EFFECTS OF AROCLOR 1254 AND ACETONE PRETREATMENT
ON THE MUTAGENICITY OF DIMETHYLNITROSAMINE

by

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ABSTRACT

Pretreatment of mice with acetone enhances the microsomal N-demethylation of dimethylnitrosamine (DMN) at low substrate concentrations (< 5 mM), while pretreatment with Aroclor 1254 represses this activity at the low concentrations and enhances it at high (> 50 mM) DMN concentrations. To relate activity of DMN-N-demethylase to the mutagenicity of DMN, liver microsomes were isolated aseptically from mice 18 hours after acetone pretreatment (3 ml/kg i.p.), 5 days after Aroclor 1254 (500 mg/kg i.p.), or the appropriate controls, and incubated with Salmonella typhimurium (TA 1535), NADPH, and DMN (1, 3, or 70 mM) for 30 minutes. After a 48 hour incubation on minimal media, revertants per plate were determined. Microsomes from acetone pretreated mice bioactivated DMN to a mutagen at significantly higher (p < 0.001) levels when incubations were performed at 1 mM DMN. Aroclor 1254-induced microsomes exhibited a decreased ability to convert DMN to a mutagen at 1 mM and 3 mM DMN (p < 0.05) and a significantly higher ability at 70 mM DMN. These and published reports suggest multiple microsomal enzymes for DMN bioactivation and that acetone may enhance the enzyme that operates at environmentally important levels of DMN.
INTRODUCTION

The ubiquity of N-nitroso compounds has been well established. They are present in urban air, agricultural chemicals, cutting oils, and cosmetics (Mirvish, 1977). They are used as intermediates in chemical syntheses and as anti-oxidants in rubber production. Nitrosamines have also been found in a variety of foods, including spinach, cheese, fruits, and also in cigarette smoke (Montesano and Bartsch, 1976).

Their general structure is:

\[
\begin{array}{c}
R \\
\hline
N-N=O \\
R'
\end{array}
\]

where the R groups can be alkyl groups, amides, substituted aromatic rings, or a heterocyclic moiety. Nitrosamines are formed by reacting nitrite with a nitrosatable amine in an acidic environment. Some compounds subject to nitrosation include secondary, tertiary, and quaternary amines, ureas, and carbamates. The required ingredients for nitrosamine formation are thus found in the human diet; nitrites are used to preserve meat, or can be formed by intestinal bacteria from nitrates present in saliva or green vegetables. The amine can be supplied by dietary meat (particularly...
fish), drugs, pesticides, or substances normally found in the blood and urine, such as piperidine or pyrrolidine. The stomach then provides the acidic environment for nitrosamine formation to take place (Mirvish, 1975). This process has been demonstrated in human volunteers who consumed a meal of bacon, spinach, and beer (Fine et al., 1977).

While diet is implicated as the source of much of the nitrosamine to which humans are exposed, it can also afford a measure of protection against its effects. Ascorbic acid, or vitamin C, blocks the nitrosation reaction by changing nitrite to nitric oxide. It also has an effect on dimethylnitrosamine (DMN) itself, in that it will inhibit both in vivo bacterial mutagenesis and formation of lung adenomas (Guttenplan, 1977; Mirvish, 1977). As a result, Mirvish has suggested that ascorbate be added to easily nitrosated drugs to prevent in vivo formation of nitrosamines.

The toxic effects of different N-nitroso compounds are largely organotropic. Diethylnitrosamine (DEN) and DMN both produce centrilobular liver necrosis (Magee and Barnes, 1967). DMN can also induce necrosis of the renal tubules and testes, particularly in rats fed a protein-deficient diet (Montesano and Bartsch, 1976). Low chronic doses of DMN (50 ppm in the diet, 42 weeks) will produce liver tumors (Magee and Barnes, 1956) while a higher dose for a short time (DMN 100 ppm in the diet, 4 weeks) causes kidney tumors.
DEN can also produce liver tumors following a single administration (Magee and Barnes, 1967). Dibutyl nitrosamine specifically causes bladder cancer (Okada, 1976), and nitrosoureas produce central nervous system tumors (Kleihues and Magee, 1973). Esophageal and respiratory tract tumors are also common with other nitrosamines (Montesano and Bartsch, 1976).

