5 ml of tissue solubilizer (NCS Amersham). The bladder was added to the urine samples. The total weights of the liver and the intestine (plus contents) were obtained and a weighed portion (200-300 mg) was also digested with NCS. After the digestion of the tissues was complete (6-12 hours) the solutions were neutralized with glacial acetic acid (30 µl/ml tissue solubilizer), 16 ml of scintillation cocktail (OCS, Amersham) was added and the vials were counted in a liquid scintillation counter (Beckman Model LS-230) using the channels ratio method to correct for quenching. Severely quenched samples were corrected by the addition of an internal standard ([^{3}H]toluene). Blood was collected in lithium-heparin tubes and 50 µl aliquots of whole blood were digested at 50°C with 0.6 ml of tissue solubilizer for 30 min. To decolorize the digests, 0.2 ml of a freshly prepared solution of benzoyl peroxide in toluene (0.2 g/ml) was added and the mixtures were incubated for an additional 30 min. After cooling, 19 ml of scintillation fluid was added and the vials were counted as described above. All samples were counted in duplicate for a period of time sufficient to reduce the counting error to less than 2%. The efficiency of tritium counting under these conditions varied from 16 to 38%. The total dpm per tissue was expressed as a percentage of the total amount of radioactive material injected. These results are shown in Table 3. [3-^{3}H]2,4-DNT was excreted rapidly into the urine (17.8% of the dose in 30 min; Table 3), but levels of radioactivity in blood, urine and small intestine increased during the 30 min period of observation. The contents of radioactive material in the large intestine and in adipose tissue (epidydimal fat) were decreasing while those in the remaining tissues (lungs, kidneys, heart, spleen, and brain) remained relatively constant during 30 min after administration. Close to 50 percent of the total radioactivity injected could be accounted for in tissues and body fluids (Table 3).

A limited number of experiments were performed to compare the effect of the vehicle and the route of administration. Table 4 shows the results of experiments designed to compare the intraperitoneal (i.p.) to the oral (p.o.) route of administration, using corn oil as the vehicle. Beyond 30 min, the total amount of radioactivity that could be accounted for in the tissues and body fluids was smaller with the p.o. than with the i.p. route of administration and this difference was mostly due to the smaller amounts in the liver, adipose tissue and in the small intestine. At the earlier time points (10-15 min and 30 min), the differences between the i.p. and p.o. routes were not significant.

When tricaprylin was used as the vehicle, the differences between the i.p. and the p.o. route of administration were more obvious (Table 5). At all time points studied, the total recovery of radioactivity was much lower with p.o. than with i.p. administration. Most striking were the much lower amounts

Table 3. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. ADMINISTRATION OF [3-^{3}H]2,4-DINITROTOLUENE INTO A/JAX MICE.

Time after Administration (min)	Percentage of Total Radioactivity Injected[a]				
	Blood[b]	Urine[c]	Liver	Lungs	Kidneys
5	2.01 ± 0.95	0.51 ± 0.62	9.13 ± 6.28	0.33 ± 0.15	2.37 ± 1.22
10	2.60 ± 0.14	1.43 ± 1.08	9.00 ± 1.75	0.23 ± 0.06	2.60 ± 0.90
15	3.40 ± 0.56	4.63 ± 2.40	8.70 ± 1.93	0.23 ± 0.06	3.13 ± 0.40
20	5.00 ± 2.86	8.33 ± 2.29	11.63 ± 3.74	0.33 ± 0.15	2.60 ± 0.26
30	5.77 ± 4.80	17.80 ± 4.81	6.43 ± 2.99	0.30 ± 0.17	2.63 ± 0.85

	Small Int.[d]	Large Int.[d]	Heart	Spleen	Adipose
5	8.5 ± 2.8	4.37 ± 1.58	0.20 ± 0.17	0.43 ± 0.06	6.57 ± 4.07
10	11.1 ± 4.2	3.60 ± 1.47	0.17 ± 0.06	0.20 ± 1.10	7.00 ± 2.25
15	13.0 ± 5.3	3.10 ± 1.31	0.10 ± 0.00	0.17 ± 0.12	4.97 ± 0.99
20	11.5 ± 5.6	2.93 ± 1.10	0.13 ± 0.06	0.02 ± 0.10	3.97 ± 0.67
30	22.1 ± 5.7	1.30 ± 0.52	0.10 ± 0.00	0.10 ± 0.00	3.27 ± 1.40

	Brain	All Tissues + Fluids
5	0.27 ± 0.15	34.7 ± 8.1
10	0.23 ± 0.06	38.2 ± 8.4
15	0.27 ± 0.06	41.8 ± 8.6
20	0.23 ± 0.06	47.0 ± 6.5
30	0.23 ± 0.06	47.9 ± 24.0

[a]Mean ± S.D. (N=3); all samples were counted for a period of time sufficient to reduce the counting error to less than 2%.
[b]Total radioactivity in blood based on 7% of the body weight.
[c]Urine and bladder.
[d]Tissue plus contents.

Table 4. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. AND P.O. ADMINISTRATION OF [^{3}H]2,4-DINITROTOLUENE, IN CORN OIL, TO A/J MICE.

	i.p.	p.o.	i.p.	p.o.	i.p.	p.o.	i.p.	p.o.
Tissue/Fluid	10 min.[d]	15 min.	30 min.	30 min.	40 min.	45 min.	50 min.	50 min.
Blood[a]	2.0 ± 0.4[e]	1.7 ± 0.4	3.2 ± 0.4	2.1 ± 1.0	2.5 ± 0.7	1.0 ± 0.7	1.9 ± 0.3	1.9 ± 0.4
Urine[b]	1.8 ± 1.2	3.7 ± 1.3	12.6 ± 3.7	24.0 ± 10.3	21.8 ± 8.8	22.5 ± 3.3	23.8 ± 0.2	31.9 ± 1.8
Liver	4.9 ± 1.9	4.8 ± 2.1	8.1 ± 0.3	3.5 ± 2.8	6.7 ± 1.3	2.5 ± 1.0	5.2 ± 1.5	2.8 ± 0.2
Lungs	0.2 ± 0.1	0.4 ± 0.3	0.3 ± 0.1	0.3 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
Kidneys	1.4 ± 1.0	1.1 ± 0.3	3.1 ± 0.3	1.3 ± 0.8	3.0 ± 0.4	0.5 ± 0.4	1.9 ± 0.3	0.7 ± 0.4
Adipose	4.5 ± 1.0	0.2 ± 0.0	4.4 ± 2.1	0.4 ± 0.4	2.7 ± 1.4	0.1 ± 0.0	2.8 ± 1.6	0.4 ± 0.1
Small Int.[c]	12.5 ± 1.7	23.9 ± 8.7	17.7 ± 6.6	15.6 ± 3.8	21.6 ± 3.0	16.2 ± 3.5	27.3 ± 11.5	19.0 ± 5.7
Large Int.[c]	3.3 ± 3.0	0.9 ± 0.3	1.7 ± 0.2	0.6 ± 0.4	1.1 ± 0.2	0.7 ± 0.5	1.8 ± 0.9	4.2 ± 4.7
TOTAL	31.2 ± 3.0	35.8 ± 11.7	52.0 ± 6.8	48.5 ± 7.7	62.5 ± 6.9	42.3 ± 2.0	66.2 ± 14.2	60.8 ± 13.2

[a] Total radioactivity in blood based on 7% of the body weight

[b] Urine and bladder

[c] Tissue plus contents

[d] Time after administration

[e] Mean ± SD (N=3) percentage of injected

Table 5. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. AND P.O. ADMINISTRATION OF [^{3}H]2,4-DINITROTOLUENE, IN TRYCAPRYLIN, TO A/J MICE.

	15 min.[d]		30 min.		45 min.		60 min.	
Tissue/Fluid	i.p	p.o.	i.p.	p.o.	i.p.	p.o.	i.p.	p.o.
Blood[a]	2.1 ± 0.3[e]	1.6 ± 0.8	2.0 ± 0.6	0.9 ± 0.4	1.9 ± 0.4	1.2 ± 0.7	1.7 ± 0.1	1.4 ± 0.7
Urine[b]	6.7 ± 5.5	1.6 ± 0.4	13.0 ± 9.1	4.4 ± 2.0	33.8 ± 1.7	4.4 ± 2.2	32.5 ± 3.9	8.6 ± 4.9
Liver	6.1 ± 1.3	3.1 ± 1.8	5.5 ± 2.7	2.1 ± 0.2	5.7 ± 0.5	1.4 ± 0.5	4.5 ± 0.9	3.0 ± 2.3
Lungs	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	1.0 ± 0.0	0.2 ± 0.1	1.0 ± 0.1
Kidneys	3.4 ± 2.7	0.3 ± 0.2	2.6 ± 2.2	0.4 ± 0.2	2.5 ± 1.3	0.4 ± 0.1	1.9 ± 1.1	0.5 ± 0.2
Adipose	2.9 ± 0.8	0.1 ± 0.0	5.4 ± 3.2	0.1 ± 0.0	2.9 ± 1.2	0.1 ± 0.1	2.3 ± 1.7	0.1 ± 0.1
Small Int.[c]	5.5 ± 0.7	13.7 ± 0.3	8.6 ± 4.6	10.0 ± 5.2	14.8 ± 2.7	7.5 ± 4.9	15.6 ± 3.1	16.0 ± 7.1
Large Int.[c]	1.1 ± 0.2	1.4 ± 1.7	0.7 ± 0.3	2.1 ± 2.0	0.6 ± 0.1	0.4 ± 0.3	0.5 ± 0.1	4.3 ± 4.6
TOTAL	27.5 ± 7.7	21.9 ± 2.0	38.0 ± 15.4	19.9 ± 1.5	62.4 ± 4.5	15.5 ± 6.2	57.4 ± 2.9	30.3 ± 13.

[a] Total radioactivity in blood based on 7% of the body weight

[b] Urine and bladder

[c] Tissue plus contents

[d] Time after administration

[e] Mean ± SD (N=3) percentage of injected

excreted in the urine, and the lower content of radioactivity in the liver and adipose tissue with the p.o. route of administration.

It may be concluded that, when corn oil was used as the vehicle, uptake and excretion of [3-^{3}H]2,4-DNT was somewhat higher with i.p. than p.o. administration, especially at the later time points. With tricaprylin, this difference was much more accentuated, clearly indicating that with i.p. administration both tissue uptake and urinary excretion was much more rapid than with p.o. administration (Tables 3 and 4). For these reasons, and also because tricaprylin is the vehicle used in the lung tumor bioassay (also with i.p. administration), all subsequent experiments were done using tricaprylin as the vehicle.

b) Effect of different doses of i.p. administered 2,4-DNT

The results of studies on the effects of different doses of 2,4-DNT on its tissue distribution and excretion are shown in Tables 6 and 7. In all these studies, 2,4-DNT was administered i.p. with tricaprylin as the vehicle and radioactivity in tissues and fluids was quantitated as described in section A.2.a. In blood, liver, lungs, kidneys and large intestine, the levels of radioactivity were relatively unaffected by doses of 2,4-DNT ranging from 0 (tracer only) to 100 mg/kg (Table 6). Maximum excretion in the urine was observed at a dose of 1 mg/kg with 60% of the radioactivity appearing in the urine 1 hour after injection. At 10 and 100 mg/kg urinary excretion was much lower (40.5 and 14.1%, respectively, after 1 hour). At doses of 0-10 mg/kg the radioactivity in adipose tissue decreased with time (Table 6). At all doses the content of radioactive material in the small intestine increased with time with maximum uptake at 1 mg/kg, the dose at which maximum excretion in the urine was observed (Table 6). At 100 mg/kg, uptake in the small intestine was lowest, again paralleling the much lower excretion in the urine at this dose. Total radioactivity that could be accounted for in tissues and fluids was highest (87.9%) at 1 mg/kg and lowest (32.2%) at 100 mg/kg (Table 7).

It was concluded that with doses of 1-100 mg/kg, rates of urinary excretion were highest with 1 mg/kg and lowest with 100 mg/kg (Table 6), a result which was not unexpected, as metabolizing enzymes become more and more saturated at the higher doses. The relatively low rate of urinary excretion at "0-dose" (tracer only) is possibly due to a relatively high uptake and slow release in adipose tissue or in other tissues not analyzed. The urine is the major route of elimination as very low amounts of radioactivity were found in the large intestine during the 1 hour period of study (Table 6).

There is no preferential uptake or sequestering of radioactive material in the lungs, the target organ in the lung tumor bioassay (Table 6), or in any other tissue, with the exception of the small intestine which is the major site of absorption.

Because urinary excretion of radioactivity during a period of 1 hr. after i.p. administration of various doses of [3-^{3}H]2,4-DNT was not complete (Table 6), and since the rate of excretion depended on the dose injected, a series of experiments were performed in order to determine the tissue distribution and rates of excretion of 2,4-DNT during time periods up to 4 hrs after i.p. injection. The protocol for these experiments was as described in Section A.2.a., except for the collection of urine. Immediately after injection, mice were placed individually into glass beakers. After 1, 2, 3 or 4 hrs, the mice were removed and their tissues and fluids were collected as

Table 6. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE.

Time after Administration (min)	Dose (mg/kg) 0	1	10	100	Dose (mg/kg) 0	1	10	100
	Blood				Urine			
15	2.1 ± 0.3[a]	3.2 ± 0.4	2.0 ± 0.4	1.6 ± 0.4	6.7 ± 5.5	6.4 ± 1.7	6.2 ± 1.0	2.0 ± 0.5
30	2.0 ± 0.6	3.5 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	13.0 ± 9.1	25.0 ± 1.4	18.5 ± 1.2	4.7 ± 1.7
45	1.9 ± 0.4	2.8 ± 0.4	1.8 ± 0.3	1.7 ± 0.4	33.8 ± 1.7	45.8 ± 6.2	29.1 ± 4.0	10.3 ± 2.1
60	1.7 ± 0.1	2.2 ± 0.2	1.9 ± 0.8	2.3 ± 0.4	32.5 ± 3.9	60.0 ± 3.9	40.5 ± 4.9	14.1 ± 2.1
	Liver				Lungs			
15	6.1 ± 1.3	6.9 ± 1.7	5.1 ± 1.3	3.2 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
30	5.5 ± 2.7	6.4 ± 0.9	5.3 ± 1.5	3.6 ± 0.3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
45	5.7 ± 0.5	3.7 ± 0.6	4.4 ± 0.8	4.3 ± 0.9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1
60	4.5 ± 0.9	2.7 ± 0.6	4.1 ± 1.1	4.6 ± 0.9	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
	Kidneys				Adipose			
15	3.4 ± 2.7	2.2 ± 0.3	2.1 ± 0.5	1.4 ± 0.2	2.9 ± 0.8	2.7 ± 1.7	3.5 ± 0.4	3.0 ± 0.7
30	2.6 ± 2.2	2.7 ± 0.5	1.9 ± 0.2	2.0 ± 0.3	5.4 ± 3.2	3.9 ± 2.0	3.4 ± 1.1	4.7 ± 0.6
45	2.5 ± 1.3	1.8 ± 0.1	1.6 ± 0.3	1.9 ± 0.4	2.9 ± 1.2	2.8 ± 1.2	1.5 ± 0.3	4.4 ± 1.6
60	1.9 ± 1.1	1.3 ± 0.3	1.3 ± 0.1	1.7 ± 1.7	2.3 ± 1.7	1.4 ± 0.7	1.8 ± 1.1	4.1 ± 2.0
	Small Intestine				Large Intestine			
15	5.5 ± 0.7	6.0 ± 0.9	6.2 ± 1.5	4.2 ± 1.0	1.1 ± 0.2	1.4 ± 0.2	1.1 ± 0.3	2.2 ± 0.2
30	8.6 ± 4.6	14.0 ± 2.0	9.2 ± 1.6	4.0 ± 0.5	0.7 ± 0.3	0.9 ± 0.2	0.8 ± 0.2	1.9 ± 0.3
45	14.8 ± 2.7	16.7 ± 2.3	12.8 ± 2.2	5.2 ± 2.0	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	2.4 ± 0.6
60	15.6 ± 3.1	16.3 ± 3.8	17.3 ± 4.2	3.0 ± 0.4	0.5 ± 0.1	0.6 ± 0.4	0.5 ± 0.1	2.5 ± 0.3

[a]Mean ± SD (N=4) percentage of injected

Table 7. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Time after Administration (min)	Dose (mg/kg)			
	0	1	10	100
15	27.5 ± 7.7[a]	29.0 ± 4.2	26.3 ± 4.3	17.8 ± 2.4
30	38.0 ± 15.4	52.4 ± 3.7	41.0 ± 1.7	22.8 ± 1.3
45	62.4 ± 4.5	73.1 ± 5.4	51.9 ± 6.2	30.5 ± 5.7
60	57.4 ± 2.9	87.9 ± 6.6	67.5 ± 7.9	32.2 ± 4.9

[a]Mean ± SD (N=4) percentage of injected

before. The beaker was rinsed twice with 2 ml of water and the rinses were combined with the urine and bladder from the animals and an aliquot of this was counted.

The results of these experiments are shown in Table 8. With the exception of the large intestine, all tissues, including blood, showed declining levels of radioactivity during the 4 hr period. In the large intestine levels of radioactivity appeared to increase, with the highest level (10.1%) 4 hrs after a dose of 1 mg/kg. This indicates that at later time periods the feces is a more important route of elimination than at earlier times. Excretion into the urine remains the major route of elimination even at 4 hrs after administration. Rates of excretion were quite variable and, especially at 1 and 10 mg/kg showed no consistent pattern, with large standard deviations at 3 and 4 hrs (Table 8). In some of the mice in these groups 75-92% of the injected radioactivity could be recovered in the urine after 3-4 hrs. It is therefore likely that the variability in the amount of radioactivity present in the urine after 3-4 hrs is related to variable extents of incompleteness of the urine collection. It is also clear that the lack of consistency in the total amount of radioactivity that can be recovered from tissues and fluids (Table 9), especially at 1 and 10 mg/kg, is largely due to the same problem with urine collection. Nevertheless, it can be concluded that 4 hrs after i.p. administration, 70-80% of the dose has been absorbed and/or excreted (Table 9) and that rates of absorption and excretion depend on the dose administered.

c) Effect of different doses of p.o. administered 2,4-DNT

It was evident from the initial studies (Section A.2.a.) that the route of administration (i.p. or p.o.) drastically affected the tissue distribution and elimination of 2,4-DNT in that elimination was much slower after p.o. than after i.p. administration (Table 5). The distribution and elimination of different doses of p.o.-administered 2,4-DNT were therefore followed for time periods up to 24 hrs, using the methods described before the analysis of total radioactivity in the tissues (Section A.2.a.). The results of these experiments are shown in Tables 10 and 11. For time periods of 4 hrs and beyond, mice were placed individually in metabolic cages in order to permit the separate collection of urine and feces. Total amounts of radioactive material in the blood (Table 10) did not differ significantly from amounts observed after i.p. administration (Table 8). Amounts in the liver, kidneys and lungs (Table 10) were also similar to those observed after i.p. injection (Table 8). Adipose tissue had very low or non-detectable amounts of radioactive material. As expected, amounts of radioactive material in the small intestine were much higher after p.o. (Table 10) than after i.p. administration (Table 8), but after 8 hrs most of a dose of 100 mg/kg had been absorbed from this organ, as levels in the large intestine did not increase significantly at this time or later (Table 10). As we found before (Table 5), concentrations of radioactive material in the urine were much lower after p.o. (Table 10) than after i.p. administration (Table 8). The failure to account for more than 50-60% of the administered dose (100 mg/kg) in the urine, even after 24 hrs (Table 10), is probably due to the incompleteness of the urine collection. Even though metabolic cages were used, the small volume of urine excreted during the experimental period, probably precludes an accurate quantitative collection. Nevertheless, at 8 hrs after p.o. administration, approximately the same amount (66.0 ± 5.1%, Table 10) was excreted as at 4 hrs after i.p. administration

Table 8. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Time after Administration (hr)	Dose (mg/kg) 1	10	100	Dose (mg/kg) 1	10	100
	Blood			Urine		
1	1.6 ± 0.7[a]	1.5 ± 0.2	2.1 ± 0.6	38.4 ± 8.2	51.5 ± 4.5	33.7 ± 6.1
2	1.3 ± 0.9	1.0 ± 0.1	1.7 ± 0.6	68.7 ± 9.7	63.6 ± 13.8	55.9 ± 12.5
3	1.5 ± 0.7	1.3 ± 0.2	1.2 ± 0.6	51.6 ± 22.8	54.4 ± 26.4	59.8 ± 12.0
4	1.7 ± 0.7	1.3 ± 0.3	1.0 ± 0.3	52.5 ± 21.0	60.1 ± 21.5	70.0 ± 25.1
	Liver			Lungs		
1	3.5 ± 0.9	2.6 ± 0.7	3.1 ± 0.6	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
2	1.8 ± 0.4	2.5 ± 1.0	2.3 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
3	1.4 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
4	1.3 ± 0.3	1.0 ± 0.2	2.2 ± 2.8	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	Kidneys			Adipose		
1	1.1 ± 0.2	1.1 ± 0.3	1.6 ± 0.4	1.0 ± 0.4	0.7 ± 0.3	1.9 ± 0.4
2	0.5 ± 0.1	0.5 ± 0.1	1.1 ± 0.3	0.3 ± 0.3	0.3 ± 0.1	1.3 ± 0.5
3	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
4	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
	Small Intestine			Large Intestine		
1	14.2 ± 1.5	12.7 ± 0.9	7.0 ± 1.1	0.5 ± 0.1	0.5 ± 0.1	0.9 ± 0.2
2	16.4 ± 5.0	12.0 ± 2.7	8.9 ± 2.3	2.2 ± 2.0	0.4 ± 0.1	0.7 ± 0.3
2	10.6 ± 5.0	7.2 ± 3.2	7.4 ± 0.5	6.2 ± 1.4	4.9 ± 1.9	1.1 ± 0.8
4	3.9 ± 1.7	8.1 ± 1.4	6.3 ± 2.5	10.1 ± 2.4	3.9 ± 3.3	2.1 ± 0.6

[a] Mean ± SD (N=4) percentage of injected.

Table 9. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Time after Administration (hr)	Dose (mg/kg)		
	1	10	100
1	60.3 ± 10.1[a]	70.8 ± 5.4	50.4 ± 5.8
2	91.2 ± 12.1	80.1 ± 14.0	72.0 ± 14.4
3	71.8 ± 18.1	69.2 ± 24.0	70.9 ± 11.0
4	69.4 ± 18.1	74.8 ± 18.9	81.7 ± 25.1

[a]Mean ± SD (N=4) percentage of injected

Table 10. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVE MATERIAL AFTER P.O. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Time after Administration (hr)	Dose (mg/kg)			Dose (mg/kg)		
	1	10	100	1	10	100
		Blood			Urine	
1	0.5 ± 0.2[a]	1.2 ± 0.5	0.9 ± 0.4	9.3 ± 2.0	9.5 ± 1.3	6.6 ± 2.3
2	0.7 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	20.6 ± 3.0	16.5 ± 4.3	17.6 ± 2.1
3	0.8 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	34.1 ± 10.6	31.3 ± 8.1	23.3 ± 1.9
4	0.8 ± 0.2	1.0 ± 0.1	0.5 ± 0.1	31.1 ± 4.9	33.6 ± 3.0	28.5 ± 5.2
8	n.d.[b]	n.d.	0.6 ± 0.1	n.d.	n.d.	66.0 ± 5.1
16	n.d.	n.d.	0.8 ± 0.1	n.d.	n.d.	57.1 ± 6.2
24	n.d.	n.d.	0.9 ± 0.0	n.d.	n.d.	52.7 ± 13.7
		Liver			Lungs	
1	0.9 ± 0.7	2.0 ± 1.3	1.4 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
2	1.2 ± 0.5	0.7 ± 0.3	2.4 ± 1.7	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
3	1.3 ± 0.3	1.8 ± 0.9	0.9 ± 0.1	0.1 ± 0.0	0.1 ± 0.9	0.1 ± 0.0
4	1.5 ± 0.7	1.9 ± 0.6	1.2 ± 0.6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
8	n.d.	n.d.	1.0 ± 0.4	n.d.	n.d.	0.1 ± 0.0
16	n.d.	n.d.	0.5 ± 0.0	n.d.	n.d.	0.1 ± 0.0
24	n.d.	n.d.	0.6 ± 0.1	n.d.	n.d.	0.1 ± 0.0
		Kidneys			Adipose	
1	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.0	0.1 ± 0.0	0.2 ± 0.1
2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.0	0.1 ± 0.1	0.1 ± 0.1
3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.0	0.1 ± 0.1	0.1 ± 0.0
4	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.0	0.1 ± 0.1	0.1 ± 0.0
8	n.d.	n.d.	0.2 ± 0.1	n.d.	n.d.	0.0
16	n.d.	n.d.	0.1 ± 0.0	n.d.	n.d.	0.0
24	n.d.	n.d.	0.0	n.d.	n.d.	0.0

Table 10 (continued)

Time after Administration (hr)	Dose (mg/kg)			Dose (mg/kg)		
	1	10	100	1	10	100
	Small Intestine			Large Intestine[c]		
1	22.4 ± 10.2	15.2 ± 9.2	20.7 ± 3.5	0.7 ± 0.2	0.3 ± 0.1	0.9 ± 0.7
2	19.4 ± 9.1	8.6 ± 2.7	14.6 ± 3.2	0.8 ± 0.3	0.5 ± 0.3	0.6 ± 0.3
3	15.0 ± 6.1	13.5 ± 5.7	26.5 ± 5.0	1.4 ± 1.0	1.7 ± 1.2	0.6 ± 0.4
4	11.9 ± 7.1	11.6 ± 4.3	16.0 ± 9.6	2.1 ± 1.8	2.0 ± 1.0	1.4 ± 0.4
8	n.d.	n.d.	4.3 ± 2.3	n.d.	n.d.	2.1 ± 3.0[b]
16	n.d.	n.d.	0.6 ± 0.1	n.d.	n.d.	1.1 ± 0.5[b]
24	n.d.	n.d.	0.6 ± 0.1	n.d.	n.d.	3.1 ± 0.9[b]

[a] Mean ± S.D. (N=4) percentage of injected

[b] n.d. = not determined

[c] Tissue (+ contents) and feces

(70.0 ± 25.1%, Table 8). Total amounts of radioactive material in tissues and fluids recovered after 1-4 hrs (Table 11), were much lower than the corresponding amounts recovered after i.p. administration (Table 9). At 8 hrs after a dose of 100 mg/kg, however, total amounts of radioactive material recovered (77.3 ± 5.6%, Table 11) approach those observed in 4 hrs after i.p. injection (81.7 ± 25.1%, Table 9).

Because in the above experiments (Table 10) on the elimination of p.o.-administered 2,4-DNT only some 30% of doses of 1 and 10 mg/kg could be recovered in the urine 4 hours after administration, these experiments were extended to 8 hours in order to establish whether excretion would be more complete and to provide a comparison with a similar experiment using 100 mg/kg (Table 10). The data obtained in these experiments are shown in Table 12. The tissue distribution of radioactivity was similar to that observed after 8 hours with a dose of 100 mg/kg (Table 10). Amounts excreted in the urine after 8 hours (52-55%, Table 12) were substantially higher than those excreted after 4 hours (Table 10) and approached the amounts excreted with a dose of 100 mg/kg (66%, Table 10). The total amount recovered (65%, Table 12) was higher than that recovered at 4 hours (47-52%, Table 11) but was somewhat lower than the corresponding amount recovered with a dose of 100 mg/kg (77.3%, Table 11). Longer time periods were not studied because, at a dose of 100 mg/kg, this did not result in higher recoveries (Table 11).

d) Rates of 2,4-DNT elimination from tissues

In this series of experiments 2,4-DNT was reisolated from the tissues after i.p. administration. [U-ring-^{14}C]2,4-DNT was used as a recovery marker.

Young adult (6-8 weeks) male A/J mice were injected i.p. with 0.25 ml of a solution of [3-^{3}H]2,4-DNT (10 µCi/ml), in tricaprylin containing the appropriate amount of 2,4-DNT to give doses of 1, 10 or 100 mg/kg. After various time periods (15-20 min) the mice were anesthetized with diethyl ether and blood was collected from the jugular vein and kept on ice. Immediately thereafter the liver, lungs and small intestine were removed, weighed and placed in ice-cold phosphate-buffered saline (5 ml for liver and small intestine and 2 ml for lungs). After homogenization (Polytron, Brinkman Instruments) a 0.1 ml aliquot of each homogenate was removed and digested at 50°C with 1 ml of NCS (Amersham). Aliquots (50 µl) of whole blood were

Table 11. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER P.O. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Time After Administration	Dose (mg/kg)		
	1	10	100
1	34.5 ± 11.9[a]	29.7 ± 8.2	31.0 ± 5.2
2	43.1 ± 8.3	27.6 ± 7.5	36.4 ± 3.8
3	53.2 ± 9.9	54.3 ± 10.0	46.6 ± 12.0
4	47.7 ± 4.6	52.4 ± 2.3	48.1 ± 10.8
8	n.d.[b]	n.d.	77.3 ± 5.6
16	n.d.	n.d.	60.3 ± 6.6
24	n.d.	n.d.	58.2 ± 14.2

[a] Mean ± SD (N=4) percentage of injected

[b] n.d. = not determined

Table 12. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVE MATERIAL 8 HOURS AFTER P.O. ADMINISTRATION OF DIFFERENT DOSES OF 2,4-DINITROTOLUENE TO A/J MICE

Tissue/Fluid	Mean Percentage (± S.D.) of Injected Dose[a]	
	1 mg/kg	10 mg/kg
Blood	0.7 ± 0.1	0.6 ± 0.1
Urine	52.5 ± 5.3	55.1 ± 5.1
Liver	0.8 ± 0.0	0.6 ± 0.0
Lungs	0.1 ± 0.0	0.1 ± 0.1
Kidneys	0.3 ± 0.0	0.2 ± 0.1
Adipose	0.1 ± 0.1	0
Small Intestine	6.4 ± 3.9	4.0 ± 1.8
Large Intestine	4.3 ± 1.2	4.6 ± 0.5
Total	65.0 ± 7.3	65.3 ± 4.9

[a] N=4

digested with 0.6 ml of NCS. The tissue digests were neutralized with glacial acetic acid (30 µl/ml NCS) and the blood digests were decolorized at 50°C in the presence of 0.2 ml of a freshly prepared solution of benzoyl peroxide in toluene (0.2 g/ml). After cooling, all samples were counted in a total volume of 20 ml of OCS (Amersham) scintillation fluid.

Homogenates and blood samples were kept on ice until after completion of extraction. Aliquots (1 ml of tissue homogenate and 100-200 µl of blood made up to 1 ml with phosphate-buffered saline) were removed and small amounts of [^{14}C]2,4-DNT (5000 cpm/sample) and carrier 2,4-DNT (200 µg/sample) were added to each sample to serve as recovery markers. The samples were then extracted with diethyl ether (2 x 3 ml/sample), the ether extracts were dried at 37°C under a stream of nitrogen and the residue was chromatographed (Eastman Kodak Silica Gel Chromatogram Sheets, 20 x 20 cm, 100 µm thickness, with Fluorescent Indicator) using n-hexane: ethyl acetate (4:1) as the solvent system. Under these conditions 2,4-DNT migrated with an R_f of 0.8 and preliminary experiments indicated that [^{3}H]2,4-DNT was well separated from its more polar radioactive metabolites, all of which had lower R_f-values. The advantage of the Eastman thin-layer sheets is that they can be eluted like paper so that scraping the silica gel off the plate is avoided. Areas of 2,4-DNT were visualized under U.V. light, marked off with a pencil and were then cut out and eluted with 2 ml of methanol. The eluates were counted with 19 ml of OCS using standard double label counting techniques. Recoveries of [^{14}C]2,4-DNT usually varied between 60 and 80% and these values were used to correct the total amount of ^{3}H in the sample for losses incurred during the extraction and chromatographic procedures. The corrected dpm ^{3}H was then expressed as a percentage of the total dpm ^{3}H present in the extracted samples.

The results of these experiments are shown in Table 13. At a dose of 1 mg/kg very little (<8%) 2,4-DNT could be reisolated from the liver at all time points (Table 13). The pattern of metabolism of 2,4-DNT in the small intestine was similar at all doses; i.e. rapidly increasing rates of metabolism with rates at 60 min similar to those observed for liver. The higher amounts that could be reisolated from the small intestine at the earlier time points probably reflect to a large extent the uptake of the injected material. Thus it may be concluded that both the liver and small intestine of the strain A mouse are major sites of metabolism of 2,4-DNT and that 60 min after single doses of 1-10 mg/kg, metabolism of this compound is virtually complete in these organs. At 100 mg/kg, 88-89% of the dose is metabolized by these organs in 60 min (Table 13).

At all doses and time points much higher amounts of 2,4-DNT could be reisolated from the blood and lungs than from the liver and small intestine

Table 13. ELIMINATION OF 2,4-DNT AND ITS METABOLITES AFTER I.P. ADMINISTRATION

Tissue	[^{3}H]2,4-DNT (percentage of total [^{3}H] per gram tissue or ml blood) 15 min	30 min	45 min	60 min	120 min
			1 mg/kg		
Blood	37.8 ± 11.2[a]	23.7 ± 7.9	27.8 ± 4.4	27.3 ± 3.1	n.d.[b]
Liver	7.8 ± 4.0	2.1 ± 1.1	1.7 ± 0.7	3.5 ± 2.9	n.d.
Lungs	38.5 ± 6.6	43.8 ± 14.5	47.4 ± 14.0	41.0 ± 17.2	n.d.
Small Intestine	34.3 ± 11.5	10.8 ± 3.3	7.9 ± 1.7	3.5 ± 1.9	n.d.
			10 mg/kg		
Blood	57.7 ± 10.1	38.0 ± 2.7	36.5 ± 7.7	7.1 ± 2.9	n.d.
Liver	2.7 ± 2.4	5.4 ± 2.3	7.1 ± 3.8	3.6 ± 2.2	n.d.
Lungs	41.4 ± 4.8	35.7 ± 9.9	30.7 ± 3.8	14.2 ± 10.5	n.d.
Small Intestine	44.3 ± 10.0	21.1 ± 2.7	7.0 ± 1.0	3.2 ± 1.3	n.d.
			100 mg/kg		
Blood	38.3 ± 8.7	79.3 ± 13.3	45.0 ± 11.2	50.0 ± 13.5	44.1 ± 6.7
Liver	13.9 ± 3.2	31.2 ± 14.1	19.1 ± 6.3	12.1 ± 2.6	13.0 ± 8.6
Lungs	38.6 ± 4.0	90.4 ± 2.1	27.1 ± 1.1	44.9 ± 4.8	37.3 ± 6.1
Small Intestine	29.9 ± 6.1	41.3 ± 18.2	19.6 ± 7.9	11.1 ± 3.9	4.4 ± 1.8

[a] Mean ± SD (N=4)

[b] n.d. = not determined

(Table 13). With the exception of the value obtained for 10 mg/kg at 60 min (7.1 ± 2.8%, Table 13), the rate of metabolism of 2,4-DNT in blood was relatively constant over the time period studied, probably being more a reflection of the rate of uptake from the peritoneal cavity than a measure of the rate of metabolism in extravascular tissues. Except for a difference seen at 1 mg/kg, the extent of 2,4-DNT metabolism observed in the lungs paralleled that seen in blood (Table 13). Since the lungs always contain some blood, a likely explanation of this observation is that the lungs, in contrast to the liver and the small intestine, are not actively metabolizing 2,4-DNT. This explanation would be consistent with the observed absence of lung tumorigenicity (Table 2), assuming that metabolism of 2,4-DNT is a necessary prerequisite for the expression of its carcinogenicity. It is possible, however, that carcinogenic intermediates were formed in extrapulmonary tissues, but that their concentration reaching the lungs was too small or that these intermediates were inactivated by further extrapulmonary metabolism before reaching the lungs.

A limited number of similar experiments were performed with p.o. administration of a dose of 100 mg/kg. The results of these experiments are shown in Table 14. Previous results showed that the absorption and elimination of 2,4-DNT was much slower after p.o. than after i.p. administration (Table 10). As expected, the small amounts absorbed at the early time points, were fully metabolized, since no unchanged 2,4-DNT could be reisolated from blood up to 1 hour, and from liver and lungs up to 30 min (Table 14). At later time points, when more 2,4-DNT was absorbed, unchanged 2,4-DNT appeared in the blood (at 2 hours) and in the liver and lungs (at 0.75 hours). In the small intestine large amounts of unchanged 2,4-DNT could be reisolated, but these amounts decreased from 85.4% at 0.25 hours to 21.6% at 4 hours. These results are different from similar studies with a similar dose (100 mg/kg) of i.p.-administered 2,4-DNT (Table 13) in that, with the exception of the small intestine, much larger amounts of unchanged 2,4-DNT could be reisolated from the tissues after i.p. - than after p.o. administration. The most likely explanation is that a large dose of i.p.-administered 2,4-DNT is rapidly absorbed, but metabolizing enzymes become saturated, leaving relatively large amounts of unchanged 2,4-DNT in the tissues, while p.o.-administered 2,4-DNT is absorbed slowly so that almost complete metabolism can take place. In contrast, in the small intestine, the rate of 2,4-DNT metabolism is much faster after i.p. than after p.o. administration.

e. <u>The effect of 2,4-DNT pretreatment and of 2,6-DNT treatment on the distribution, elimination and metabolism of 2,4-DNT</u>

Because human exposure to DNT is likely to be of a prolonged nature, it is possible that such exposure alters the pharmacokinetics of a single dose. Therefore, the effect of pretreatment of mice with 2,4-DNT on the distribution, elimination and metabolism of 2,4-DNT was examined. Mice were pretreated for 3 weeks with 3 equal i.p. injections (125 mg/kg/injection) per week for a total dose of 1125 mg/kg. The distribution and elimination of [^{3}H]2,4-DNT was then examined for 2-6 hrs after i.p. injection. In addition, unmetabolized [^{3}H]2,4-DNT was reisolated from blood, liver, lungs and small intestine, using the methodology described before (Section A.2.d.). The results of these experiments are shown in Table 15. Compared to the results obtained previously with a similar, but single dose of 2,4-DNT (Table 8), the distribution of

Table 14. ELIMINATION OF 2,4-DNT AND ITS METABOLITES AFTER P.O. ADMINISTRATION OF 100 mg/kg TO A/J MICE

Time After Administration (hrs)	[^{3}H]2,4-DNT (percentage of total [^{3}H] per gram tissue or ml blood)			
	Blood	Liver	Lungs	Small Intestine
0.25	0	0	0	85.4 ± 12.1[a]
0.50	0	0	0	60.5 ± 8.4
0.75	0	11.9 ± 15.4	18.8 ± 21.1	51.6 ± 27.6
1.00	0	5.6 ± 7.9	7.9 ± 7.4	68.6 ± 18.5
2.00	7.0 ± 1.5	5.9 ± 5.8	-[b]	44.2 ± 3.1
3.00	10.7 ± 4.1	6.9 ± 2.8	7.0 ± 9.7	33.0 ± 14.0
4.00	10.8 ± 2.5	-	-	21.6 ± 24.3

[a] Mean ± S.D. (N=4)

[b] Data incomplete

radioactivity in the tissues is not significantly different (Table 15), with the exception of somewhat higher amounts of radioactive material in the large intestine. At 4 and 6 hrs, amounts in the urine are lower than at 2 hrs (Table 15). This is probably due, in part, to losses of urine, as these animals were not placed in metabolic cages for these experiments. As judged by the amount of radioactive material in the tissues (Table 15), it appears that a 3-week pretreatment period does not result in a decrease in the rate of elimination of i.p.-injected [^{3}H]2,4-DNT. Whether the rate of elimination is actually increased is not known, since time periods earlier than 2 hrs were not studied. The metabolism of 2,4-DNT appears to be virtually complete within 2 hrs, as only small amounts of [^{3}H]2,4-DNT could be reisolated from blood, liver, lungs and small intestine at all time periods (Table 15). Thus, it may be concluded that the metabolism of 2,4-DNT after pretreatment is at least as rapid as that observed before (Table 13) without pretreatment.

Because technical grade DNT consists principally of a mixture of 2,4-DNT and 2,6-DNT, it is possible that these components influence each other's pharmacokinetic parameters. For this reason the effect of a relatively large dose of 2,6-DNT on the distribution and elimination of 2,4-DNT was examined. The [^{3}H]2,4-DNT was a tracer amount only, injected i.p. together with the 2,6-DNT. The distribution and elimination of total radioactivity was followed for 4 hrs and the results are shown in Table 16. With the exception of differences in the small intestine, these results are similar to those obtained before with a dose of 100 mg/kg of 2,4-DNT. In the small intestine the rates of uptake at 1-4 hrs are somewhat higher than those observed for 2,4-DNT (Table 8), implying that, at this dose, 2,6-DNT facilitates the absorption of 2,4-DNT. However, the differences are too small to be reflected in higher levels of radioactivity in other tissues or fluids. It may be concluded that, at a dose of 125 mg/kg, 2,6-DNT does not significantly influence the overall pattern of distribution and elimination of 2,4-DNT.

A study similar in design to the one just described, was performed in order to determine the effect of 2,6-DNT on the rate of metabolism of 2,4-DNT. 2,4-DNT was reisolated from blood, liver, lungs and small intestine using the methods described in Section A.2.d. The results of these experiments are shown in Table 17. As was seen before with 100 mg/kg of 2,4-DNT (Table 13), metabolism of 2,4-DNT became more complete at the later time points and the liver and small intestine were more active in this respect than the blood and the lungs. Rates of metabolism in the latter 2 tissues were similar. The major difference between the present data (Table 17) and those obtained with a dose of 100 mg/kg of 2,4-DNT (Table 13) is the more rapid overall rates of metabolism in the present study. This is especially striking in the blood and lungs. The reason for this is not clear, but it appears possible that 2,6-DNT accelerates the clearance of 2,4-DNT metabolites from the tissues.

3. In vivo metabolism of 2,4-DNT

Mice were injected i.p. with 1, 10 or 100 mg/kg of 2,4-DNT along with a tracer amount (2-3 μCi) of [^{3}H]2,4-DNT. The animals were sacrificed after 15, 30, 45, 60 and 120 min, and their bladder (including contents) were removed. For each dose and time point 4 mice were used and their urines were combined and stored at -20°C until workup. For workup, the tissue was removed and the total radioactivity in the urine was determined. The volume was then

Table 15. THE EFFECT OF PRETREATMENT WITH 2,4-DINITROTOLUENE ON THE DISTRIBUTION AND ELIMINATION OF I.P. ADMINISTERED [^{3}H]2,4-DINITROTOLUENE[a]

Tissue/Fluid	Time after administration [^{3}H]2,4-DNT (hrs) 2	4	6
	Percentage of injected (mean ± S.D., n=4)		
Blood	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.1
Urine	51.7 ± 16.4	38.3 ± 8.8	25.7 ± 10.5
Liver	1.4 ± 0.3	0.9 ± 0.1	0.7 ± 0.3
Lungs	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Kidneys	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Adipose	0.3 ± 0.1	0.1 ± 0.1	0.0
Small Intestine	7.5 ± 0.9	2.5 ± 0.1	2.3 ± 1.1
Large Intestine	6.0 ± 1.9	8.1 ± 1.4	7.6 ± 1.5
Total	68.1 ± 15.4	51.0 ± 10.3	37.5 ± 11.9
	Percentage [^{3}H]2,4-DNT recovered (mean ± S.D., n=4)[b]		
Blood	1.5 ± 2.4	3.3 ± 4.9	0.5 ± 0.8
Liver	0.0	3.9 ± 7.9	0.1 ± 0.3
Lungs	4.3 ± 0.3	0.4 ± 0.5	4.4 ± 4.8
Small Intestine	0.8 ± 0.3	0.4 ± 0.4	5.1 ± 8.9

[a] Mice were pretreated for 3 weeks with 3 equal i.p. injections/week of 2,4-dinitrotoluene (125 mg/kg/injection).