The organ-specific reactions of these compounds seem to be dependent on two properties:

1. The ability of an organ to metabolize a compound to a reactive intermediate.
2. The ability of an organ to repair damage to DNA.

**Metabolism**

Nitrosamines are procarcinogens; they are not carcinogenic in their original form, but must be metabolized by enzymes of the mixed function oxidase system (MFO) (Magee et al., 1976). The pathway of metabolism of nitrosamines has been described as follows (Guttenplan, 1977):

\[
\begin{align*}
\text{dialkyl-nitrosamine} & \xrightarrow{\text{MFO}} \text{hydroxyl-nitrosamine} & \xrightarrow{\text{H}^+} \text{alkyl diazonium ion} \\
\text{alkonium ion (ultimate mutagen)} & \xrightarrow{\text{N}_2} \text{monoalkyl-nitrosamine} & \text{alkyl diazonium ion}
\end{align*}
\]
Hydroxylation of the α-carbon, followed by spontaneous cleavage, is believed to be the primary mechanism of degradation. In the case of DMN, the N-methyl group is hydroxylated (the α-carbon). The subsequent degradation of the hydroxylated species liberates the methyl group in the form of formaldehyde. So the terms N-demethylation or DMN-N-demethylase are frequently used to define the initial reaction or enzyme involved in the metabolism of DMN. There is also evidence of β, ω, and ω-1 hydroxylation in longer chain nitrosamines (Blattman, 1977), suggesting the involvement of an enzyme system similar to that found in fatty acid catabolism (Kruger, 1972).

Of the aldehydes, carboxylic acids, and other by-products of metabolism, the most important in terms of cellular damage appears to be an alkyl carbonium ion which can alkylate nucleophilic sites of tissue constituents as described below. It can also form superoxide radicals from membrane lipids, which most likely cause the observed degranulation of the rough endoplasmic reticulum and interference with protein synthesis (Magee and Barnes, 1967).

Alkylation of cellular constituents has been observed following the in vivo administration of N-nitroso compounds. The reactive species formed by nitrosamines (e.g., DMN) and nitrosoureas (e.g., methylnitrosourea, or MNU) is an SN1 electrophile (Lawley, 1974; Guttenplan, 1977)
which will attack various groups on RNA, DNA, and proteins. Some of the alkylation products include N-7-methylguanine, N-3-methyladenine, N-3-methylguanaine, and 0-6-methylguanaine, along with lesser amounts of other substituted bases (Lawley, 1976). The N-7-methylation of guanine is the predominant reaction, and was thought to be the most significant effector of somatic damage until Ludlum (1970) pointed out that N-7-methylguanaine pairs the same as guanine.

Loveless (1969) first suggested that the 0-6 substitution of guanaine was the important event leading to DMN-induced carcinogenesis. This reaction masks one of the sites involved in hydrogen bonding with cytosine. The resulting tautomeric shift to the enol form (Goth and Rajewsky, 1974) permits pairing with thymine. The difference between 0-6-methylguanaine and N-7-methylguanaine can be further illustrated by the relative carcinogenicities of four alkylating compounds. Dimethylsulfate (DMS) and methylmethane-sulfonate (MMS) alkylate DNA equally as well as DMN and MNU, but do not produce nearly as many tumors (Kleihues and Magee, 1973; Magee et al., 1976). Lawley (1976) has suggested that the difference is in their electrophilic character. DMS and MMS are SN2 alkylation agents, and produce more N-7-methylguanaine than 0-6-methylguanaine. The ratio of 0-6-methylguanaine to N-7-methylguanaine is 0.004. DMN and MNU are SN1 electrophiles, and produce more 0-6-methylguanaine. The ratio of
0-6-methylguanine to N-7-methylguanine is 0.1 for these two potent carcinogens. The amount of N-3-methylguanine is about the same for both classes of alkylating agents, and does not seem to be important in tumor formation.

In addition to alkylated DNA bases, a cell will also acquire alkylated RNA bases, which could halt translation (Shank, 1975), or cause misincorporation of amino acids into proteins. These proteins may be histones, which influence gene regulation, and therefore cell differentiation, or enzymes which replicate DNA. The proteins themselves could be alkylated (Turberville and Craddock, 1971), altering their tertiary structure. Some of the amino acids methylated by DMN include histidine, serine, and lysine (Magee, 1977).

In vitro DMN-N-demethylase assays with tissue slices have shown the most active DMN-metabolizing organ to be the liver, followed by the kidney and esophagus. Intestine and respiratory tract tissues had low levels of activity, corresponding with their low susceptibilities to the tumorigenic effects of DMN. Further data are offered by a study of in vitro metabolism of DEN by hamster lung tissue. This study showed DEN to be metabolized at a rate forty times greater than DMN. In the corresponding in vivo experiment, a few doses of DMN produced liver tumors, while similar doses of DEN caused death from respiratory tumors before there was any pathology of the liver or kidneys (Montesano and Magee, 1974). As a result, in vitro
metabolism has been frequently used as an indicator of tumorigenic potential.

The DMN activation capacity of various tissues can be measured by several in vitro tests. They include bacterial mutagenesis, DMN-N-demethylase as measured by formation of formaldehyde, and content of cytochrome P-450, the terminal oxidase of the microsomal biotransformation system (Czygan et al., 1973). The levels of activity in these assay systems can be increased by pretreatment of test animals with different microsomal inducing agents which will be discussed below.

The enzyme system referred to as "cytochrome P-450" is a heterogeneous class of porphyrin enzymes located in the endoplasmic reticulum (ER) of the liver and to a lesser extent, other tissues. When the ER is isolated by homogenization and centrifugation, it is termed microsomes. Thus the term "microsomal cytochrome P-450." The microsomal cytochrome P-450s can be induced by a variety of drugs and other xenobiotics which have historically been divided into two general classes: the phenobarbital group, and the polycyclic hydrocarbon, or cytochrome P-448 group, which includes 3-methylcholanthrene (3-MC) and benzo(a)pyrene. Both groups of enzymes are induced by Aroclor 1254 (Alvares, Bickers, and Kappas, 1973). In addition, a particular compound may be classified according to the cytochrome which is involved in its metabolism. The amount of this
Cytochrome present is then used as an index of the metabolic potential of a microsomal preparation (Alvares et al., 1973).

Cytochrome P-450 can bind carbon monoxide, forming a complex which dissociates when exposed to monochromatic light at 450 nm. Cytochrome P-450 oxidative metabolism is inhibited when the cytochrome is complexed with carbon monoxide, but enzymatic activity can be restored by exposure to this monochromatic light (Rosenthal and Cooper, 1967).

To examine the extent of cytochrome P-450 involvement in DMN metabolism, Czygan et al. (1973) studied the effect of carbon monoxide on DMN-N-demethylation and DMN-induced mutagenesis of S. typhimurium. They found both to be inhibited, and that both activities were best recovered by exposure to light at a wavelength of 450 nm.

A later paper from the same laboratory (Czygan et al., 1974) showed a linear relationship between cytochrome P-450 content and mutagenesis, and Frantz and Malling (1975) found a linear relationship between mutagenesis and formaldehyde formation from DMN. So it would appear that there is an interdependence between the metabolic capabilities of a microsomal system, as measured by cytochrome P-450 content, and the end products of this metabolism, formaldehyde formation and mutagenesis.