[b] 2,4-DNT was reisolated and the amount was expressed as a percentage of the total amount of radioactivity in the tissue or fluid.

Table 16. EFFECT OF ADMINISTRATION OF 2,6-DINITROTOLUENE (125 mg/kg) ON THE DISTRIBUTION AND ELIMINATION OF [^{3}H]2,4-DNT

Time After Administration (min)	Mean % (± SD) of Injected [^{3}H]2,4-DNT (N=4)				
	Blood	Urine	Liver	Lungs	Kidneys
15	1.1 ± 0.2	4.7 ± 0.3	4.8 ± 2.3	0.2 ± 0.0	1.3 ± 0.2
30	1.6 ± 0.4	16.2 ± 2.0	5.3 ± 1.2	0.3 ± 0.1	2.0 ± 0.2
45	1.4 ± 0.2	30.9 ± 3.7	4.0 ± 1.1	0.2 ± 0.0	1.6 ± 0.1
60	1.4 ± 0.2	34.1 ± 3.1	3.5 ± 0.6	0.2 ± 0.0	1.4 ± 0.2
120	1.0 ± 0.1	62.4 ± 5.7	3.6 ± 1.6	0.1 ± 0.0	0.6 ± 0.1
180	0.6 ± 0.1	67.5 ± 1.8	4.1 ± 2.9	0.1 ± 0.0	0.3 ± 0.0
240	0.7 ± 0.2	37.2 ± 19.1	1.7 ± 0.8	0.3 ± 0.0	0.3 ± 0.0

	Adipose	Small Intestine	Large Intestine	Total[a]
15	1.1 ± 0.3	4.7 ± 0.3	1.1 ± 0.2	19.6 ± 4.7
30	2.4 ± 1.4	10.0 ± 1.1	1.6 ± 0.2	39.3 ± 2.8
45	1.5 ± 0.9	10.8 ± 2.8	0.8 ± 0.2	51.2 ± 6.7
60	1.0 ± 0.2	11.8 ± 1.7	0.8 ± 0.1	54.1 ± 4.7
120	0.6 ± 0.2	14.8 ± 2.5	0.6 ± 0.4	83.7 ± 6.2
180	0.1 ± 0.0	12.0 ± 3.2	1.1 ± 0.7	85.8 ± 7.0
240	0.1 ± 0.1	5.1 ± 1.6	5.6 ± 1.8	50.7 ± 18.1

[a] Total of all fluids and tissues.

Table 17. EFFECT OF 2,6-DNT (125 mg/kg) ON THE ELIMINATION OF [^{3}H]2,4-DNT AND ITS METABOLITES

Time After Administration (min)	[^{3}H]2,4-DNT (% of total per g tissue or ml blood)			
	Blood	Liver	Lungs	Small Intestine
15	36.0 ± 1.9[a]	23.5 ± 5.1	30.0 ± 2.3	25.4 ± 1.1
30	16.7 ± 6.4	11.4 ± 0.5	12.5 ± 4.4	14.2 ± 6.7
45	15.1 ± 0.5	8.7 ± 1.8	15.8 ± 5.8	4.0 ± 1.0
60	12.5 ± 3.4	4.6 ± 2.0	9.5 ± 2.2	3.0 ± 1.9

[a] Mean ± SD (N=4)

adjusted to 1 ml with distilled water and each sample was extracted twice with 3 ml of ethyl acetate:acetone (3:1). The organic phases were combined and dried at 40°C under a stream of nitrogen. The resulting residue constituted the organic solvent-extractable (unconjugated or free) fraction. The aqueous phase was adjusted to pH 6.8 by addition of 0.1 ml of 0.75 M phosphate buffer, pH 6.8, after which β-glucuronidase (400 units/ml, Type VII, Sigma) was added. The samples were covered, incubated at 37°C for 16 hours, and then extracted as before. This was termed the glucuronide (glucs) fraction. Both the free and glucuronide fraction were reconstituted in 0.4 - 0.6 ml of water: methanol (1:1), containing a mixture of authentic standards (10 µg/ml of each), and filtered through 0.22 µm Swinnex filter (Millipore). An aliquot (5-10 µl) was removed for the determination of total radioactivity (Table 18) and another aliquot (50-100 µl) was used for analysis by high-pressure liquid chromatography (HPLC). The system used for chromatography was modified from that described by Rickert and Long.[15] The standards used, their retention times and the system used are summarized in Table 19.

Amounts of unconjugated (free) metabolites ranged from 2.8 to 9.2% of the total present in the urine, while the glucuronide fraction constituted 2.4 - 7.5% of the total (Table 18). Thus, most of the metabolites were not extractable with organic solvents, even after β-glucuronidase hydrolysis, indicating their polar nature. Preliminary analysis of a few samples of spent urine by HPLC revealed that the large majority of the remaining radioactivity in the urine eluted early in the chromatogram, together with the 3 carboxylic acid standards (4Ac2NBAcid, 2,4-DNBAcid and 2A4NBAcid, Table 19). It is evident from the data presented in Table 20 that some of these acids partitioned in the organic phase. Together with the 3 standard acids, at least 2 additional peaks appeared in the chromatograms which were not separated from the standards under the conditions of elution. This group of compounds was therefore tentatively termed "Acids" (Table 20). The proportion of Acids increased with dose and time but was lower after 120 min than after 60 min (at 100 mg/kg). 2,4-DAT could be detected at the higher doses, but only at the later time points (Table 20). In almost all cases, 2,4-DNBAlc was the major metabolite of the free fraction and it constituted a larger proportion of the total at the higher doses. Substantial amounts of 4A2NT and smaller amounts of 2A4NT were detected. Unchanged 2,4-DNT was detected in appreciable amounts at 15 min, but much smaller amounts were found at the later time points (Table 20). Radioactive material associated with the remainder of the standards (Table 19) was negligible and the proportion unaccounted for eluted in areas of the chromatogram other than those of the standards. Total recovery in chromatographic runs ranged from 79 to 92%. It may be concluded that i.p.-injected 2,4-DNT undergoes mostly oxidative metabolism of the methyl moiety and some reductive metabolism of the nitro moieties of the molecule. A comparison with the urinary metabolites of 2,4-DNT in the rat[15] is difficult because the HPLC analysis was done on whole urine in the case of the rat.

The results of the study on the urinary 2,4-DNT metabolites in the glucuronide fraction after an i.p. dose of 100 mg/kg are shown in Table 21. Compared to the corresponding metabolites in the free fraction (Table 20) the following observations may be made. The proportion of the major metabolite, 2,4-DNBAlc, was approximately the same (60-74%) in both the free and glucuronide fraction. While the fraction termed "Acids" is not likely to be conjugated with glucuronic acid, similar to the situation seen in the free

Table 18. RADIOACTIVE MATERIAL IN THE UNCONJUGATED (FREE) AND GLUCURONIDE (GLUCS) FRACTIONS OF MOUSE URINE AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF [^{3}H]2,4-DINITROTOLUENE

Time After Administration (min)	Percentage of Total Radioactivity in Urine[a]					
	1 mg/kg		10 mg/kg		100 mg/kg	
	Free	Glucs	Free	Glucs	Free	Glucs
15	3.9	4.1	5.1	6.5	7.5	3.2
30	2.8	3.4	9.2	2.4	8.8	2.4
45	4.2	4.8	9.0	3.0	3.5	6.9
60	4.0	3.4	5.0	3.8	5.7	7.5
120					3.6	5.0

[a] Each data point is derived from the pooled urines of 4 mice.

Table 19. HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF 2,4-DINITROTOLUENE METABOLITES[a]

Metabolite	Abbreviation	Retention Time (min)
4-(N-acetyl)amino-2-nitrobenzoic acid	4Ac2NBAcid	2.64
2,4-Dinitrobenzoic acid	2,4-DNBAcid	4.95
2-Amino-4-nitrobenzoic acid	2A4NBAcid	6.17
2,4-Diaminotoluene	2,4-DAT	9.74
2-Amino-4-nitrobenzyl alcohol	2A4NBAlc	14.67
2-(N-acetyl)amino-4-nitrotoluene	2Ac4NT	17.57
2,4-Dinitrobenzyl alcohol	2,4-DNBAlc	18.87
4-Amino-2-nitrotoluene	4A2NT	19.6
2-Amino-4-nitrotoluene	2A4NT	20.2
4-(N-Acetyl)amino-2-nitrotoluene	4Ac2NT	21.1
2,4-Dinitrotoluene	2,4-DNT	23.3

[a] The chromatographic system consisted of a Beckman Model 334 Liquid chromatograph with a Model 160 U.V.-detector and a Model C-RIA integrator. Fractions were collected using an ISCO Model 328 fraction collector which was interfaced with the Model 160 detector. The column used was a 4.6 x 25 mm Ultrasphere ODS (Altex) which was eluted using 2 consecutive linear gradients, the first from 15 to 73% methanol in 0.005 M phosphate buffer over 17 min, followed by the second from 73 to 100% methanol over the next 13 min.

Table 20. DISTRIBUTION OF ORGANIC SOLVENT-EXTRACTABLE URINARY METABOLITES AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF [^{3}H]2,4-DINITROTOLUENE TO A/J MICE

Time after Administration (min)	Dose (mg/kg)	Percentage of Extractable Radioactivity					
		Acids	2,4-DAT	2,4-DNBAlc	4A2NT	2A4NT	2,4-DNT
15	1	-	-	12.7	8.8	1.6	2.4
	10	-	-	53.6	45.9	5.4	4.0
	100	2.8	-	61.3	0.4	-	9.5
30	1	-	-	18.9	12.7	3.3	1.0
	10	2.2	-	46.8	42.9	7.0	1.5
	100	1.1	-	63.5	0.4	0.1	0.4
45	1	3.6	-	21.9	19.0	2.5	0.4
	10	2.8	-	31.6	36.7	4.1	0.3
	100	15.3	1.5	41.3	1.4	0.8	2.0
60	1	3.5	-	31.4	40.9	4.3	1.0
	10	4.3	0.8	47.1	15.3	7.3	0.3
	100	16.5	1.8	70.4	1.9	1.8	2.2
120	100	3.8	14.4	43.8	0.9	0.4	0.1

[1] Acids, mixture of carboxylic acids (see text); 2,4-DAT, 2,4-diaminotoluene; 2,4-DNBAlc, 2,4-dinitrobenzylalcohol; 4A2NT, 4-amino-2-nitrotoluene; 2A4NT, 2-amino-4-nitrotoluene; 2,4-DNT, 2,4-dinitrotoluene.

Table 21. DISTRIBUTION OF URINARY METABOLITES IN THE GLUCURONIDE FRACTION AFTER I.P. ADMINISTRATION OF 100 mg/kg OF [^{3}H]2,4-DNT TO A/J MICE

Metabolite[a]	Percentage of total amount chromatographed				
	15 min[b]	30 min	45 min	60 min	120 min
"Acids"	19.7	19.9	11.3	12.6	17.6
2,4-DAT	22.6	-	0.7	1.3	2.0
2A4NBAlc	4.9	3.4	2.9	4.6	0.2
2Ac4NT	-	5.4	1.7	2.5	0.1
2,4-DNBAlc	47.2	60.3	67.6	61.5	74.7
4A2NT	1.1	0.7	1.6	1.2	1.0
2A4NT	-	0.6	0.6	0.5	0.2
4Ac2NT	-	-	0.1	0.3	0.2
2,4-DNT	-	-	0.2	0.2	0.1
Unknown	5.4	10.5	13.3	15.4	4.0

[a] "Acids", mixture of carboxylic acids; 2,4-DAT, 2,4-diaminotoluene; 2A4NBAlc, 2-amino-4-nitrobenzyl alcohol; 2Ac4NT, 2-(N-acetyl)amino-4-nitrotoluene; 2,4-DNBAlc, 2,4-dinitrobenzyl alcohol; 4A2NT, 4-amino-2-nitrotoluene; 2A4NT, 2-amino-4-nitrotoluene; 4Ac2NT, 4-(N-acetyl)amino-2-nitrotoluene; 2,4-DNT, 2,4-dinitrotoluene.

[b] Time after administration of 2,4-DNT.

fraction, a small portion of these acids partitioned into the organic phase, yielding 11-19% of the total amount chromatographed (Table 21). 2A4NBAlc, which could not be detected in the free fraction (Table 20), was present in detectable amounts (0.2 - 4.6%) in the glucuronide fraction. Similarly, small amounts of 2Ac4NT and 4Ac2NT could be found in the glucuronide fraction (Table 20) but not the free fraction (Table 20). Amounts of the other metabolites, including unknowns, were approximately the same in both fractions. It may be concluded that, with the exception of 2A4NBAlc, the urinary metabolites of 2,4-DNT in the free and glucuronide fractions are similar, both qualitatively, as well as in terms of the relative amounts.

The in vivo metabolism of 2,4-DNT was also studied after p.o. administration. Mice were given 100 mg/kg of 2,4-DNT by gavage, along with a tracer amount (2-3 μCi) of [^{3}H]2,4-DNT. The animals were sacrificed after 1-24 hours and their bladders (including contents) were removed. For each time period 4 mice were used and their urines were combined and stored at -20°C until workup. The workup procedure was essentially as described above after i.p. administration.

The results of these studies are presented in Tables 22 and 23. Amounts of unconjugated (free) metabolites ranged from 5.5 to 10.2% of the total present in the urine, while the glucuronide fraction constituted 21-34.4% of the total (Table 22). Thus, the majority of the metabolites were not extractable with organic solvents, even after β-glucuronidase hydrolysis. Solvolysis of the spent urine yielded negligible organic solvent-extractable radioactivity, indicating the absence of sulfate conjugates. The data in Table 22 differ from similar data obtained after i.p. administration (Table 18) in that the amounts of metabolites in both the free and glucuronide fractions are 2-3 times higher after p.o. than after i.p. administration. As was found before after i.p. administration (see above), HPLC analysis of the spent urine showed that the radioactivity remaining in the urine eluted early, together with the 3 carboxylic acid standards. Based on a few such analyses, it may be concluded that amounts of unextractable carboxylic acid metabolites are lower after p.o. than after i.p. administration.

Comparing metabolites in the free fraction, some major differences between p.o. and i.p. administration are seen. First, amounts of Acids, when expressed as a percentage of the total amount of radioactivity per fraction, are higher after p.o. (Table 22), than after i.p.-administration (Table 20). Second, whereas no 2A4NBAlc was found after i.p. administration of 2,4-DNT (Table 20), substantial amounts (6.1 - 22.5%) could be isolated from the free fraction after p.o. administration (Table 23). Third, while 2,4-DNBAlc was the major identifiable free metabolite after i.p. administration (41.3 - 70.4%, Table 20), this compound represented a relatively small proportion of this fraction (0.6 - 7.7% at 1-8 hours, Table 23) after p.o. administration. Fourth, while no acetylated neutral metabolites could be found after i.p. administration of 2,4-DNT (Table 20), 2Ac4NT was identified in both the free and the glucuronide fractions (Table 23) after p.o. administration (Table 23). Differences between the free and the glucuronide fractions also pertain to these metabolites: amounts of Acids were lower in the glucuronide fraction, amounts of 2A4NBAlc were very low in the glucuronide fraction, and amounts of 2,4-DNBAlc were much higher in the glucuronide than in the free fraction. As a result, unidentified radioactive material ("Other," Table 23) in the glucuronide fraction constituted a smaller proportion of the total than that in the free fraction.

Table 22. RADIOACTIVE MATERIAL IN THE UNCONJUGATED (FREE) AND GLUCURONIDE (GLUCS) FRACTIONS OF MOUSE URINE AFTER P.O. ADMINISTRATION OF 100/mg/kg OF [^{3}H]2,4-DINITROTOLUENE

	Percentage of Total Radioactivity in Urine[a]		
Time After Administration (hrs)	Free	Glucs	Free + Glucs
1	6.5	20.5	26.5
2	6.2	28.2	34.4
3	5.5	26.4	31.9
4	5.6	21.3	26.9
8	6.8	25.0	31.8
16	10.2	14.5	24.7
24	6.5	14.5	21.0

[a] Each data point is derived from the pooled urines of 4 mice.

Table 23. DISTRIBUTION OF URINARY METABOLITES IN THE FREE AND GLUCURONIDE FRACTIONS AFTER P.O. ADMINISTRATION OF [^{3}H]2,4-DINITROTOLUENE (100 mg/kg) TO A/J MICE

Time after Administration (hours)	Fraction	Percentage of Extractable Radioactivity								
		Acids	2,4-DAT	2A4NBAlc	2Ac4NT	2,4DNBAlc	4A2NT	2A4NT	2,4-DNT	Others
1	Free	47.6	1.1	7.3	0.6	2.6	0.7	0.1	--	40.0
	Glucs	49.2	0.5	1.0	0.9	26.6	1.2	0.5	--	20.1
2	Free	41.2	1.3	18.1	0.9	2.1	1.1	0.3	0.2	34.7
	Glucs	22.5	0.4	0.4	0.6	57.2	0.9	0.4	--	17.6
3	Free	32.4	1.7	18.6	0.4	0.6	0.7	0.5	0.3	44.9
	Glucs	14.9	0.4	0.9	3.1	60.4	1.1	1.5	--	17.7
4	Free	26.4	1.7	22.5	0.4	4.1	0.8	0.6	0.1	43.4
	Glucs	18.7	0.6	0.8	3.7	54.0	0.9	0.6	--	20.7
8	Free	39.4	0.8	22.5	3.0	7.7	0.5	0.6	--	25.5
	Glucs	22.5	0.4	0.5	4.5	56.3	0.8	0.5	--	14.5
16	Free	37.2	0.6	6.1	1.3	19.4	0.6	0.4	0.4	34.1
	Glucs	24.2	0.5	0.7	3.6	49.7	0.5	0.4	--	20.5
24	Free	34.4	1.1	14.1	3.0	21.6	0.8	0.5	0.7	24.7
	Glucs	23.0	0.5	0.7	1.0	46.6	0.9	0.7	--	26.6

Amounts of the other metabolites (2,4-DAT, 2Ac4NT, 4A2NT, 2A4NT) were minute in both fractions.

It may be concluded that p.o.-administered 2,4-DNT, when compared to i.p.-administered 2,4-DNT, undergoes less complete reductive metabolism to unconjugated metabolites and is, in part, acetylated (presence of 2A4NBAlc and 2Ac4NT) and undergoes more complete oxidative metabolism to glucuronides (high amounts of 2,4-DNBAlc, Table 23).

4. In vitro metabolism of 2,4-DNT

In vitro metabolism studies were performed with liver- and lung microsomes, intestinal explants and cecal contents. Liver and lung microsomes were prepared from 8-10 week-old male A/J mice. Animals were killed by cervical dislocation. All subsequent steps were carried out at 0-4°C. Livers and lungs were removed, washed in phosphate-buffered saline (PBS), minced, and, after several additional washes with PBS, homogenized in 3-4 volumes of PBS, using a Polytron homogenizer. After centrifugation for 20 min at 9000 x g, the supernatant (S-9 fraction) was further centrifuged at 105,000 x g for 60 min to obtain the microsomal pellet. Microsomes were resuspended in a small volume of PBS containing 30% (v/v) glycerol and stored at -80°C until use. Microsomal proteins were determined by the Lowry procedure.

[^{3}H]2,4-DNT (0.5 µCi) and 2,4-DNT (100 or 200 µM final concentration) were incubated with liver microsomes (0.8-1 mg microsomal protein/dish) in 60 mm tissue culture dishes in 2.5 ml of 0.1 M phosphate buffer, pH 7.4, containing 70 µM $MgCl_2$ and 0.56 µM NADPH. Dishes were placed in air-tight chambers which were gassed (to 3 psi) with either oxygen, air, or nitrogen, and rocked at 5 cycles/min, so that, in the case of explants, the tissues were alternately exposed to the medium and the atmosphere. For incubation with tissues, the small and large intestine were removed, opened longitudinally and washed free of contents. Explants (0.5 mm^2 each) were placed in the culture dishes (2 explants/dish, opposite each other) with the epithelium oriented towards the gaseous phase. For incubations with cecal contents, the combined contents of ceca from 4 mice was suspended in the incubation medium and distributed equally among 4 dishes. All incubations were performed at 37°C.

At the end of the incubation period the medium was extracted twice with ethyl acetate:acetone (2:1). The combined extracts were evaporated at 40°C under a stream of nitrogen and the residue was dissolved in methanol:water (1:1) containing a mixture of authentic 2,4-DNT metabolites. The metabolites were then separated and quantitated by high-pressure liquid chromatography (HPLC) as described above in Section A.3. and in Table 19.

Incubation of 100 µM 2,4-DNT with liver microsomes in oxygen or air resulted in metabolism of 41-43% of the substrate, with 2,4-dinitrobenzylacohol (2,4-DNBAlc) being the major metabolite (Table 24). The only other metabolite that could be identified under these conditions was 4-amino-2-nitrotoluene (4A2NT) in amounts equal to 3.3 - 3.4% of the total metabolites. As before (Tables 20 and 23), "Acids" were incompletely separated and represented <4% of the total. Doubling of the substrate concentration (to 200 µM) resulted in an approximate 50% decrease in the extent of metabolism (16 - 22.1% of the substrate metabolized, Table 24), indicating that saturating conditions were

Table 24. METABOLISM OF 2,4-DNT BY LIVER MICROSOMES FROM A/J MICE

Expt. No.	Incubation atmosphere	Substrate conc'n (μM)	Incubation Time (min)	Amount of metabolite (dpm x 10^{-2}/mg microsomal protein)[a]					Unknown Metabolites(%)	Total % DNT metabolized
				Acids	2,4-DNBAlc	4A2NT	2A4NT	4Ac2NT		
1	oxygen	100	60	11 (0.8)	151 (28.6)	19.5 (3.3)	--	--	10.5	43.2
2	air	100	60	21.4 (3.9)	186 (34.1)	19.2 (3.4)	--	--	0.2	41.6
3	air	200	30	--	46.4 (12.7)	3.4 (1.0)	1.8 (0.5)	--	1.8	16.0
4	air	200	60	--	58.0 (19.7)	2.7 (0.9)	2.3 (0.8)	0.7 (0.2)	0.5	22.1
5	nitrogen	100	60	--	--	--	--	--	2.1	2.1
6	nitrogen	200	30	--	8.6 (1.1)	2.3 (0.3)	1.5 (0.2)	--	3.4	5.0
Control experiments										
7	boiled microsomes									
	a) oxygen	100	60	8.3 (2.8)	--	--	--	--	1.2	4.0
	b) air	100	60	--	5 (1.7)	--	--	--	5.9	7.6
	c) nitrogen	200	30	--	--	--	--	--	2.1	2.1
8	no microsomes air	200	60	--	0.9 (0.7)	0.7 (0.5)	--	--	1.0	2.2
9	no NADPH air	200	30	--	2.7 (2.0)	0.9 (0.7)	--	-	--	2.7

[a] Number in parentheses represents percentage of total 2,4-DNT metabolites. All numbers represent the average of duplicate experiments.

approached at 100 μM. In addition, very small amounts of two other metabolites (2-amino-4-nitrotoluene, 2A4NT, and 4-(N-acetyl)amino-2-nitrotoluene, 4Ac2NT) were detected under these conditions. In an atmosphere of nitrogen, virtually no metabolism took place (Table 24). Also, boiling the microsomes, or omitting microsomes or NADPH from the incubation mixture resulted in a very small extent of metabolism (Table 24). It may be concluded that the oxidative metabolism of 2,4-DNT (to 2,4-DNBAlc) is dependent on oxygen and on NADPH and is, therefore, most likely mediated by cytochrome P-450.

Additional experiments were performed to study the in vitro metabolism of [^{3}H]-2,4-DNT in liver and lung microsomes under identical conditions. The results (Table 25) indicate that unlike liver microsomes, lung microsomes were poor in metabolizing 2,4-DNT. In the presence of air, the major metabolite was 2,4-dinitrobenzyl alcohol which represented 2.6% of the total metabolites. The reductive metabolites constituted only 0.5 to 0.6% of the total metabolites. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is a known inducer of microsomal monoxygenases in strain A mice. Since microsomal metabolism of 2,4-DNT is principally oxidative and possibly requires monoxygenases, we studied the effect of a known inducer of monoxygenases, such as TCDD, on liver and lung microsomal metabolism of 2,4-DNT. TCDD treatment of strain A mice (i.p., 0.4 mg/kg for 24 hours) resulted in a 94% increase in hepatic microsomal metabolism of 2,4-DNT. The increase in the total metabolism of 2,4-DNT was accompanied by an approximately two fold increase in the amount of 2,4-dinitrobenzyl alcohol and amino metabolites including 4-amino 2-nitrotoluene and 2-amino-4-nitrotoluene. This indicates that oxidative metabolism of 2,4-DNT is possibly mediated by microsomal monoxygenases and is inducible. Unlike liver microsomes, lung microsomal metabolism was not inducible by TCDD.

Incubation of intestinal explants (Table 26) with 2,4-DNT in the presence of oxygen or air resulted in the formation of 2,4-DNBAlc, 4A2NT, 2A4NT, and 4Ac2NT, all in approximately equal amounts. An atmosphere of nitrogen did not seem to have a different effect, as only 2,4-DNBAlc became nondetectable. The small- and large intestine formed approximately the same amounts of these metabolites (Table 26). Cecal contents formed much larger amounts of 4A2NT and 2A4NT in the presence of nitrogen than in the presence of oxygen or air. These data indicate that both intestinal tissue and cecal contents are capable of metabolizing 2,4-DNT.

B. 2,6-DINITROTOLUENE

1. Purity of 2,6-Dinitrotoluene and [3-^{3}H]2,6-Dinitrotoluene

The purity of 2,6-dinitrotoluene (2,6-DNT) was verified by HPLC using the Waters system described before (Section A.1.) A 10 μl aliquot of a solution of 2,6-DNT in methanol (1000 ppm) was chromatographed on a C18-μBondapak (Waters) column. The column was eluted isocratically at 2100 psi using water:methanol (1:1) as the solvent. Two U.V.-absorbing peaks were eluted, a major peak with a retention time of 11.54 min and a minor peak with a retention time of 13.70 mins. The minor peak comprised 3% while the major peak comprised 97% of the material injected, as concluded from integration of the peak areas. It was therefore concluded that 2,6-DNT was at least 97% pure.

Table 25. METABOLISM OF 2,4-DNT BY LIVER AND LUNG MICROSOMES FROM A/J MICE: EFFECT OF TCDD TREATMENT

Incubation Conditions	Incubation Time (mins)	Acids	Amount of the Metabolite[a] (dpm X 10^{-3}/mg microsomal protein) 2,4-DNBAlc	4A2NT	2A4NT	4Ac2NT	% Unknown	% DNT Metabolized
Control Liver Microsomes + NADPH + ^{3}H-DNT	30	-	77.9 (11.9)	12.4 (1.9)	4.8 (0.71)	-	2.5	17
Control Lung Microsomes + NADPH + ^{3}H-DNT	30	-	49.3 (2.6)	9.9 (0.5)	5.5 (0.29)	-	4.3	8
TCDD Liver Microsomes + NADPH + ^{3}H-DNT	30	-	152.7 (22.8)	27.3 (4.0)	8.0 (1.2)	-	3.5	33
TCDD Lung Microsomes +NADPH + ^{3}H-DNT	30	-	61 (2.0)	19.6 (0.6)	15.4 (0.24)		4.8	8.3
Boiled Liver Microsomes + NADPH + ^{3}H-DNT	30	-	14.8 (0.74)	9.1 (0.4)	-	-	2.9	4
Boiled Lung Microsomes + NADPH + ^{3}H-DNT	30		4.28 (0.27)	-	-	-	2.4	3

Data in parenthesis show the percentage of total metabolites. Numbers represent the average of duplicate determinations with 20-30% variation.

Table 26. METABOLISM OF 2,4-DNT BY INTESTINAL EXPLANTS AND CECAL CONTENTS OF A/J MICE

Tissue/ Material	Incubation Atmosphere	Amount of metabolite (dpm X 10^{-2})[a]								Unknown Metabolites (%)	Total % DNT Metabolized
		Acids	2,4-DNBAlc		4A2NT		2A4NT		4AC2NT		
Small intestine	oxygen	--	27	(1.6)	21.0	(1.2)	18.0	(1.1)	12.0 (0.7)	3.4	8.0
	air	--	25	(4.2)	16.0	(2.7)	17.0	(2.8)	9.0 (1.9)	4.4	16.0
	nitrogen	--	--		24.0	(1.5)	20.0	1.3	12.0 (0.8)	1.1	4.7
Large intestine	oxygen	--	13	(0.9)	16.0	(1.1)	21.0	(1.4)	15.0 (1.0)	4.6	9.0
	air	--	--		5.4	(1.8)	5.9	(2.0)	8.8 (3.0)	2.1	10.0
	nitrogen	--	--		14.0	(2.9)	6.0	(1.3)	6.0 (1.2)	1.6	7.0
Cecal contents	oxygen	--	--		35.0	(7.0)	35.0	(7.0)	7.8 (1.0)	9.0	24.0
	air	--	5.0	(0.4)	23.0	(2.0)	18.0	(1.5)	9.0 (0.7)	0.4	5.0
	nitrogen	--	17.5	(2.5)	172.0	(25.2)	166.0	(24.9)	24.0 (3.5)	4.9	61.0

[a] Numbers in parenthesis represent percentage of total 2,4-DNT metabolites. Values were obtained with single incubations of 100 μM 2,4-DNT.

For the verification of the radiochemical purity of [3-^{3}H]2,6-DNT, approximately 40,000 cpm of this material was cochromatographed with 2,6-DNT as described above. Determination of the radioactivity in 0.5 ml eluate fractions of the entire chromatogram revealed that 99.7% of the total amount of radioactive material injected cochromatographed with the major peak, representing 2,6-DNT. Therefore, the [3-^{3}H]2,6-DNT was greater than 99.7% radiochemically pure.

2. Tissue Distribution and Elimination Studies

a) Effect of different doses of i.p. administered 2,6-DNT

For this series of experiments, [3-^{3}H]2,6-DNT was used as tracer and the protocol was as described for similar studies with 2,4-DNT (Section A.2.a.). The results of these studies are shown in Tables 27 and 28. Similar to our previous findings with 2,4-DNT (Table 6), the levels of radioactivity in blood, liver, kidneys and lungs were relatively unaffected by the dose of 2,6-DNT administered. In the liver the levels of radioactivity were somewhat lower at 100 mg/kg than at the other doses (Table 27). As expected, uptake of radioactivity by the small intestine increased with time and was slowest at the highest dose. However, all values in this tissue, especially at 0-10 mg/kg, were much lower (Table 27) than those observed for 2,4-DNT (Table 6). On the other hand, excretion in the urine at these doses was not much slower (Table 27), especially not after 60 min, than that observed for 2,4-DNT (Table 6). A possible explanation may be that, although the initial uptake of 2,6-DNT is slower than that of 2,4-DNT, once absorbed, its rate of elimination is only slightly lower than, or equal to that of 2,4-DNT. Similar to the findings with 2,4-DNT, radioactivity in the lungs was very low and did not change appreciably with time or dose (Table 27). As expected, total radioactivity recovered from tissues and fluids was highest (70.5%) at the lowest dose and lowest (38.6%) at the highest dose (Table 28).

Because the urinary excretion of radioative material during a period of 1 hr after i.p. administration of 2,6-DNT was not complete (Table 28), a series of experiments was performed in order to determine the tissue distribution and rates of excretion of 2,6-DNT during time periods up to 4 hrs after i.p. injection. The results of these experiments are shown in Table 29. With the exception of the large intestine, all tissues, including blood, showed declining levels of radioactivity during the 4 hr period. In the large intestine the levels of radioactivity appeared to increase at 1 and 10 mg/kg, with no change at 100 mg/kg. This was also observed with 2,4-DNT, except that the initial levels (<2 hr) were somewhat lower and that the later levels (<2 hr) were somewhat higher with 2,4-DNT (Table 8). This may indicate that the fecal route of elimination may be more important for 2,4-DNT than for 2,6-DNT, even though in both cases the urine is by far the most important route of elimination. As before with 2,4-DNT, the rate of excretion of radioactivity into the urine was quite variable (Table 29). Again, this can be attributed largely to shortcomings in the method of urine collection. It is therefore difficult to make comparisons between 2,4-DNT and 2,6-DNT regarding their rates of excretion in the urine. Nevertheless, it appears that there are no large differences in the rates of absorption and excretion of 2,4-DNT (Tables 8 and 9) and those of 2,6-DNT (Tables 29 and 30). As before with 2,4-DNT, the rate

Table 27. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time after Administration (min)	Dose (mg/kg) 0[a]	1	10	100	Dose (mg/kg) 0[a]	1	10	100
	Blood				Urine			
15	1.9 ± 0.5[b]	1.6 ± 1.3	2.5 ± 0.6	1.6 ± 0.6	1.8 ± 1.6	2.2 ± 0.4	3.3 ± 0.6	2.3 ± 1.3
30	3.1 ± 1.1	2.1 ± 0.4	5.5 ± 3.4	2.3 ± 0.6	5.1 ± 4.7	12.4 ± 2.0	12.0 ± 6.0	5.8 ± 1.0
45	2.6 ± 0.1	2.3 ± 0.3	5.2 ± 3.2	2.1 ± 1.1	21.9 ± 6.0	28.1 ± 4.2	27.7 ± 8.9	9.1 ± 1.6
60	2.6 ± 0.3	2.4 ± 0.3	3.6 ± 0.7	3.1 ± 0.8	47.0 ± 9.1	36.0 ± 15.9	42.4 ± 14.5	16.8 ± 1.6
	Liver				Lungs			
15	5.0 ± 0.5	3.8 ± 0.8	4.3 ± 0.5	3.0 ± 0.6	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.5
30	5.5 ± 2.6	5.9 ± 0.6	4.5 ± 1.2	2.9 ± 1.7	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
45	6.0 ± 1.2	5.9 ± 0.9	6.0 ± 1.7	3.2 ± 1.4	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 1.1
60	4.6 ± 0.5	6.0 ± 2.1	5.7 ± 2.0	4.9 ± 1.5	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
	Kidneys				Adipose			
15	2.3 ± 0.6	1.7 ± 0.4	1.7 ± 0.3	2.2 ± 1.3	2.6 ± 0.9	3.7 ± 1.0	2.5 ± 1.0	2.7 ± 1.5
30	2.7 ± 1.1	3.5 ± 0.9	3.5 ± 0.9	2.1 ± 0.3	2.7 ± 1.5	4.5 ± 1.4	4.1 ± 0.9	3.6 ± 1.0
45	3.0 ± 1.2	3.3 ± 0.2	3.1 ± 0.7	2.2 ± 1.0	2.1 - 0.9	2.4 ± 1.7	2.4 ± 1.7	3.3 ± 2.9
60	2.9 ± 0.5	3.0 ± 0.3	3.1 ± 0.6	2.5 ± 0.1	2.0 ± 0.3	3.2 ± 0.7	3.2 ± 0.7	4.5 ± 3.0
	Small Intestine				Large Intestine			
15	4.5 ± 1.4	3.8 ± 0.6	4.1 ± 0.3	4.0 ± 2.4	2.1 ± 0.5	2.8 ± 0.5	2.7 ± 0.3	1.7 ± 0.7
30	4.7 ± 1.8	4.6 ± 2.9	5.7 ± 1.0	3.5 ± 0.6	3.5 ± 1.9	2.6 ± 0.3	2.5 ± 1.0	1.8 ± 0.5
45	6.4 ± 0.2	5.8 ± 0.4	6.2 ± 1.4	4.4 ± 1.5	1.9 ± 0.3	2.0 ± 0.2	2.1 ± 0.6	3.9 ± 2.4
60	9.2 ± 1.0	7.8 ± 1.5	6.8 ± 1.7	4.5 ± 0.8	1.5 ± 0.2	1.6 ± 0.3	1.9 ± 0.1	1.8 ± 0.3

[a]0 - Tracer dose only

[b]Mean ± SD (N=4) percentage of injected.

Table 28. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time after Administration (min)	Dose (mg/kg)			
	0[a]	1	10	100
15	20.3 ± 3.2[b]	19.7 ± 3.3	20.0 ± 3.2	17.9 ± 4.7
30	27.3 ± 9.2	37.1 ± 4.9	38.0 ± 5.4	22.2 ± 2.6
45	51.6 ± 13.3	50.2 ± 4.7	53.3 ± 12.9	28.0 ± 5.7
60	70.5 ± 10.5	60.5 ± 17.8	67.6 ± 18.3	38.6 ± 4.6

[a] Tracer dose only
[b] Mean ± SD (N=4) percentage of injected

Table 29. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVITY AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time after Administration (hrs)	Dose (mg/kg) 1	10	100	Dose (mg/kg) 1	10	100
		Blood			Urine	
1	2.3 ± 0.2[a]	2.8 ± 0.3	2.7 ± 0.2	46.5 ± 8.0	34.0 ± 3.1	20.1 ± 2.0
2	1.8 ± 0.3	1.0 ± 0.1	2.3 ± 0.4	74.0 ± 5.4	66.1 ± 10.4	52.5 ± 8.6
3	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	79.1 ± 17.1	61.9 ± 18.4	65.1 ± 20.2
4	0.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	87.6 ± 11.3	55.2 ± 11.7	49.1 ± 21.9
		Liver			Lungs	
1	4.9 ± 0.9	5.9 ± 1.2	5.0 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
2	4.0 ± 1.5	2.7 ± 0.1	3.7 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2
3	1.8 ± 0.9	1.3 ± 0.2	1.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
4	1.1 ± 0.3	1.4 ± 0.3	0.9 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
		Kidneys			Adipose	
1	2.9 ± 0.3	2.5 ± 0.1	2.1 ± 0.3	2.4 ± 1.2	2.7 ± 0.5	2.9 ± 0.4
2	1.1 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	0.9 ± 0.8	0.7 ± 0.2	0.7 ± 0.1
3	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
4	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.3	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
		Small Intestine			Large Intestine	
1	8.8 ± 0.8	6.6 ± 1.0	5.2 ± 0.6	1.8 ± 0.6	1.3 ± 0.2	1.6 ± 0.3
2	7.9 ± 0.5	8.9 ± 1.5	7.0 ± 1.5	0.8 ± 0.1	1.5 ± 1.1	1.0 ± 0.1
3	7.1 ± 2.4	8.0 ± 0.6	5.8 ± 1.7	1.6 ± 0.4	1.0 ± 0.3	1.6 ± 1.0
4	5.4 ± 3.4	4.4 ± 1.3	6.2 ± 1.9	2.3 ± 1.3	2.4 ± 0.7	1.2 ± 1.0

[a] Mean ± SD (N=4) percentage of injected

Table 30. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER I.P. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time After Administration (hrs)	Dose (mg/kg)		
	1	10	100
1	69.8 ± 8.3[a]	56.0 ± 3.6	40.1 ± 3.5
2	91.4 ± 7.2	82.3 ± 10.4	68.7 ± 7.9
3	91.6 ± 14.7	75.1 ± 19.0	75.5 ± 19.4
4	98.4 ± 12.8	64.5 ± 12.0	58.7 ± 20.7

[a] Mean ± SD (N=4) percentage of injected

of elimination was dose-related, with the most rapid and complete rates being observed at 1 mg/kg (Table 30).

b) Effect of different doses of p.o. administered 2,6-DNT

The distribution and elimination of different doses of p.o.-administered 2,6-DNT were studied for time periods up to 8 hours. The earlier study with 2,4-DNT (Tables 10 and 11) has been extended to 24 hours at the 100 mg/kg dose. Because the rate of elimination of 2,4-DNT in the urine did not increase beyond 8 hours (Table 10) and because there were no large differences between the elimination of 2,4-DNT and that of 2,6-DNT after i.p. administration, the time periods in the present studies were restricted to 8 hours. The results of these experiments are shown in Tables 31 and 32. Amounts of radioactive material in blood (Table 31) were somewhat lower than those observed after i.p. administration (Table 29). Similarly, amounts in liver, kidneys, adipose tissue and large intestine (Table 31) were lower than after i.p.-administration (Table 29). As expected, amounts of radioactive material in the small intestine were higher after p.o.- (Table 31) than after i.p. administration (Table 29), but decreased with time. Amounts in the lungs (Table 31), however, were very low and similar to those observed before (Table 29). Amounts in the large intestine increased slightly with time (Table 31), but the urinary route clearly remained the major mode of elimination of p.o.-administered 2,6-DNT, as amounts excreted increased up to 8 hours (Table 31). As was found before with 2,4-DNT (Table 10), the rate of elimination of 2,6-DNT was much slower after p.o. administration (Table 31) than after i.p. administration (Table 29). Total amounts of radioactive material recovered in tissues and fluids after 1-8 hours were much lower (Table 32) than the corresponding amounts recovered after i.p. administration (Table 30). A similar situation was observed before with 2,4-DNT (Table 11).

c) Rates of 2,6-DNT elimination from tissues

The elimination of 3 different doses (1, 10 and 100 mg/kg) of i.p. administered 2,6-DNT from blood, liver, lungs and small intestines was followed for time periods up to 2 hr after administration. The methodology used in these sets of experiments was essentially as that described for similar studies with 2,4-DNT (Section A.2.d.). As before, small amounts of [^{14}C]2,6-DNT (5000 cpm/sample) and 2,6-DNT (200 µg/sample) were added to blood or tissue samples to serve as recovery markers.