Sipes et al. (1973) used isopropanol and its metabolite, acetone, to increase DMN demethylase levels in rats. While DMN metabolism is supposed to be cytochrome
P-450 dependent (Czygan et al., 1973), cytochrome P-450 content was not elevated as with other inducers of these enzymes (Alvares et al., 1973). Also unaffected was the activity of ethylmorphine demethylase, which is a characteristic substrate of the cytochrome P-450 dependent mixed function oxidase system.

Acetone increases the in vitro hydroxylation of aromatic compounds (Anders, 1972), possibly through a membrane solubility effect in which the spatial relationship between enzymes, cytochromes, and cofactors is altered to make the system more efficient. Activity could then increase without creating more components. Since the first step in DMN metabolism is thought to be α-carbon hydroxylation (Guttenplan, 1977; Fahmy and Fahmy, 1975), and the presence of aromatic compounds inhibits DMN metabolism (Guttenplan, Hutterer, and Garro, 1976), a similar effect might be expected with DMN. Such is not the case, however, as acetone and isopropanol inhibit the activity of DMN-N-demethylase in vitro (Sipes et al., 1973). Also, acetone induction of demethylase is not observed after a period of time allowing for intracellular distribution (4 hours), but is seen at 16 hours, suggesting the synthesis of new enzymes (Sipes et al., 1973).

However, Slocumb (1978) has presented evidence that the inhibitors of protein synthesis, cycloheximide and actinomycin D, do not prevent enhanced DMN-N-demethylase
activity when administered thirty minutes before or simultaneously with acetone. This argues against acetone enhancement of de novo protein synthesis, and leaves the actual mechanism of action unclear.

While alcohols have been used as inducers of DMN metabolism (Maling et al., 1975; Sipes et al., 1973), other workers have studied the effects of alcohol metabolism inhibitors on DMN metabolism in vitro and in vivo. Pyrazole is a competitive inhibitor of alcohol dehydrogenase, and 3-amino-1,2,4-triazole (AT) is a noncompetitive inhibitor of catalase. Both inhibit the formation of formaldehyde and methanol from DMN (Lake et al., 1975), while demethylation of aminopyrine, another cytochrome P-450 compound, is not affected at all by pyrazole, and is one-third as inhibited by AT as is DMN demethylation (Phillips et al., 1974). So it would seem that if cytochrome P-450 is involved in DMN demethylation, it is a species different from the one involved in the metabolism of aminopyrine or ethylmorphine.

The secondary relationship of formaldehyde formation to mutagenesis, toxicity, and general metabolism is also apparent. Guttenplan et al. (1976) noted that in mice given a variety of inducing agents (3-MC, Aroclor 1254, phenobarbital) and inhibitors of DMN metabolism (SKF 525-A, benzo(a)pyrene) mutagenesis was affected from 6 to 20 times more than formaldehyde formation. Lake et al. (1974) found that while phenobarbital and 20-methyl cholanthe (20-MC)
both increased DMN demethylase activity in rats, only 20-MC significantly lowered the LD$_{50}$. Somogyi et al. (1972) showed that pregnenolone-16-carbonitrile pretreatment decreases DMN-N-demethylase activity without decreasing the rate of DMN elimination from the blood of test rats. Phillips et al. (1975) used phenobarbital and 20-MC to increase DMN-N-demethylase levels in rats without increasing the rate of elimination of DMN from the blood.

The confusion and seeming contradictions noted above were summed up in a controversy regarding induction of DMN-N-demethylation by 3-MC. Some investigators (Frantz and Malling, 1975; Guttenplan et al., 1976) reported an increase in activity with pretreatment, and others reported a decrease (Vankatesan, Arcos, and Argus, 1968; Venkatesan, Argus, and Arcos, 1970). Arcos et al. (1977) recently explained this as the result of enzymic forms of DMN demethylase, one of which is active between 0 and 4 mM DMN, and the other which is dominant from 50 to 200 mM DMN.