The results of these experiments are shown in Table 33. The extent of 2,6-DNT elimination from blood and tissues was dose-dependent; i.e., larger amounts of unchanged 2,6-DNT could be reisolated from blood and tissues at doses of 10 and 100 mg/kg than at 1 mg/kg. Similarly, as expected, the amounts of unchanged 2,6-DNT that could be reisolated depended on the time elapsed after administration. At 1 and 10 mg/kg, very little 2,6-DNT was present in blood (<1.4%) and lungs (<5.3%). The amounts of 2,6-DNT in blood and lungs after a dose of 10 mg/kg were somewhat higher, but were, nevertheless, lower than those in liver and small intestine. Thus, similar to the situation observed with 2,4-DNT (Table 13), the elimination of 2,6-DNT from blood paralleled that from the lungs. A major difference however, was that, compared to 2,4-DNT, much lower amounts of unchanged 2,6-DNT (Table 33) could be reisolated from these 2 tissues, at all doses and time points. It also appears that the metabolism of 2,6-DNT in the liver was much more rapid than that of

Table 31. TISSUE DISTRIBUTION AND EXCRETION OF RADIOACTIVE MATERIAL AFTER P.O. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time after Administration (hrs)	Dose (mg/kg) 1	10	100	Dose (mg/kg) 1	10	100
		Blood			Urine	
1	0.7 ± 0.2[a]	0.4 ± 0.2	1.2 ± 0.2	9.1 ± 2.4	7.4 ± 0.8	9.1 ± 1.1
2	0.6 ± 0.2	0.4 ± 0.1	1.0 ± 0.2	18.0 ± 4.2	16.5 ± 4.2	17.8 ± 3.5
4	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	33.6 ± 16.6	25.2 ± 10.0	24.3 ± 3.1
8	0.8 ± 0.2	0.8 ± 0.0	0.8 ± 0.1	53.7 ± 6.9	53.5 ± 7.9	48.6 ± 7.5
		Liver			Lungs	
1	2.3 ± 1.1	0.7 ± 0.3	0.9 ± 0.4	0.3 ± 0.2	0.0	0.3 ± 0.2
2	0.9 ± 0.2	0.9 ± 0.5	1.1 ± 0.4	0.1 ± 0.0	0.2 ± 0.2	0.3 ± 0.2
4	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
8	1.1 ± 0.2	1.1 ± 0.2	0.6 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
		Kidneys			Adipose	
1	1.0 ± 0.4	0.3 ± 0.2	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
4	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
8	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
		Small Intestine			Large Intestine	
1	9.5 ± 5.6	16.9 ± 9.6	13.6 ± 8.3	0.5 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
2	9.6 ± 3.6	12.5 ± 9.0	8.9 ± 4.6	0.5 ± 0.1	0.8 ± 0.3	0.5 ± 0.1
4	6.9 ± 5.4	6.7 ± 3.2	7.3 ± 2.8	1.1 ± 0.2	0.8 ± 0.2	0.7 1 0.1
8	3.6 ± 2.2	3.0 ± 1.3	7.0 ± 2.7	1.2 ± 0.6	1.7 ± 0.5	1.1 ± 0.1

[a] Mean ± S.D. (N=4) percentage of injected.

Table 32. TOTAL RADIOACTIVITY IN TISSUES AND FLUIDS AFTER P.O. ADMINISTRATION OF DIFFERENT DOSES OF 2,6-DINITROTOLUENE TO A/J MICE

Time After Administration (hrs)	Dose (mg/kg)		
	1	10	100
1	23.0 ± 4.5[a]	26.1 ± 9.6	23.7 ± 7.3
2	30.2 ± 7.6	31.5 ± 6.0	30.0 ± 5.7
3	43.3 ± 12.7	34.4 ± 11.7	33.9 ± 1.4
4	61.0 ± 7.1	60.6 ± 7.5	58.5 ± 7.8

[a] Mean ± SD (N=4) percentage of injected

Table 33. ELIMINATION OF 2,6-DNT AND ITS METABOLITES AFTER I.P. ADMINISTRATION

Tissue	[^{3}H]2,6-DNT (percentage of total [^{3}H] per gram tissue or ml blood)[a]				
	15 min	30 min	45 min	60 min	120 min
			1 mg/kg		
Blood	0.3 ± 0.4	1.4 ± 1.3	0.0	0.0	
Liver	5.9 ± 7.3	4.1 ± 4.3	0.0	0.0	
Lungs	5.3 ± 7.2	2.8 ± 5.6	0.0	0.0	
Small Intestine	28.2 ± 5.9	18.0 ± 3.8	13.1 ± 1.6	6.1 ± 2.8	
			10 mg/kg		
Blood	0.4 ± 0.8	0.0	0.0	0.0	
Liver	11.3 ± 7.8	5.1 ± 2.8	0.0	0.0	
Lungs	1.9 ± 2.7	1.3 ± 2.2	0.0	0.0	
Small Intestine	68.0 ± 18.3	28.4 ± 14.4	14.9 ± 11.4	6.0 ± 0.7	
			100 mg/kg		
Blood	17.1 ± 6.9	5.7 ± 5.2	5.0 ± 2.4	1.4 ± 2.8	0.0
Liver	20.9 ± 3.1	16.8 ± 3.7	14.4 ± 1.8	6.4 ± 3.7	0.1 ± 0.1
Lungs	2.6 ± 3.5	1.8 ± 3.4	1.9 ± 3.3	1.2 ± 2.4	0.0
Small Intestine	51.4 ± 6.5	37.6 ± 9.9	23.1 ± 7.4	13.1 ± 3.6	0.7 ± 0.8

[a] Mean ± SD (N=4)

2,4-DNT by this organ; e.g., at 1 and 10 mg/kg, no 2,6-DNT could be reisolated after 45 min (Table 33). In contrast, amounts of unchanged 2,6-DNT isolated from the small intestine were remarkably similar to those of unchanged 2,4-DNT (Table 13). It appears then, that the extent of in vivo metabolism of 2,6-DNT by the small intestine is similar to that of 2,4-DNT, but that first-pass liver metabolism of 2,6-DNT is much faster than that of 2,4-DNT, resulting in very little unchanged 2,6-DNT appearing in the circulation. As observed before with 2,4-DNT, the extent of blood metabolism resembles the extent of lung metabolism. However, at 100 mg/kg amounts of unchanged 2,6-DNT were significantly lower in the lungs than in the blood, so that, in contrast to the situation with 2,4-DNT, the lungs may possess a limited capacity to metabolize 2,6-DNT. The elimination of p.o. doses of 1, 10 and 100 mg/kg of 2,6-DNT from blood, liver, lungs, small intestine and large intestine (including feces) was followed for time periods up to 8 hours after administration. The methodology used in these sets of experiments was essentially as that described for the reisolation of i.p. administered 2,6-DNT. As before, small amounts of [^{14}C]2,6-DNT (5000 cpm/sample) and 2,6-DNT (200 µg/sample) were added to blood or tissue homogenates to serve as recovery markers.

The results of these experiments are shown in Table 34. Previous results showed that the absorption and elimination of 2,6-DNT was much slower after p.o. than after i.p. administration (Table 31). The present studies were therefore extended to 8 hours after administration. Contrary to previous experiments of this nature, the large intestine, including feces, was included for analysis in this series of experiments because it was found that relatively large amounts of unchanged 2,6-DNT could be reisolated from the small intestine, even after relatively long periods of time (4-8 hours). As observed before with p.o.-administered 2,4-DNT (Table 14), the small amounts absorbed were fully metabolized as judged by the very low amounts of 2,6-DNT that could be reisolated from blood and liver at all time points and doses. Relatively large amounts of unchanged 2,6-DNT (35-64%) could be reisolated from the small intestine at all time points and doses. These results are different from the results obtained after i.p. administration of 2,6-DNT (Table 33). In those studies the metabolism of 2,6-DNT by the small intestine was virtually complete after 1 hr at all doses. Similar differences in extents of 2,4-DNT metabolism by the small intestine were observed (Table 14), which were also related to the route of administration. The reasons for these differences remain to be investigated. In contrast to the small intestine, virtually no unmetabolized 2,6-DNT could be reisolated from the large intestine at 4 or 8 hours after administration. Possibly, 2,6-DNT is efficiently metabolized by the epithelium of the large intestine and/or by its bacterial flora.

3. In vitro metabolism of 2,6-DNT

Shortly before the studies on the metabolism of 2,6-DNT were initiated, a preliminary report appeared, which indicated that 2,6-DNT (>99.9% pure) was a very potent hepatocarcinogen in the male Fischer-344 rat.[10] Inasmuch as 2,6-DNT is negative in the strain A mouse lung tumor bioassay (Table 2) and the liver and the intestine appear to be primary sites of 2,6-DNT metabolism (Tables 33 and 34), it was of interest to determine whether differences in 2,6-DNT metabolism by these organs could account for differences in the tumorigenic response in the two species. Therefore, the in vitro metabolism of

Table 34. ELIMINATION OF 2,6-DNT AND ITS METABOLITES AFTER P.O. ADMINISTRATION

Tissue	1 hr.	2 hr.	3 hr.	4 hr.	8 hr.
	[^{3}H]2,6-DNT (percentage of total [^{3}H] per gram tissue or ml blood)[a]				
	1 mg/kg				
Blood	0	0	0	0	0
Liver	0				
Lungs	11.6 ± 19.1	14.5 ± 29.1	16.7 ± 33.4	20.9 ± 25.5	35.9 ± 29.6
Small Intestine	63.9 ± 5.5	47.6 ± 32.3	38.7 ± 16.6	39.0 ± 16.9	35.0 ± 19.0
Large Intestine[b]	-	-	-	0.2 ± 0.4	1.6 ± 1.8
	10 mg/kg				
Blood	0	0	0	0	-
Liver	0	0	0	37.3 ± 7.3	-
Lungs	31.7 ± 21.9	0	6.2 ± 12.4	8.3 ± 16.6	-
Small Intestine	51.2 ± 17.5	54.6 ± 14.9	35.2 ± 11.0	63.2 ± 25.5	-
	100 mg/kg				
Blood	0	0	0	0	0
Liver	7.0 ± 12.4	0	10.7 ± 15.4	0	0
Lungs	1.6 ± 3.2	9.6 ± 16.6	7.7 ± 10.1	0	18.8 ± 24.7
Small Intestine	48.6 ± 3.7	49.7 ± 1.8	51.9 ± 14.0	54.9 ± 5.3	39.4 ± 14.1
Large Intestine[b]	-	-	-	1.4 ± 1.7	1.6 ± 0.6

[a] Mean ± SD (N=4).

[b] Feces was collected separately and combined with large intestine (+ contents) before workup.

2,6-DNT by both hepatocytes and cecal contents was studied in the A/J mouse and in the Fischer-344 rat.

Before the study on the in vitro metabolism of 2,6-DNT by hepatocytes could be initiated, it was necessary to determine its toxicity in this system. Hepatocytes were isolated from strain A mice mice using the collagenase perfusion method.[16] Approximately 1 x 10^6 cells were plated out in L15 medium containing 10% fetal bovine serum (FBS). After allowing the cells to attach for 2 hr, the medium was changed to L15 with 1% FBS, and 20 µl of dimethylsulfoxide (DMSO) containing the test compound was added. After 24 hr toxicity was measured using trypan blue exclusion of the cells[16], and their lactate dehydrogenase (LDH)-release into the medium[17] as endpoints. The results are shown in Table 35. Significant toxicity was observed at 2,6-DNT concentrations >50 µg/ml, by both criteria. In subsequent metabolism experiments, nontoxic concentrations (<50 µg/ml or <274 µM) were used. Hepatocytes were isolated from the livers of Fischer-344 rats and A/J mice essentially as described by Klaunig et al.[16], using the two step hepatic vein perfusion technique. Hank's calcium-and magnesium-free solution containing 0.5 mM ethylene glycol-bis(β-aminoethyl) N,N-tetraacetic acid and 0.05 M HEPES and collagenase (100 µ/ml Leibovitz L-15 medium) were used as perfusate. Only those preparations with a cell viability greater than 90%, as judged by trypan blue exclusion were used. Hepatocyte suspensions were plated in 20 x 100 mm plastic dishes (approximately 20 x 10^6 cells/dish) and cells were allowed to attach at 37°C in the presence of 5 ml of L-15 medium containing 10% fetal bovine serum (Hyclone). After 4 hr the medium, containing unattached cells, was replaced with 5 ml of L-15 medium containing 1% fetal bovine serum, 220 µM 2,6-DNT and a trace amount (12 µCi/ml) of [^{3}H]2,6-DNT. Both 2,6-DNT and [^{3}H]2,6-DNT were first dissolved in a small volume of dimethyl sulfoxide (DMSO) so that the final DMSO concentration in the medium was <1%.

Incubations were carried out at 37°C in humidified air for varying time periods. Cells were harvested for analysis of DNA binding and the medium was stored until analysis of metabolites by h.p.l.c. Cecal contents from Fischer-344 rats or A/J mice were removed and homogenized in 0.01 M phosphate buffer, pH 7.4, at 4°C. Homogenates were centrifuged at 9,000 x g for 20 min at 4°C, and the resulting supernatants were kept frozen at -80°C until use. For metabolism studies, the incubation mixture, in a final volume of 1 ml of 0.01 M phosphate buffer, pH 7.4, contained 20 µCi [^{3}H]2,6-DNT, 240 µM 2,6-DNT, 1 mM NADPH and cecal enzymes (1 - 1.2 mg protein). After 30 min of incubation at 37°C in an atmosphere of nitrogen, the mixture was frozen and stored at 20°C until analysis by h.p.l.c. H.p.l.c. analysis of 2,6-DNT metabolites was performed essentially as described by Long and Rickert[18] with some modifications. One ml of medium was extracted twice with two volumes of ethyl acetate:acetone (2:1). The combined organic phases were dried at 40°C under a stream of nitrogen, and the resulting residue was termed the unconjugated fraction. The aqueous phase was adjusted to pH 6.8 by the addition of 0.1 ml of 0.75 M phosphate buffer, pH 6.8, and the samples were incubated with β-glucuronidase (400 units/ml, Type VII, Sigma) at 37°C for 16 hr and then extracted as above. This fraction constituted the glucuronide fraction. Both the glucuronide and unconjugated fractions were reconstituted in 0.5 ml of water:methanol (1:1). Using the Waters (Milford, MA) h.p.l.c. system described before (Section A.1), an aliquot was analyzed for metabolites by cochromatography with a mixture of authentic standards. The mixture of standards consisted of the following metabolites (10 µg/ml each): 2,6-DNBAcid,

Table 35. CYTOTOXICITY OF 2,6-DNT IN PRIMARY MONOLAYER CULTURES OF HEPATOCYTES FROM STRAIN A/J MICE

Concentration (µg/ml)	Trypan Blue Exclusion (% viable cells)[a]	LDH Release[b] (I.U./liter)
2	86.0	20
10	93.0	17
50	72.0	64
100	35.7	376
200	4.0	418
500	0	421

[a] Essentially all cells were viable in untreated or DMSO-treated cultures.

[b] "Spontaneous" release of LDH into the medium varied from 205-215 I.U./ml and was deducted in all cases.

2,6-DNBAlc, 2Ac6NT, 2A6NT, and 2,6-DNT. Metabolites were separated at ambient temperature on a 4.6 x 25 cm column (Ultrasphere ODS, Altex). A constant flow rate of 1.0 ml/min and a 30 min linear gradient methanol in 0.005 M potassium phosphate buffer, pH 7.4, to 100% methanol were used. Eluted fractions (1.0 ml each) were collected into counting vials and the radioactivity in each fraction was quantitated by standard scintillation methods. Recovery of sample radioactivity varied from 38.6 to 99.8% of the amount injected.

Metabolites of 2,6-DNT were separated as shown in Figure 1A, and the separations obtained were essentially the same as those reported by Long and Rickert.[18] In all of the media from incubations with hepatocytes early-eluting, polar material was detected which co-eluted in part with standard 2,6-DNBAcid (Figure 1B). No attempts were made to further resolve this material and these fractions were therefore combined and referred to as "polar" metabolites (Table 36). We have observed a similar phenomenon in our studies on the in vivo metabolism of 2,4-DNT by the A/J mouse (Table 24). In addition, unknown material eluted between standard 2,6-DAT and 2,6-DNBAlc (fractions 7-12, Figure 1B) and in some cases this material amounted to a sizeable proportion of the total metabolites in the glucuronide fraction (Table 36). This compound(s) was also formed by cecal enzymes under anaerobic conditions (Table 37), so that it is possible that it represents a reduced, or partially reduced metabolite.

Total extractable metabolites (unconjugated and glucuronides) formed by hepatocytes from the A/J mouse were similar to those from the Fischer-344 rat (35.3 - 54.7%, Table 36). Metabolism by hepatocytes from Fischer-344 rats was similar to that by hepatocytes from A/J mice (Table 36), both quantitatively and qualitatively. 2,6-DNBAlc was the major identifiable metabolite, constituting 57.5 to 85.5 of the total in all cases. Significant amounts of polar material (4.7 - 38.7%) were found in the glucuronide fraction, with lower amounts (8.4 - 9.7%) present in the unconjugated fraction. Small amounts (1.2 - 5.0%) of 2A6NT were isolated from the unconjugated fraction, and substantial amounts (13 - 20%) of an unknown compound, eluting between polar material and 2,6-DNBAlc, could also be detected.

In both species, anaerobic metabolism of 2,6-DNT by extracts of cecal contents showed principally reduced products and the results were similar in the two species, both qualitatively and quantitatively (Table 37). 2A6NT represented the major metabolite, constituting 6.4 - 9.6% of the total. In addition, small amounts (1.3 - 4.6 %) of 2A6NT could be detected and a very minor proportion (0.4 - 0.7%) of total metabolites represented 2,6-DAT. Unknown metabolites comprised 5.3 - 6.5% of the total in the mouse, but only a trace amount in the rat (0.7%, Table 37). Thus, as was seen with 2,4-DNT (Tables 24-26), 2,6-DNT is metabolized to mainly oxidized products in the liver and to reduced products by the intestinal microflora.

4. In vivo and in vitro DNA-binding studies

As was indicated above in Section B.3., differences in metabolism may account for differences in the carcinogenic response to 2,6-DNT in Fischer-344 rats and A/J mice. Similarly, because it is thought that covalent binding to DNA is a requirement for the initiation of chemical carcinogenesis[19], it is possible that differences in the extent of covalent binding of 2,6-DNT to

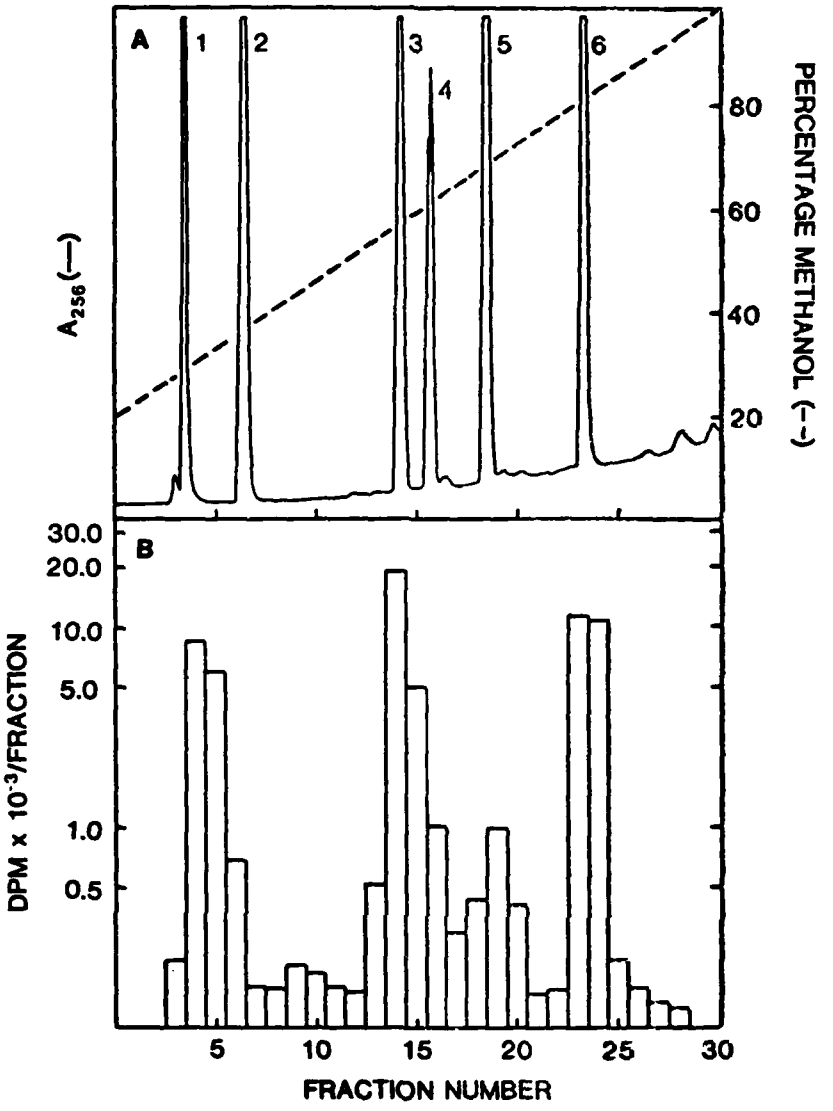

Fig. 1A FIG. 1. HPLC elution profile of standard 2,6-DNT metabolites (A), and a representative profile of unconjugated radioactive 2,6-DNT metabolites (B) obtained after incubation of mouse hepatocytes with 2,6-DNT. In panel A, peak 1 is 2-6-DNBAcid; peak 2 is 2,6-DAT, peak 3 is 2,6-DNBAlc, peak 4 is 2Ac6NT. The solvent program for HLPC consisted of a 30-min lineargradient of 20% methanol in 0.005 M potassium phosphate buffer, pH 7.4 to 100% methanol. Other conditions for HPLC, as well as those for the incubation of 2,6-DNT with hepatocytes, are described in the text.

Table 36. METABOLISM OF [^{3}H]2,6-DNT BY CULTURED HEPATOCYTES FROM A/J MICE AND FISCHER-344 RATS

Source of hepatocytes/ Experiment no.[a]	Fraction (% of total)[b]	pmol/10^6 cells/24 h (% of total isolated per fraction)					
		Polar	Unknown	2,6-DNBAlc	2A6NT	Total Metabolites	2,6-DNT
Mouse 1	U (38.0)	649 (8.5)	---[c]	6,387 (83.8)	94 (1.2)	7,130 (93.5)	493 (6.5)
	G (16.7)	875 (24.0)	474 (13.0)	2,299 (63.0)	---	3,648	---
2	U (29.9)	1,362 (9.7)	---	8,050 (57.5)	566 (4.0)	9,978 (71.2)	4,023 (28.8)
	G (5.4)	726 (24.5)	136 (4.6)	2,100 (70.9)	---	2,962	---
Rat 1	U (46.8)	1,380 (8.9)	---	9,760 (62.7)	822 (5.3)	11,962 (76.9)	3,615 (23.1)
	G (6.9)	615 (21.7)	576 (20.0)	1,666 (58.5)	---	2,848	---
2	U (38.0)	2,301 (8.4)	---	23,504 (85.5)	371 (1.3)	26,176 (95.2)	1,312 (4.8)
	G (15.2)	4,391 (38.7)	---	6,807 (61.3)	---	11,198	---

[a] Hepatocytes were isolated from A/J mice and Fischer-344 rats. In mouse experiment 1 and in the rat experiments the [^{3}H]2,6-DNT concentrations were 12 µCi/ml and 220 µM, respectively, while these were 5 µCi/ml and 274 µM, respectively in mouse experiment 2.

[b] U, unconjugated fraction; G, glucuronide fraction

[c] ---, non detectable

Table 37. IN VITRO METABOLISM OF [^{3}H]2,6-DNT BY CECAL ENZYMES FROM A/J MICE AND FISCHER-344 RATS

Source of cecal enzyme experiment no. [a]	p/mol/mg protein/30 min (% of total isolated)					
	Unknown	2,6-DAT	2Ac6NT	2A6NT	Total Metabolites	2,6-DNT
A/J mouse						
1	327 (6.5)	25 (0.5)	131 (2.6)	322 (6.4)	805 (16.0)	4,222 (84.0)
2	344 (5.3)	39 (0.6)	182 (2.8)	597 (9.2)	1,162 (17.9)	5,325 (82.1)
Fischer-344 rat						
1	56 (0.7)	56 (0.7)	366 (4.6)	731 (9.2)	1,209 (15.2)	6,738 (84.8)
2	---[b]	19 (0.4)	63 (1.3)	462 (9.6)	544 (11.3)	4,267 (88.7)

[a] An extract of cecal contents was prepared and incubated anaerobically for 30 min with [^{3}H]2,6-DNT (20 μuCi/ml) and 2,6-DNT (240 μM).

[b] ---, none detectable.

tissue DNA may contribute to the difference in sensitivity between the Fischer-344 rat and the A/J mouse to 2,6-DNT-induced carcinogenesis. The in vivo DNA binding to various organs of both species was therefore determined. In addition, because carcinogenicity tests of 2,4-DNT have yielded conflicting results, this compound was also tested for covalent binding. A/J mice were injected i.p. with a tricaprylin solution (0.25 ml/mouse) containing 2,6-DNT or 2,4-DNT and 1 μCi of [^{3}H]2,6-DNT or [^{3}H]2,4-DNT respectively, to give a final dose of 150 mg/kg in each case. Fischer-344 rats were treated similarly except that they received 3 μCi of the radioactive tracer. The animals were sacrificed by cervical dislocation after 12 or 24 hr (2 animals/time point) and their liver, lung, small and large intestines were removed. Tissues were weighed, minced, repeatedly washed in 0.15 M KCl, and then homogenized in three volumes of 0.05 M sodium phosphate buffer, pH 6.5, containing 0.01 M EDTA and 0.01 M EGTA.

DNA was isolated from the tissue homogenates essentially as described earlier.[20] Briefly, homogenates of liver, lung, small and large intestine were first treated with RNAse (100 μg/ml homogenate) overnight at 37°C in a shaking water bath. Following RNAse digestion, homogenates were adjusted to 1.5%(w/v) sodium dodecyl sulfate and were then treated with 200 μg/ml of autodigested proteinase K (type XI, Sigma) for 2.5 hr at 37°C. DNA was then isolated by hydroxylapatite chromatography.[20] Samples were dialyzed overnight against distilled water, and DNA was estimated by measuring the absorbance at 260 nm. The A260/A280 ratio of DNA isolated in this manner varied from 1.78 to 1.84. Radioactivity associated with the purified DNA was quantitated by standard liquid scintillation methods.

I.p. administration of 2,6-DNT to A/J mice resulted in covalent binding to liver DNA, but under the experimental conditions no binding could be detected in the other organs examined (Table 38). In these experiments only amounts greater than 100 dpm above background in the purified DNA were considered indicative of significant binding. Binding examined 24 hr after administration of 2,6-DNT was approximately 6 to 7-fold higher in the rat than in the mouse and relatively low binding levels were detectable in the lungs and small intestine (14.9 - 22.7 pmol/mg DNA, Table 38). In the mouse, binding of 2,4-DNT to hepatic DNA was approximately the same as that of 2,6-DNT (42.6 - 58.9% vs 25.9 - 31.9 pmol/mg DNA) and, in contrast to the findings with the 2,6-isomer, 2,4-DNT was covalently bound to DNA of the lungs, small intestine, and large intestine of this species (9.7 - 39.0 pmol/mg DNA). In the rat binding of 2,4-DNT to hepatic DNA was 4 to 5-fold higher than in the mouse (215.4 and 226.8 vs 42.6 and 58.9 pmol/mg DNA, respectively) and, similar to the situation in the mouse, binding levels of 2,4-DNT to DNA from extrahepatic tissues were 3 to 4-fold lower than those of 2,4-DNT to hepatic DNA (Table 38).

To elucidate the role of cecal enzymes in binding of 2,6-DNT to DNA, two sets of experiments were performed. In the first set hepatocytes were incubated with 2,6-DNT (240 μM) and [^{3}H]2,6-DNT (20 μCi/ml) for a period of 24 hr as described above. In the second set of experiments, [^{3}H]2,6-DNT and 2,6-DNT were first incubated with cecal enzymes as described above. After extraction of the metabolites the extract was taken to dryness and the residue was reconstituted in a small volume of DMSO, which then served as the substrate for incubation with hepatocytes as described above. After 24 hr the cells were harvested and DNA was isolated as described above. Similar sets of experiments

Table 38. IN VIVO COVALENT BINDING OF [^{3}H]2,6-DNT and [^{3}H]2,4-DNT IN A/J MICE AND FISCHER-344 RATS.

Compound/Strain/ experiment (time)[a]		Covalent binding (pmol/mg DNA)			
		Liver	Lung	Small Intestine	Large Intestine
2,6-DNT					
A/J mouse	1 (12 h)	19.4	---[b]	---	---
	2 (12 h)	16.7	---	---	---
	3 (24 h)	31.9	---	---	---
	4 (24 h)	25.9	---	---	---
Fischer-344 rat					
	1 (24 h)	131.1	14.9	---	18.9
	2 (24 h)	259.9	22.7	---	16.6
2,4-DNT					
A/J mouse	1 (24 h)	42.6	23.1	17.1	11.0
	2 (24 h)	58.9	39.0	9.7	11.1
Fischer-344 rat					
	1 (24 h)	215.4	58.3	60.9	45.0
	2 (24 h)	226.8	58.5	66.2	75.0

[a] Each rat received an i.p. injection of [^{3}H]2,6-DNT or [^{3}H]2,4-DNT (3 μCi/rat) along with 2,6-DNT or 2,4-DNT, respectively, to give a dose of 150 mg/kg. Mice were treated similarly, except that each mouse received 1 μCi of radioactive tracer. Time indicates hours elapsed between injection and sacrifice.

[b] ---, none detectable.

were performed using liver microsomes and calf thymus DNA. Liver microsomes were prepared and stored at -20°C as described before (Section A.4). The incubation mixture consisted of 1 ml of 0.01 M potassium phosphate, pH 7.4, containing 1 mM NADPH, 1 mg microsomal protein, 1 mg calf thymus DNA, and the substrate, which consisted of either a mixture of 2,6-DNT (240 µM final concentration) and [^{3}H]2,6-DNT (100 µCi/ml), or the residue of the extract of a preincubation mixture of the substrate with cecal enzymes, as described above. Both types of substrate were added dissolved in DMSO. The reactions were performed in petri dishes which were placed in an air-tight chamber. The chamber was gassed with air at 3 p.s.i. and then placed on a rocker platform. After incubation for 30 min at 37°C with rocking (5 cycles/min), DNA was isolated from the mixtures as described above and covalent binding of radioactive material to DNA was quantitated.

In vitro binding of 2,6-DNT to calf thymus DNA, catalyzed by rat liver microsomes, yielded a low level of binding (1.2 - 1.3 pmol/mg DNA, Table 39), and this binding was approximately doubled when the substrate was preincubated with an extract from cecal contents. No covalent binding could be detected when hepatocytes from either the Fischer-344 rat or the A/J mouse were incubated with 2,6-DNT, but when 2,6-DNT was preincubated with the respective extracts from cecal contents, before addition to the hepatocytes, binding levels became almost as high (89.4 - 138.5 pmol/mg DNA, Table 39) as those observed in vivo (Table 38), and they were approximately the same in mouse and rat hepatocytes.

Covalent DNA binding in vivo in the A/J mouse is detectable only in the liver and not in any of the extrahepatic tissues (Table 38). Our studies on the absorption and elimination of 2,6-DNT in the A/J mouse have clearly shown that the lung and the intestines are active in 2,6-DNT metabolism[21], even though they are not target organs in the lung tumor bioassay (Table 2). In contrast, the Fischer-344 rat, whose extrahepatic tissues also metabolize 2,6-DNT[22], exhibits detectable DNA binding in the lungs and the large intestine, and binding in the liver is much higher than that in the A/J mouse (Table 38). Thus, the apparent high susceptibility of the rat liver to 2,6-DNT-induced carcinogenesis[10] and the absence of carcinogenicity of 2,6-DNT in the strain A mouse lung tumor bioassay (Table 2) may be related to quantitative differences in the covalent binding of 2,6-DNT to DNA. The reasons for this quantitative difference are unknown, but are not likely to be related to pharmacokinetic parameters since, after oral administration, 2,6-DNT is rapidly eliminated, almost exclusively via the urine, in both the Fischer-344 rat[18] and the A/J mouse[21]. It appears more likely that intestinal metabolism plays a role. Cecal extracts from the A/J mouse and the Fischer-344 rat metabolize 2,6-DNT in a similar manner, only forming reduced metabolites (Table 38). The results of genotoxicity studies with germfree and conventional Fischer-344 rats have suggested a role of the intestinal microflora in the covalent binding of DNT to hepatic DNA[23], and this was confirmed in experiments in which hepatic macromolecular covalent binding of 2,6-DNT in Fischer-344 rats was found to change with diet-induced changes in the intestinal microflora.[24] Our present in vitro studies unequivocally established the obligatory role of the intestinal microflora in the metabolic pathways leading to the covalent binding of 2,6-DNT to hepatic DNA of both species (Table 39). The exact mechanism responsible for the difference in this binding between the two species (Table 38) will probably have to await the elucidation of the nature of the covalent adducts formed and their relative

Table 39. IN VITRO COVALENT BINDING OF [^{3}H]2,6-DNT TO CALF THYMUS AND HEPATOCYTE DNA FROM A/J MICE AND FISCHER-344 RATS

Preparation[a]	Pre-incubation conditions[b]	Covalent binding (pmol/mg DNA)
Fischer-344 rat		
Liver microsomes and calf thymus	none	1.2
		1.3
	cecal enzymes	3.1
		2.0
Hepatocytes	none	---[c]
		---
	cecal enzymes	89.4
		138.5
A/J mouse		
Hepatocytes	none	---
		---
	cecal enzyme	136.0
		97.0

[a] Liver microsomes were incubated with calf thymus DNA, [^{3}H]2,6-DNT (100 µCi/ml) and 2,6-DNT (240 µM) for 30 min, as described in Materials and Methods. Primary monolayers of hepatocytes were incubated for 24 h with similar concentrations of substrate, as described in Material and Methods.

[b] Before incubation with microsomes or hepatocytes, the substrate was incubated with cecal enzymes as described in Materials and Methods.

[c] ---, none detected

rates of removal. In the Fischer-344 rat, existing evidence indicates that terminal half-lives of DNA-bound 2,6-DNT in the liver ranges from 5.1 to 7.9 days[22], and that, by analogy to the situation with various aromatic amines (reviewed in ref. 25), a sulfate ester derivative of a hydroxylamine metabolite of 2,6-DNT may be the reactive intermediate that binds to DNA.[26]

Compared to 2,6-DNT, 2,4-DNT has very low genotoxic activity[23] and it is believed not to be carcinogenic in the Fischer-344 rat.[27] Our present studies have shown that 2,4-DNT is not carcinogenic in the strain A mouse lung tumor bioassay (Table 2). The pattern of binding of 2,4-DNT to liver DNA is similar to that observed with 2,6-DNT, except that higher levels are seen (Table 38). Kedderis et al.[26] and Rickert et al.[22] found that the binding of 2,6-DNT to liver DNA of Fischer-344 rats after a single dose was approximately 2-fold higher than that of 2,4-DNT. In contrast to binding in the liver, binding of 2,4-DNT to extrahepatic tissues of the Fischer-344 rat is higher than that of 2,6-DNT (Table 38). The extrahepatic metabolism of 2,4-DNT is well-documented[28,29] (Tables 25-26) and, in the intestine, mainly reduced products are formed, either by the microflora[29] (Tables 25-26) or by the epithelium itself (Table 26). It is possible that the pathways leading to covalent binding of 2,4-DNT in extrahepatic tissues are more active than those for 2,6-DNT, or that the rate of removal of DNA adducts from extrahepatic tissue is faster with 2,6-DNT than with 2,4-DNT. This latter possiblity could be related to the nature of the genotoxic lesion or DNA adduct produced, which may be such that repair enzymes are more active in removing 2,6-DNT-induced damage than that caused by 2,4-DNT. For other carcinogens there is ample evidence that various adducts are removed at different rates (reviewed in ref 25). Finally, the similarity of the covalent binding of 2,4-DNT and 2,6-DNT to hepatic DNA of both species emphasizes that, in all likelihood, factors other than the initial extent of covalent DNA binding determine the ultimate outcome of the carcinogenic process initiated by these chemicals.

SECTION 3. LUNG TUMOR RESPONSE IN A/J MICE TO CHEMICALS ADMINISTERED INTRAPERITONEALLY AND ORALLY

With the exception of an early study in which polycyclic hydrocarbons were found to be more carcinogenic after intravenous administration than when given subcutaneously[2], there had been no systematic comparison of the lung tumor response in strain A mice to chemicals given by various routes. Therefore, we conducted a study to compare the ability of a series of compounds from different chemical classes (polycyclic hydrocarbon, nitrosamine, carbamate, hydrazine, dinitrotoluene and diaminotoluene) to induce lung tumors in A/J mice when given by at least two routes: i.p. and orally (p.o.) An i.p.-p.o. comparison seemed appropriate since most long-term chronic bioassays have employed the p.o. route and data comparisons between the lung tumor bioassay and long-term bioassays are desirable. In addition, it seemed appropriate to test the dinitrotoluene compounds; 2,4-DNT and 2,6-DNT, by the p.o. route since other data indicated that these compounds are metabolized principally by the bacterial flora in the bowel.[22,29] Finally, we decided to test both 2,4-diaminotoluene and 2,6-diaminotoluene in the lung tumor bioassay since these compounds are metabolites of the corresponding dinitrotoluenes. 2,4-Diaminotoluene is a hepatocarcinogen in the rat[30,31] and in female mice.[32] 2,6-Diaminotoluene is not carcinogenic in either the mouse or the rat.[33]

PROCEDURES

Animals and Housing

The source of strain A/J mice and the conditions for housing the animals are described in Section 1, Animals and Housing.

Chemicals

Reagent grade (>95% pure) 3-methylcholanthrene (MCA), benzo(a)pyrene (BP), diethylnitrosamine (DENA), urethan, 1,2-dimethylhydrazine (DMH), and ethylnitrosouea (ENU) were purchased from Sigma Chemical Co., St. Louis, MO. The diaminotoluene compounds; 2,4-diaminotoluene (2,4-DAT; >98% pure) and 2,6-diaminotoluene (2,6-DAT; >97% pure) were supplied by Dr. Mary C. Henry, U.S. Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, MD. The chemicals were stored at 4°C in the dark.

With sterile technique, the chemicals were administered either i.p. by injection or p.o. (gavage) as 0.1 ml/dose in water or in tricaprylin (glycerol trioctanoate, Eastman Kodak, Rochester, N.Y.). Solutions were freshly prepared immediately before administration. Amber colored bottles were used to protect the chemicals from fluorescent light.

Preliminary Toxicology

Maximum tolerated doses (MTD) for BP, ENU, DMH, 2,4-DNT, 2,6-DNT, the 2:1 mixture of 2,4-DNT and 2,6-DNT, 2,4-DAT and 2,6-DAT were determined. Serial twofold dilutions of each chemical or the mixture were administered either i.p. or p.o. to groups of eight mice (four males and four females). The MTD was the maximum dose that all eight mice tolerated (survived) after receiving 3 i.p. or p.o. treatments over a 1-week period. Animals were held for 1 month before experimental groups were initiated in order to detect delayed toxicity.

After i.p. administration, MCA and urethan have been shown to induce lung tumors in strain A mice[14], and DENA induced lung tumors in adult SWR mice.[34] Therefore, in the present study, the doses used for MCA, urethan, and DENA were selected from the published data.

Bioassays

Each compound was tested at three dose levels: the MTD, 0.5 MTD, and 0.2 MTD, with either approximately 32 or 52 mice (equal number of males and females) per dose. MCA, BP, urethan, DMH, and ENU were given in a single injection; DENA was administered once weekly for 4 weeks; and both the dinitrotoluenes and diaminotoluenes were given either three times per week for 8 weeks (i.p.) or two times per week for 12 weeks (p.o.). Control groups consisted of untreated and vehicle (water or tricaprylin)-treated mice. Animal weights were obtained every 2 weeks during the injection period and at monthly intervals thereafter.

Mice given MCA, BP, DENA, urethan, DMH, or ENU were killed 24 weeks after initiation of the bioassay. Animals that received either 2,4-DNT; 2,6-DNT; the 2:1 mixture of 2,4-DNT and 2,6-DNT; 2,4-DAT or 2,6-DAT were killed after 30 weeks. The lungs were fixed, tumors counted and a selected number of tumors sampled for histopathological evaluation and confirmation of adenoma as described in Section 1, Bioassays. In addition, the liver, kidneys, spleen, intestines, thymus, stomach, and the salivary and endocrine glands were examined grossly. If gross lesions were observed, they were examined histologically for the presence of neoplasms.

The lung tumor response (percentage of mice that developed lung tumors and the number of lung tumors per mouse) in experimental and control groups was compared as described in Section 1, Bioassays.

Lung Tumor Response in Controls

Table 40 presents results on the occurrence of lung adenomas in untreated and vehicle-treated A/J mice. The tumor responses in male and female mice were not significantly different ($p > 0.05$); therefore, results from the two sexes were combined. Data from untreated mice represent the "spontaneous" occurrence of lung tumors in A/J mice and agree closely with the data in Table 1, and with earlier results on mice of equivalent age.[3-5] The tumor responses in mice administered tricaprylin or distilled water either i.p. or p.o. were not significantly different ($p > 0.05$) from those in the untreated mice indicating that the occurrence of lung tumors was not affected by the injections of vehicle.

Lung Tumor Response in Chemically-Treated Mice:

Data on bioassays of the ten chemicals following i.p. or p.o. administration are summarized in Table 41. As for the controls, the tumor responses in male and female mice were not significantly different ($p > 0.05$); therefore, data from the two sexes were combined. MCA, at a total dose of 100 mg/kg, was highly toxic when given i.p. but not p.o. The animals were dying as late as 23 weeks after receiving the single injection. All other chemicals were relatively nontoxic at the doses administered except DMH, which induced 50% mortality when given p.o. at a dose of 25 mg/kg.

When compared to the appropriate vehicle controls, MCA, DENA, urethan, and ENU produced significant ($p < 0.05$) and dose-related increases in the lung tumor response when given either i.p. or p.o. BP was carcinogenic at all doses when given i.p. and at the highest dose after p.o. administration. DMH was positive at the high and middle p.o. doses and at the highest dose following i.p. administration. 2,6-DAT was positive at the high dose only when given i.p., but not after p.o. administration. 2,4-DNT; 2,6-DNT; the 2:1 mixture of 2,4-DNT and 2,6-DNT and 2,4-DAT were inactive by both routes and at all dose levels.

The tumor responses to MCA, BP, the highest dose of DENA, and the middle doses of both ENU and DMH varied as a function of the route of administration (Table 41). This finding was most evident for the polycyclic hydrocarbons. At all doses, MCA and BP induced significantly more lung tumors when given i.p. as compared to p.o. For example, the tumor response in animals receiving the middle dose of MCA or the highest dose of BP by the i.p. route exceeded that by the p.o. route by factors of 12 and 13, respectively. Only two animals survived the highest i.p. dose of MCA (100 mg/kg); therefore, lung tumor responses to 100 mg/kg MCA given either i.p. or p.o. could not be compared. DENA (200 mg/kg) and ENU (50 mg/kg) induced 1.45 (DENA) and 2 (ENU) times as many tumors per lung when given i.p. as compared to p.o. In contrast, DMH, at the dose of 12.5 mg/kg, produced significantly more tumors per lung when administered p.o. The lung tumor response to urethan did not vary significantly by either route of administration.

Table 40. LUNG TUMOR RESPONSE IN UNTREATED AND VEHICLE-TREATED A/J MICE[a]

Treatment	Route	Number of Treatments	Duration of Experiment (weeks)	Survivors/ Initial[b]	Mice With Lung Tumors (%)	Average Number Lung Tumors/ Mouse
Untreated	-	-	24	175/175	30	0.35 ± 0.06[c]
Untreated	-	-	30	94/96	30	0.38 ± 0.10
Tricaprylin	i.p.	1	24	32/32	25	0.28 ± 0.08
Tricaprylin	i.p.	24	30	52/52	29	0.37 ± 0.09
Tricaprylin	p.o.	1	24	98/100	23	0.28 ± 0.06
Tricaprylin	p.o.	24	30	45/50	27	0.31 ± 0.08
Water	i.p.	1	24	46/50	26	0.28 ± 0.07

[a] Table contains control data for bioassays of the compounds described in Section 2 (see Table 2).

[b] Combined data from males and females.

[c] Values are mean ± standard error.

Table 41

LUNG TUMOR RESPONSE IN A/J MICE TO EIGHT CHEMICALS ADMINISTERED EITHER INTRAPERITONEALLY OR ORALLY

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[a] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
MCA (i.p.)[b]	T[c]	24	100[d]	32	2	100	199
			50	32	31	100	139 ± 9.39[e,f]
			20	32	28	100	57 ± 3.09[e,f]
MCA (p.o.)	T	24	100	24	18	100	6 ± 0.98[e]
			50	32	32	100	12 ± 1.14[e]
			20	32	31	90	2 ± 0.26[e]
BP (i.p)	T	24	100	32	32	100	13 ± 1.06[e,f]
			50	32	31	100	5 ± 0.60[e,f]
			20	33	33	67	1 ± 0.16[e,f]
BP (p.o.)	T	24	100	32	32	69	1.00 ± 0.24[e]
			50	32	31	45	0.71 ± 0.16
			20	32	32	35	0.45 ± 0.13

Table 41 (continued)

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[a] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
DENA (i.p.)	T	24	200	32	28	100	16 ± 2.22[e,f]
			100	34	34	100	6 ± 0.58[e]
			40	30	21	85	2 ± 0.31[e]
DENA (p.o.)	T	24	200	32	31	100	11 ± 0.80[e]
			100	32	30	97	5 ± 0.53[e]
			40	32	32	100	2 ± 0.18[e]
Urethan (i.p.)	W	24	1000	32	29	100	31 ± 1.43[e]
			500	32	27	100	13 ± 0.79[e]
			200	38	38	92	5 ± 0.63[e]
Urethan (p.o.)	W	24	1000	51	51	100	28 ± 1.08[e]
			500	32	32	100	13 ± 0.68[e]
			200	32	32	78	4 ± 0.50[e]

Table 41 (continued)

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[a] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
ENU (i.p.)	T	24	100	32	31	100	20 ± 1.39[e]
			50	32	32	100	10 ± 1.89[e,f]
			20	32	32	78	2 ± 0.26[e]
ENU (p.o.)	T	24	100	32	32	100	17 ± 1.93[e]
			50	32	30	97	5 ± 1.56[e]
			20	32	31	87	2 ± 0.21[e]
DMH (i.p.)	W	24	25	31	31	42	0.71 ± 0.18[e]
			12.5	32	32	25	0.28 ± 0.09
			5	33	33	27	0.30 ± 0.09
DMH (p.o.)	W	24	25	32	16	44	0.75 ± 0.25[e]
			12.5	32	32	56	0.75 ± 0.14[e,f]
			5	32	32	50	0.53 ± 0.10

Table 41 (continued)

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[d] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
2,4-DAT (p.o.)	T	24	750	32	28	29	0.36 ± 0.12
			375	32	27	41	0.56 ± 0.12
			150	32	28	43	0.46 ± 0.11
2,4-DAT (i.p.)	T	24	750	32	29	17	0.28 ± 0.13
			375	32	31	29	0.35 ± 0.11
			150	33	32	41	0.47 ± 0.11
2,6-DAT (p.o.)	T	24	375	32	29	59	0.59 ± 0.14
			187	32	28	39	0.43 ± 0.11
			75	32	28	32	0.36 ± 0.11
2,6-DAT (i.p.)	T	24	375	32	29	59	0.78 ± 0.14[e]
			187	32	32	34	0.44 ± 0.13
			75	32	32	34	0.44 ± 0.13

Table 41 (continued)

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[a] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
2,4-DNT (i.p.)[g]	T	30	3000	52	50	26	0.28 ± 0.07
			1500	52	52	19	0.21 ± 0.06
			600	53	52	44	0.46 ± 0.08
2,4-DNT (p.o.)	T	30	6000	52	44	23	0.30 ± 0.09
			3000	52	48	31	0.31 ± 0.07
			1200	52	47	28	0.34 ± 0.08
2,6-DNT (i.p.)	T	30	3000	52	47	30	0.40 ± 0.10
			1500	52	51	45	0.53 ± 0.09
			600	52	50	34	0.40 ± 0.09
2,6-DNT (p.o.)	T	30	6000	52	38	34	0.47 ± 0.12
			3000	52	50	22	0.26 ± 0.07
			1200	52	49	18	0.27 ± 0.09

Table 41 (continued)

Compound (Route)	Vehicle	Duration of Experiment (weeks)	Total Dose[a] (mg/kg)	No. of Animals		Survivors with Lung Tumors	
				Initial	Survivors	%	No. Tumors/Mouse (Mean ± S.E.)
2,4-DNT:2,6-DNT (2:1)[g] (i.p.)	T	30	4800	52	40	23	0.25 ± 0.08
			2400	52	50	28	0.32 ± 0.08
			960	52	48	33	0.37 ± 0.08
2,4-DNT:2,6-DNT(2:1) (p.o.)	T	30	6000	52	48	33	0.40 ± 0.09
			3000	52	48	35	0.40 ± 0.08
			1200	52	48	35	0.40 ± 0.08

[a] Total cumulative dose per animal

[b] MCA, 3-methylcholanthrene; BP, benzo(a)pyrene; DENA, diethylnitrosamine; ENU, ethylnitrosourea; DMH, dimethylhydrazine; DNT, dinitrotoluene; DAT, diaminotoluene.

[c] T, tricaprylin; W, distilled water

[d] MCA, BP, urethan, ENU and DMH were given in a single administration; DENA was administered once weekly for 4 weeks; the diaminotoluenes and dinitrotoluenes were given either 3 times per week for 8 weeks (i.p.) or 2 times per week for 12 weeks (p.o.).

[e] Significantly different ($p < 0.05$) from corresponding vehicle treated control (Table 1).

[f] Significantly different ($p < 0.05$) from corresponding p.o. or i.p. group.

[g] The i.p. data for these groups are also given in Table 2.

Table 42. LESIONS OTHER THAN LUNG TUMORS NOTED IN I.P.-P.O. STUDY

Compound	Total dose (mg/kg)	Route	No. of animals with lesions/ No. of animals	Site	Histologic appearance
MCA[a]	100	i.p.	7/32	peritoneal serosa	sarcoma (poorly differentiated)
"	50	i.p.	1/32	peritoneal serosa	sarcoma (poorly differentiated)
BP	50	i.p.	1/32	hind leg	fibrosarcoma
"	50	p.o.	1/32	preputial gland	adenocarcinoma
DENA	200	p.o.	4/32	forestomach	squamous cell carcinoma
"	200	p.o.	8/32	liver	bile duct hyperplasia
"	100	p.o.	2/32	forestomach	squamous cell carcinoma
ENU	100	p.o.	1/32	spine	lymphosarcoma
DMH	5	p.o.	1/33	duodenum	undifferentiated carcinoma

[a] MCA, 3-methylcholanthrene; BP, benzo(a)pyrene; DENA, diethylnitrosamine; ENU, ethylnitrosourea; DMH, dimethylhydrazine

Lesions in Other Organs

Lesions other than lung adenomas observed at necropsy among the various chemically treated groups are summarized in Table 42. There were no grossly appearing lesions other than lung adenomas observed among the controls or in mice administered the dinitrotoluene and diaminotoluene compounds.

Histological examination of paraffin-embedded tissues from MCA-treated mice indicated that the peritoneal tumors were poorly differentiated sarcomas. These sarcomas were spread throughout the peritoneal surface of the diaphragm, liver, stomach, pancreas, intestines, and kidneys in a circumferential growth that resembled the patterns seen in mesotheliomas. However, only spindle-shaped cells were observed in areas of parenchymal invasion; epithelial-like cells forming pseudo-glands typical of mesotheliomas were not seen.

There were no grossly observable lesions other than lung adenomas in mice administered i.p. DENA. However, in animals given p.o. DENA, forestomach tumors were observed in 6 of 64 treated mice and liver lesions in 8 of 32 mice. The forestomach tumors were well-differentiated squamous cell carcinomas that varied from microinvasive papillary elevations to invasive neoplasms that penetrated the stomach wall. The grossly observable liver lesions appeared to be the result of bile duct hyperplasia. The bile ducts appeared as multiple cystic profiles that occasionally exceeded 0.5 mm in diameter. A single neoplastic nodule was observed immediately under the capsular surface of one liver. In addition, all eight livers contained multiple eosinophilic focal areas of cellular alteration that were not evident in the centrilobular portions of the liver. Cells within the foci were two to three times larger than normal surrounding hepatocytes and their nuclei were pleomorphic, enlarged, and frequently contained nuclear inclusions.

A lymphosarcoma of the spine and an undifferentiated carcinoma of the duodenum were observed in mice given p.o. ENU and DMH, respectively.

DISCUSSION

The investigations in Section 3 were undertaken to compare the carcinogenic potential of a series of compounds from different chemical classes (polycyclic hydrocarbon, nitrosamine, nitrosourea, carbamate, hydrazine, and dinitrotoluene) to induce lung tumors in A/J mice when given by the p.o. and i.p. routes.

MCA, BP, urethan, DENA, ENU, and DMH induced a significant increase in the lung tumor response when given both i.p. and p.o. On a molar dose basis, the tumor responses to i.p. administered MCA and urethan were similar to those previously reported in strain A mice.[35,36] BP, administered p.o., is approximately one-fifth as active for lung tumor induction in A/J mice (present study) than in A/HeJ mice.[37] This result was unexpected since strains A/J and A/HeJ mice have similar lung tumor responses to i.p. administered urethan.[36] Possibly the difference in tumor response is related to the use of different vehicles (tricaprylin vs corn oil[37]). DENA, given i.p. was approximately four times more active for lung tumor induction in A/J mice than in SWR (Swiss) mice.[34] The tumor response to i.p. administered ENU is nearly identical to that of an earlier report[38] in which female A/J mice were used in transplacental studies. DMH, a strong colon carcinogen, has not been reported to induce lung tumors in strain A mice, and was only marginally active at the dose levels given in the present study. Hydrazine sulfate and 1,1-DMH induced lung tumors in BALB/c and SWR mice, respectively, when administered by gavage[39,40], and at 200-fold higher doses than employed for 1,2-DMH in the present study. To our knowledge, this is the first report of the ability of MCA, urethan, DENA, and ENU to induce lung tumors in strain A mice when administered p.o. and of BP to induce lung tumors when given i.p.

The lung tumor responses to MCA, BP, DENA, ENU, and DMH were significantly different when the compounds were given i.p. as compared to p.o. This difference was particularly evident for the polycyclic hydrocarbons, MCA and BP, which were several-fold more active when given i.p. and indicates that the i.p. route is superior to the p.o. route when testing polycyclic hydrocarbons for lung tumor induction in A/J mice. However, at high doses, strongly carcinogenic polycyclic hydrocarbons may induce a higher mortality when given i.p. as compared to p.o. (see data for 100 mg/kg MCA; Table 41). It is likely that the high mortality in mice given 100 mg/kg MCA i.p. was due to the development of fibrosarcomas in the peritoneal cavity since these tumors were observed in all seven mice (Table 42) necropsied prior to death including the two animals that survived 24 weeks. Unfortunately, the other 25 mice that died during the bioassay were too decomposed for necropsy; therefore, it is not known whether they had fibrosarcomas, or any other tumors.

Studies of the distribution and elimination of MCA were undertaken to determine the basis for the observed difference in the lung tumor response when the compound is given either i.p. or p.o.[14] These studies showed that low amounts of MCA (or its metabolites) are present in all organs, both after i.p. and p.o. administration, and that these amounts are two- to threefold lower after p.o. than after i.p. administration. Approximately 50% of unchanged MCA was found in the liver and small intestine 72 hours after i.p. administration; whereas, no unchanged MCA was found at this time after p.o. administration.

These data suggest that p.o. administered MCA is either rapidly eliminated through the bowel without being absorbed and metabolized, or that it is essentially metabolized before being absorbed and the metabolites are rapidly detoxified (e.g. by conjugation) in the gastrointestinal tract. Either result would lead to lower amounts of the ultimate carcinogen reaching the lungs, the target organ in the bioassay, and possibly explain the lower tumor yield observed after p.o. administration (Table 41). Similar explanations may apply to other polycyclic hydrocarbons, such as BP, for which the lung tumor response after i.p. administration was also much higher than after p.o. administration (Table 41).

The lung tumor response to the highest dose of DENA and the middle doses of ENU and DMH also varied as a function of the route of administration. However, these differences occurred at only one dose level and require verification by additional experiments. Urethan produced a similar lung tumor response when given by both routes. Pharmacokinetic studies of the uptake, distribution, and metabolism of urethan in A/J mouse tissues are required to determine if the absence of a difference in tumor response after i.p. and p.o. administration can be explained by pharmacokinetic parameters.

As indicated in Section 1, the dinitrotoluenes; 2,4-DNT, 2,6-DNT and the 2:1 mixture of 2,4-DNT and 2,6-DNT, did not induce lung tumors in strain A/J mice following i.p. administration. Data from Section 3 indicate that the compounds were also negative for lung tumor induction when given p.o. Therefore, the availability of these compounds for metabolism directly by the microbial flora did not result in the production of metabolites that induced lung tumors in the animals.

2,6-Diaminotoluene induced a significant (albeit marginal) increase in the lung tumor response when given i.p. at a total dose of 375 mg/kg. After p.o. administration at the same dose, the compound produced an increase in the tumor response when compared to tricaprylin controls (0.59 vs 0.31 tumors per mouse), but the difference was not significant ($p > 0.05$). Therefore, 2,6-DAT should be considered as only marginally active in this model system since the tumor response was only slightly elevated when the compound was given by a single route (i.p.) and it was not dose-related. 2,4-DAT was negative after both i.p and p.o. administration. These results in the strain A/J mouse are not in agreement with other data on the diaminotoluenes. 2,4-DAT was observed to induce liver tumors in the rat[30,31] and in female mice.[32] In contrast, 2,6-DAT was not carcinogenic in either the rat or the mouse.[33]

In previous carcinogenicity studies, tumors were not observed on the surface of the liver of strain A mice after the standard 6-month bioassay, and histological studies of the liver were not performed. However, the identification of liver lesions in mice given p.o. DENA in the present study suggests that the livers of strain A mice should be examined routinely for histopathological changes at the end of the 6-month bioassay. This examination could permit the identification of chemicals that produce foci of altered hepatocytes or other lesions suggestive of their carcinogenic potential.

SECTION 4 - BIOASSAY OF DYE COMPOUNDS FOR CARCINOGENIC ACTIVITY IN THE STRAIN A MOUSE

During the third year of this contract, we tested two dye compounds: C.I. Solvent Yellow 33 and a C.I. Solvent Green 3 - C.I. Solvent Yellow 33 mixture, for carcingoenic activity in the strain A mouse lung tumor bioassay. Both dyes are incorporated into colored smoke grenades and, during the manufacture of these munitions, workers are exposed to the dyes via the dermal and inhalation routes. The strain A mouse lung tumor bioassay was chosen to evaluate the potential carcinogenic activity of the dyes since the standard route of administration for this bioassay, intraperitoneal, may allow a greater amount of the dyes to reach the target organ.

Both dyes were tested in the lung tumor bioassay under a subcontract to DAMD 17-81-C-1178, and a final report of these studies was submitted in January, 1985.[41] Neither C.I. Solvent Yellow 33 nor the mixture of C.I. Solvent Green 3 - C.I. Solvent Yellow 33 induced a significant increase in the lung tumor response when compared to controls. Therefore, both dyes were non-carcinogenic in the lung tumor bioassay.

SECTION 5 - DEVELOPMENT AND EVALUATION OF A TRANSPLACENTAL CARCINOGENESIS BIOASSAY IN STRAIN A/J MICE

Transplacental carcinogenesis is the induction of tumors in the offspring as a result of prenatal exposure to an agent circulating in the mothers system. The first report on the transplacental induction of lung tumors in mice with a chemical carcinogen (urethan) was published in 1947.[42] Since that time, several investigators have administered urethan in single or repeated doses at different times during the gestation period.[43-46] All treatment schedules resulted in the induction of lung tumors in the offspring, however, the mouse fetus was the most susceptible to lung tumor induction when the pregnant female was treated with urethan within 24 hours of parturition.[46]

Urethan, when given during this period of maximum susceptibility for the fetus has been shown to induce a greater number of tumors with a shorter latency period than in adult mice treated with a comparable dose.[46] The increased sensitivity to urethan of the fetus during the last 24 hours of gestation is most likely due to the ability of the tissues to metabolize urethan into an ultimate carcinogen; early fetal tissues cannot metabolize urethan to any measurable extent.[47]

In 1979, Armuth and Berenblum[48] used transplacental carcinogenesis for the study of initiation and promotion of lung tumors in mice. The fetuses of Balb/c mice were initiated prenatally with tritiated thymidine, followed after birth by repeated i.p. injections of phorbol. A higher lung tumor response was observed in the mice that were treated with tritiated thymidine plus phorbol than those treated with tritiated thymidine alone. Other researchers have demonstrated the phenomenon of initiation and promotion on the chemical induction of tumors in newborn mice.[49-51] The tumor response to an initiating dose of carcinogen in newborn mice could be promoted by the repeated administration of such compounds as phenobarbital[51] and phorbol.[50] Taking these earlier studies into consideration, we decided to develop a transplacental carcinogenesis model to investigate the ability of selected chemicals to initiate and/or promote the induction of lung tumors in strain A mice. The initial experiment was done with ethylnitrosourea (ENU) as the initiator and butylated hydroxytoluene (BHT) as the promoter.

PROCEDURES

Animals and Housing

Weaned virgin female A/J mice, selected from our breeding colony at the Medical College of Ohio were used. The mice were maintained as described in Section 1, Animals and Housing.

Chemicals

The initiator, ethylnitrosourea (ENU), and the promoter, butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co., St. Louis, MO. ENU was stored at 4°C in the dark, and BHT was stored at room temperature.

ENU and BHT were administered intraperitoneally (i.p.) by injection in tricaprylin. Solutions were freshly prepared each day before administration. Amber colored bottles were used to protect the chemicals from fluorescent light.

Bioassay

Weaned virgin female A/J mice were used. They were grouped together at weaning, 8 females in a large polycarbonate cage. This grouping of females has been shown to synchronize oestrus in mice.[52] At 10-12 weeks of age the mice were placed in individual cages, and a male approximately the same age was added. The females were checked for the presence of a vaginal plug the next morning and afternoon, or until a plug was noted. When the plug was detected, the female was separated from the male, and that day was considered day 1 of gestation.

To ensure sufficient numbers of newborns for the bioassay, we began with 150 females. This number of females allowed for failures to impregnate, fetal deaths and small litters. After 10 days of gestation, confirmation of pregnancy was made by gross observation and palpation. Females not pregnant were excluded from the experiment.

Timed pregnant mice were randomized and divided into three equal groups as shown in Table 43. The mice were treated on the sixteenth day of gestation with a single i.p. injection of ENU at either 10 or 2 mg/kg or they were untreated. The time of injection and the weight of the females was recorded. The animals were observed every 4 hours during the next 24 hours and when parturition occurred, the date of birth and number of young were recorded. Fifteen days after parturition the three groups with their offspring were each divided into three treatment groups designated A, B or C (Table 43). The offspring in group A received no treatment, group B received i.p. injections of the promoter (BHT) and group C received i.p. injections of the vehicle (distilled water).

Table 43. SCHEMATIC DIAGRAM OF TRANSPLACENTAL CARCINOGENESIS STUDIES IN STRAIN A/J MICE

Pregnant A/J Mice	Treatment 16th day of gestation+	15 days after Parturition	Treatment of Progeny*	Approximate No of Animals
Group 1	Untreated	A	Untreated	50
		B	Promoter	50
		C	Vehicle	50
Group 2	ENU 10 mg/kg	A	Untreated	50
		B	Promoter	50
		C	Vehicle	50
Group 3	ENU 2 mg/kg	A	Untreated	50
		B	Promoter	50
		C	Vehicle	50

+ Animals were given a single i.p. injection, 0.1 ml.

* Progeny were injected 3 X per week for 12 wks for a total of 22 injections.

RESULTS

Of the 114 A/J females that were bred, 70 females had litters. Due to cannibalism, only 8 of the 70 litters survived (see Table 44). In an effort to increase the survival rate, the pregnant mice were given nesting materials, noise was kept at a minimum and the bedding was not changed four days prior to and after birth of the litters. In spite of these efforts, the survival rate of the progeny of ENU-treated animals (approximately 11%) was too low to justify the continuation of this study.

In future studies, it was decided to employ one of the A/J X C3H hybrid strains (see Section 6) for studies in transplacental carcinogenesis. In our experience, these strains have a lower rate of cannabalism, larger litters, and are heartier than A/J mice. In addition, the target organs for the hybrid strain would include both the liver and the lung.

Table 44. STRAIN A/J TRANSPLACENTAL CARCINOGENESIS STUDY - SURVIVAL RATE OF OFFSPRING

Treatment of Female	No. females that had litters	No. litters surviving	Survival Rate (%)
ENU[a], 10 mg/kg	23	0	0
ENU, 2 mg/kg	29	3	9.4
Untreated	18	5	21.7
Total Number	70	8	11.4

[a] ENU = ethylnitrosourea

SECTION 6 - DEVELOPMENT OF A HYBRID MOUSE LUNG/LIVER MODEL FOR CARCINOGENESIS BIOASSAYS

The strain A mouse lung tumor bioassay for testing the potential carcinogenic effects of environmental chemicals was developed in 1940 by Shimkin.[2] Since 1940, more than 300 compounds from different chemical classes have been tested for carcinogenic activity by the lung-tumor-induction technique.[3-5] In general, the lung tumor bioassay has many positive features: (a) It is highly susceptible to the carcinogenic effects of the polycyclic aromatic hydrocarbons and various alkylating agents[3-5]; (b) The tumor response to relatively strong carcinogens is dose-related thus permitting quantitative comparisons of carcinogenic potential; (c) Compounds found to be positive in the lung tumor bioassay are usually, but not always, carcinogenic in other bioassays; and, (d) When compared to long-term carcinogenesis bioassays, the lung tumor test is more rapid, less expensive and less labor intensive.

In spite of these positive attributes, the strain A mouse lung tumor bioassay is not without its limitations. As indicated in this report (Section 1), the lung tumor test is relatively insensitive to liver carcinogens. For example, the potent rat liver carcinogens: diethylnitrosamine, aflatoxin B_1 and 2-acetylaminofluorene were found to be 33-, 120-, and 11,740-fold less active, respectively, than 3-methylcholanthrene for the induction of lung tumors in A mice.[5] In a recent report in which data from the mouse lung tumor bioassay were compared to long-term carcinogenesis bioassays, Maronpot, et al.[53] found that the lung tumor test correctly predicted the carcinogenicity, or lack thereof, for only 37% of 54 chemicals assayed. In addition, there were 44 percent "false positives" and 75% "false negatives." Most of the chemicals tested were liver carcinogens such as the aromatic amines. In addition, the lung tumor bioassay is not sensitive to certain bladder carcinogens or to some of the unstable, direct-acting alkylating agents.[54] In view of the limited ability of the lung tumor bioassay to detect the tumorigenic potential of many liver carcinogens, we decided to develop a mouse strain with the potential for a broader range of carcinogenic susceptibility. To accomplish this, A/J mice (high lung tumor susceptibility) were crossed with C_3HeB/FeJ mice (high liver tumor susceptibility[55,56]) to develop hybrids that could be more susceptible to both "lung" and "liver" carcinogens. The progress of these studies through August 31, 1984 will be described in this report.

PROCEDURES

Animals and Housing

Breeding colonies of both A/J mice and C_3HeB/FeJ mice were established at the Medical College of Ohio. Breeding sets of each strain were obtained initially from the Jackson Laboratories, Bar Harbor, ME. The mice were kept on corn-cob bedding in temperature (22°C) and humidity (30-60%) controlled rooms with a 12 hour light/darkness cycle. They were given Purina Certified

Diet and water ad libitum. Hygienic conditions were maintained by twice-weekly changes of the animal cages and water bottles and both were sterilized routinely. The health status of the breeding colonies and the mice used in the bioassays was periodically examined by: (a) complete gross necropsy, tabulating gross lesions, and general condition and body weight of each animal; (b) histopathological evaluation of formalin fixed tissues involving all major organs; and (c) serological tests for the presence of murine virus infections.

Chemicals

Diethylnitrosamine (DENA) was purchased from Sigma Chemical Co., St. Louis, MO, and was stored at 4°C in the dark. DENA was administered i.p. by injection as 0.1 ml/dose in water. Solutions were freshly prepared immediately before administration. Amber-colored bottles were used to protect the chemicals from fluorescent light.

Preliminary Toxicology

Previous data by other investigators indicated that DENA, at a dose of 50 mg/kg, was sufficient to induce liver tumors in C_3HeB/FeJ mice.[57] Therefore, a preliminary toxicity study was undertaken in both A/J mice and C_3HeB/FeJ mice given in a single i.p. injection at doses of 200, 100, 50, 25 and 10 mg/kg. The animals were kept for four weeks for evidence of delayed toxicity. The dose of 50 mg/kg was the highest dose that all mice tolerated (survived) during this period; therefore, this dose was selected for the assay.

Bioassay

The bioassay was conducted with four groups of mice: (a) A/J male X A/J female; (b) C_3HeB/FeJ male X C_3HeB/FeJ female; (c) A/J female X C_3HeB/FeJ male; and, (d) C_3HeB/FeJ female X A/J male. The litters of the four crosses were divided in half. One half of the litters were used in the experiments at 15 days old, and the remaining one-half were used at 6-8 weeks of age. The 15 day-old mice were divided into three treatment groups: DENA-treated (a single i.p. injection of 50 mg/kg); vehicle (distilled water)-treated; and untreated controls. These animals were sacrificed at 12, 24 and 52 weeks after treatment with DENA. The 6-8 week-old mice were divided into the following treatment groups: DENA-treated (a single i.p. injection of either 50, 25 or 10 mg/kg), vehicle (distilled water)-treated; and untreated controls. These animals were sacrificed at 24 and 52 weeks after treatment.

At necropsy the mice were weighed and body weights recorded. Lung and liver tissues were removed. The liver was weighed and fixed in toto in 10% phosphate-buffered formalin. Lungs were fixed in Tellyesniczky's solution.[3-5] The remaining body organs were examined for pathologic lesions, and any grossly visible lesions were examined by light microscopy. The number of grossly visible tumors in livers and lungs were counted by two technologists working independently.

At the time of completion of this contract, the initial experiment on the liver and lung tumor response to DENA in 15 day-old mice and 6-8 week-old mice was not completed. Carcinogen-treated and control animals from the 15 day-old series were harvested at 3 and 6 months after treatment and the liver and lung tumors enumerated. However, the 12-month data had not been obtained. Similarly, carcinogen-treated and control animals from the 6-8 week-old series were harvested at 6 months after treatment, but the 12-month data had not been collected. In this report, we will describe the results obtained through August 31, 1984, the date of completion of the contract.

A. Fifteen-Day Treatment Series

1. Diethylnitrosamine-treated mice

a. Liver tumors. Tumors were not observed on the surface of the livers of any of the carcinogen-treated parental or hybrid mice after 3 months (Table 45). However, 6 months after treatment, all parental and hybrid strains exhibited liver tumors. The tumor response was significantly higher in male mice of all strains than in females. All male mice from the C_3HeB/FeJ parental strain had liver tumors with a multiplicity of 84 tumors per mouse. Male offspring derived from the A/J female X C_3HeB/FeJ male cross had a tumor incidence of 100% and a multiplicity of 18 tumors per mouse. Male offspring from the C_3HeB/FeJ female X A/J male cross had a tumor incidence of 100% and a multiplicity of 15 tumors per mouse. The liver tumor response was lowest in the A/J parental strain since only one tumor was observed in a male mouse 6 months after treatment with DENA.

b. Lung tumors. Lung tumors were observed in all parental and hybrid strains 3 months after treatment with DENA (Table 45). The highest lung tumor response occurred in A/J mice in which the males exhibited a 70% tumor incidence (average no. = 1 tumor per mouse) and the females a 29% tumor incidence (average no. = 0.5 tumors per mouse). Tne A/J female X C_3HeB/FeJ male hybrid had a higher tumor incidence in both sexes than did the C_3HeB/FeJ female X A/J male hybrid. The tumor response in both hybrids was higher in the males than in the females. As expected, the lung tumor incidence was lowest in the C_3HeB/FeJ parental strain with only 1 in 17 animals exhibiting a single tumor.

Six months after treatment with DENA, the highest lung tumor incidence occurred in the C_3HeB/FeJ female X A/J male hybrid mice (average no. = 2.63 tumors per mouse). The tumor occurrence in the A/J parental strain was similar to that in the A/J female X C_3HeB/FeJ male cross. The tumor response was lowest in the C_3HeB/FeJ parental strain with only 1 in 14 animals exhibiting a single tumor.

Table 45. LIVER AND LUNG TUMOR RESPONSE IN PARENTAL AND HYBRID MICE FOLLOWING TREATMENT WITH DIETHYLNITROSAMINE[a]

Strain	Sex	No. of mice sampled	Time of sampling (months)	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
							% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	17	3	23.9	1.5	4.8	0 (0)	0.00 ± 0.00	70.5 (12)	1.00 ± 0.21
	F	24	3	19.1	1.0	5.3	0 (0)	0.00 ± 0.00	29.2 (7)	0.50 ± 0.20
"	M	18	6	24.4	1.1	4.8	5.5 (1)	0.06 ± 0.06	66.7 (12)	1.40 ± 0.35
	F	22	6	19.9	1.0	5.2	0 (0)	0.00 ± 0.00	81.8 (18)	1.90 ± 0.37
C_3HeB/FeJ	M	10	3	26.3	1.5	5.8	0 (0)	0.00 ± 0.00	10 (1)	0.10 ± 0.10
	F	7	3	22.1	1.3	6.1	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
"	M	4	6	33.0	2.0	6.1	100 (4)	84.25 ± 12.93	25 (1)	0.25 ± 0.25
	F	10	6	28.4	1.3	4.8	40 (4)	0.50 ± 0.22	0 (0)	0.00 ± 0.00
A/J (female) x C_3HeB/FeJ (male)	M	10	3	26.7	1.4	5.2	0 (0)	0.00 ± 0.00	60 (6)	0.90 ± 0.31
	F	8	3	21.3	1.0	4.9	0 (0)	0.00 ± 0.00	38 (3)	0.50 ± 0.27
"	M	4	6	44.1	2.4	5.5	100 (4)	18.00 ± 7.27	100 (4)	1.50 ± 0.29
	F	4	6	35.3	1.6	4.7	25 (1)	0.75 ± 0.75	100 (4)	1.25 ± 0.25
C_3HeB/FeJ (female) x A/J (male)	M	13	3	31.0	1.7	5.5	0 (0)	0.00 ± 0.00	15 (2)	0.23 ± 0.17
	F	15	3	26.0	1.4	5.5	0 (0)	0.00 ± 0.00	7 (1)	0.13 ± 0.13
"	M	27	6	39.8	2.2	5.5	100 (27)	15.19 ± 2.57	89 (24)	2.63 ± 0.33
	F	8	6	32.3	1.6	4.9	13 (1)	0.13 ± 0.13	88 (7)	2.63 ± 0.42

[a] 15 day-old parental and hybrid strains received a single i.p. dose of diethylnitrosamine at 50 mg/Kg. After 3 and 6 months, the animals were sacrificed and the surface tumors on the liver and lungs counted.

2. Vehicle-treated and untreated mice

Tables 46 and 47 illustrate the occurrence of both liver and lung tumors in vehicle (distilled water)-treated and control mice after 3 and 6 months. There was a very low incidence of both liver and lung tumors in all parental and hybrid strains at both time periods. Therefore, the occurrence of "spontaneously-arising" tumors in the hybrid and parental strains is minimal, and treatment with the vehicle did not result in the induction of tumors in either organ.

B. Six to Eight-Week Treatment Series

1. Diethylnitrosamine-treated mice

a. Liver tumors. No tumors were observed on the surface of the livers of A/J strain mice 6 months after treatment with DENA (Table 48). Male C_3HeB/FeJ mice had an average of 1.14 tumors per liver whereas females had only 0.13 tumors per liver. The liver tumor response in the two hybrid strains was 0.14 tumors per mouse. The liver tumor response in mice treated at 6-8 weeks of age was significantly lower than in mice treated at 15 days of age.

b. Lung tumors. The lung tumor response to DENA was higher in females than in males of all strains (Table 48). The response was in the order of A/J > A/J female X C_3HeB/FeJ male > C_3HeB/FeJ > C_3HeB/FeJ female X A/J male. With the exception of the C_3HeB/FeJ female X A/J male hybrid, the lung tumor responses in the 6 to 8 week-old animals were higher than in the 15 day-old animals (compare Tables 45 and 48).

2. Vehicle-treated and untreated mice

The occurrence of both liver and lung tumors in vehicle (distilled water)-treated and control mice after 6 months is illustrated in Tables 49 and 50. Only one liver tumor was observed. This tumor occurred in a C_3HeB/FeJ mouse that had received a single injection of distilled water. The lung tumor occurrence was in the order of A/J > A/J female X C_3HeB/FeJ male > C_3HeB/FeJ female X A/J male > C_3HeB/FeJ.

Table 46. LIVER AND LUNG TUMOR RESPONSE IN VEHICLE-TREATED PARENTAL AND HYBRID MICE[a]

Strain	Sex	Treatment[a]	No. of mice sampled	Time of sampling (months)	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
								% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	Water	1	3	20.3	1.0	5.3	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
	F	"	9	3	19.2	1.0	5.5	11.1 (1)	0.11 ± 0.11	0 (0)	0.00 ± 0.00
"	M	"	10	6	25.9	1.2	4.7	0 (0)	0.00 ± 0.00	17 (1)	0.10 ± 0.10
	F	"	11	6	22.2	0.9	4.4	0 (0)	0.00 ± 0.00	33 (2)	0.18 ± 0.12
C_3HeB/FeJ	M	"	11	3	27.0	1.4	5.5	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
	F	"	11	3	21.7	1.3	6.0	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
"	M	"	14	6	30.7	1.5	5.1	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
	F	"	13	6	24.7	1.4	5.6	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
A/J (female) x C_3HeB/FeJ (male)	M	"	4	3	30.0	1.4	4.6	0 (0)	0.00 ± 0.07	0 (0)	0.00 ± 0.00
	F	"	12	3	22.8	1.2	5.4	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
"	M	"	26	6	36.2	1.6	4.4	0 (0)	0.00 ± 0.00	0 (0)	0.00 ± 0.00
	F	"	23	6	29.3	1.3	4.4	0 (0)	0.00 ± 0.00	4.3 (1)	0.04 ± 0.04
C_3HeB/FeJ (female) x A/J (male)	M	"	11	3	37.2	1.6	4.5	0 (0)	0.00 ± 0.07	0 (0)	0.00 ± 0.00
	F	"	15	3	28.2	1.3	4.8	7 (1)	0.07 ± 0.07	0 (0)	0.00 ± 0.00
"	M	"	13	6	43.5	1.8	4.2	7.7 (1)	0.14 ± 0.08	0 (0)	0.00 ± 0.00
	F	"	10	6	36.1	1.5	4.2	0 (0)	0.00 ± 0.00	10 (1)	0.10 ± 0.10

[a] 15 day-old hybrid mouse study. Parental and hybrid mice were given a single i.p. injection of 0.1 ml distilled water. Three and 6 months later the mice were killed and the surface tumors on the liver and lungs counted.

Table 47. LIVER AND LUNG TUMOR RESPONSE IN UNTREATED PARENTAL AND HYBRID MICE[a]

Strain	Sex	Treatment[a]	No. of mice sampled	Time of sampling (months)	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
								% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	Untreated	14	3	22.9	1.0	4.49	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
	F	"	24	3	19.6	0.8	4.43	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
"	M	"	12	6	24.6	1.3	5.40	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
	F	"	12	6	20.2	1.0	5.23	0(0)	0.00 ± 0.00	8.3(0)	0.08 ± 0.08
C_3HeB/FeJ	M	"	8	6	35.9	1.5	4.29	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
	F	"	7	6	28.8	1.5	5.20	0(0)	0.00 ± 0.00	17(0)	0.14 ± 0.14
A/J (female) x C_3HeB/FeJ (male)	M	"	10	3	25.6	1.2	4.96	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
	F	"	10	3	20.8	1.0	5.24	0(0)	0.00 ± 0.00	10(0)	0.11 ± 0.11
"	M	"	16	6	39.1	1.6	4.11	0(0)	0.00 ± 0.00	6.3(1)	0.06 ± 0.06
"	F	"	14	6	30.9	1.3	4.27	0(0)	0.00 ± 0.00	7.1(1)	0.06 ± 0.06
C_3HeB/FeJ (female) x A/J (male)	M	"	37	3	32.8	1.6	4.94	0(0)	0.00 ± 0.00	3(1)	0.03 ± 0.03
	F	"	10	3	24.4	1.1	4.88	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
"	M	"	18	6	41.2	1.8	4.47	0(0)	0.00 ± 0.00	0(0)	0.00 ± 0.00
	F	"	7	6	31.4	1.5	4.87	0(0)	0.00 ± 0.00	14(1)	0.14 ± 0.14

[a] 15 day-old hybrid mouse study. Untreated parental and hybrid mice were sacrificed 3 and 6 months after initiation of the bioassay and the surface tumors on the liver and lungs counted.

Table 48. LIVER AND LUNG TUMOR RESPONSE IN PARENTAL AND HYBRID MICE FOLLOWING TREATMENT WITH DIETHYLNITROSAMINE[a]

Strain or Cross[+]	Sex	Treatment[a]	No. of mice sampled	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
							% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	DENA	8	31.6	1.4	4.4	0	0.00 ± 0.00	100 (8)	1.88 ± 0.35
	F	"	4	22.7	1.0	4.5	0	0.00 ± 0.00	100 (4)	4.75 ± 1.38
C_3HeB/FeJ	M	"	7	44.0	2.1	4.8	57 (4)	1.14 ± 0.46	57 (4)	1.71 ± 0.78
	F	"	8	33.6	1.5	4.7	13 (1)	0.13 ± 0.13	88 (7)	2.13 ± 0.67
A/J (female) x C_3HeB/FeJ (male)	M	"	8	41.7	1.8	4.4	1 (8)	0.13 ± 0.13	75 (6)	1.60 ± 0.65
	F	"	8	36.2	1.4	3.9	0	0.00 ± 0.00	100 (8)	3.12 ± 0.39
C_3HeB/FeJ (female) x A/J (male)	M	"	7	38.7	1.7	4.4	14 (1)	0.14 ± 0.14	29 (2)	0.50 ± 0.34
	F	"	4	32.1	1.4	4.5	0	0.00 ± 0.00	75 (3)	2.20 ± 1.11

[a] Six-to-eight week-old parental and hybrid strains received a single i.p. dose of diethylnitrosamine at 50 mg/Kg. After six months, the animals were sacrificed and the surface tumors on the liver and lungs counted.

Table 49. LIVER AND LUNG TUMOR RESPONSE IN VEHICLE-TREATED PARENTAL AND HYBRID MICE[a]

Strain or Cross+	Sex	Treatment[a]	No. of mice sampled	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
							% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	Water	7	24.5	1.1	4.5	0	0.00 ± 0.00	28 (2)	0.28 ± 0.18
	F	"	8	22.7	1.0	4.3	0	0.00 ± 0.00	37 (3)	0.62 ± 0.37
C_3HeB/FeJ	M	"	8	39.2	1.9	4.8	0	0.12 ± 0.12	0	0.00 ± 0.00
	F	"	8	33.5	1.5	4.6	0	0.00 ± 0.00	0	0.00 ± 0.00
C_3HeB/FeJ (female) x A/J (male)	M	"	6	42.0	1.8	4.3	0	0.00 ± 0.00	0	0.00 ± 0.00
	F	"	4	29.2	1.2	4.1	0	0.00 ± 0.00	25 (1)	0.25 ± 0.25
A/J (female) x C_3HeB/FeJ (male)	M	"	6	45.5	1.9	4.2	0	0.00 ± 0.00	25 (2)	0.25 ± 0.16
	F	"	4	36.0	1.5	4.2	0	0.00 ± 0.00	0	0.00 ± 0.00

[a] Six-to-8 week-old hybrid mouse study. Parental and hybrid strains received a single i.p. injection of 0.1 ml distilled water. Six months later, the animals were killed and the surface tumors on the liver and lungs counted.

Table 50. LIVER AND LUNG TUMOR RESPONSE IN UNTREATED PARENTAL AND HYBRID MICE[a]

Strain or Cross	Sex	Treatment[a]	No. of mice sampled	Mean body weight (g)	Mean liver weight (g)	Liver wt. / Body wt. X 100	Mice with liver tumors		Mice with lung tumors	
							% (No.)	No. tumors/mouse (mean ± S.E.)	% (No.)	No. tumors/mouse (mean ± S.E.)
A/J	M	Untreated	7	29.7	1.3	4.4	0	0.00 ± 0.00	43 (3)	0.43 ± 0.20
	F	"	8	22.8	1.1	4.8	0	0.00 ± 0.00	38 (3)	0.63 ± 0.38
C_3HeB/FeJ	M		7	41.3	1.9	4.6	0	0.00 ± 0.00	0	0.00 ± 0.00
	F	"	8	31.3	1.5	4.8	0	0.00 ± 0.00	0	0.00 ± 0.00
A/J (female) x C_3HeB/FeJ (male)	M	"	8	43.6	1.9	4.4	0	0.00 ± 0.00	50 (4)	0.50 ± 0.19
	F	"	8	33.3	1.6	4.8	0	0.00 ± 0.00	0	0.00 ± 0.00
C_3HeB/FeJ (female) x A/J (male)	M	"	6	38.2	1.8	4.7	0	0.00 ± 0.00	0	0.00 ± 0.00
	F	"	5	32.2	1.3	4.0	0	0.00 ± 0.00	0	0.00 ± 0.00

ya Six-to-8 week-old hybrid mouse study. Parental and hybrid strains were sacrificed six months after initiation of the bioassay.

DISCUSSION

Based upon the data obtained to date, the hybrid strain would appear promising as a model to detect both lung and liver carcinogens. Treatment of 15 day-old animals is preferable to 6-8 week-old mice due to the higher liver tumor response at 6 months. The lung tumor response to DENA in both hybrid strains is similar to the parental A/J mice suggesting that lung tumor susceptibility is a dominant trait confined to a single gene locus. In contrast, the liver tumor response to DENA was significantly higher in the C_3HeB/FeJ strain than in either of the hybrid strains suggesting that liver tumor susceptibility is controlled by more than one genetic locus.

At the present time, we are completing the initial DENA study under Army Contract No. DAMD17-85-C-5014, and are further evaluating the hybrid model for the testing of military-relevant compounds for carcinogenic activity.

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