A Hofstee plot of DMN demethylase showed three distinct $K_m$ and $V_{max}$ values which were similar in rats and mice. The low substrate concentration segment was labelled "DMN demethylase I," and was repressed by Aroclor 1254 and phenobarbital in rats and mice and by 3-MC in rats. The high substrate concentration segment was labelled "DMN demethylase II," and its activity was enhanced by Aroclor 1254 in the mouse and rat, and by phenobarbital and 3-MC in
the rat. The intermediate segment was described as saturated demethylase I masking the behavior or demethylase II below 50 mM (Arcos et al., 1977).

The existence of two forms of DMN-N-demethylase is then part of a picture which involves many cytochrome P-450-type enzymes (Haugen, van der Hoeven, and Coon, 1975), not all of which have been enumerated. With different cytochromes responsible for the production of formaldehyde, the mutagenic alkylating metabolite, and the species responsible for cytotoxicity, conflicting observations may then be accounted for (Arcos et al., 1977). The process described by Sipes et al. (1973) in which DMN-N-demethylase is increased, while cytochrome P-450 levels stay the same, could be a mixture of "cytochrome P-450s" in which acetone causes the modification of an existing species to the metabolically essential species without altering the total cytochrome content.

**Repair**

While rat liver DNA may originally be alkylated to a greater extent than other tissues such as kidney or brain (Magee et al., 1976; Goth and Rajewsky, 1974), tumors may appear in the latter organs rather than the liver. This contradiction can be explained by the presence of DNA repair mechanisms in the cell.
Several investigators have worked with nitrosoureas and their specificity for central nervous system tumors. The nitrosoureas are thought to act by the same end product alkylating agent as nitrosamines. However, they do not need to be metabolically activated to be carcinogenic. The amount of reactive endproduct in a given tissue is then not dependent on the presence of an active mixed-function oxidase system such as is found in the liver. Margison and Kleihues (1975) showed that small weekly doses (10 mg/kg) of MNU given intravenously to young rats caused 0-6-methylguanine to accumulate in the brain to a much greater extent than liver or kidney. Goth and Rajewsky's 1974 study gave similar results, in that a single dose (75 mg/kg) of ethylnitrosourea produced 0-6 and N-7 ethylation of guanine and N-3-ethyladenine in similar proportions in liver, kidney, and brain. But the elimination of 0-6-ethylguanine from brain DNA was much slower than from liver, and three times slower than elimination of N-3-ethyladenine and N-7-ethylguanine from the brain.

As mentioned previously, DMN given in low doses over a period of time will produce liver tumors, while a larger dose given for a shorter time causes kidney tumors. By measuring the rate of decrease of 0-6-methylguanine, it was determined that liver tissue exposed to high (20 mg/kg) and low (2.5 mg/kg) doses, and kidney tissue exposed to the low dose of DMN will remove damaged bases at about the same
rates. By the end of three days, most of the 0-6-methylguanine had disappeared. However, when rats were given the high dose, the kidney had only removed about 30% of the 0-6-methylguanine by fifteen hours when enzyme activity plateaued. At fifty hours, a slow decline in 0-6-methylguanine began again (Magee et al., 1976).

In further experiments, Magee and co-workers (1976) compared kidney tumor incidence at eighty weeks in animals given two intraperitoneal injections of DMN (16 mg/kg). If the doses were given 16 or 32 days apart, they produced the same tumor incidence (12%) as two doses of 16 mg/kg given separately, while if the two injections were given less than four days apart, tumorigenic response was the same as or greater than one dose of 32 mg/kg (55%).

Intracellular repair mechanisms have been shown to possess mutagenic capabilities of their own. When *E. coli* cells are exposed to ultraviolet (UV) irradiation, pyrimidine dimers are formed in DNA. These lesions can then be repaired by three mechanisms:

1. Photoreactivation, where dimers are enzymatically cleaved when cells are exposed to light between 310 and 400 nm.

2. Excision repair, which causes a single strand nick in the DNA and removes the dimer along with a few bases on either side of it. The gap is then filled using the opposite strand as a template. In
recombination repair, daughter strand DNA is also used to replace the area around the dimer, and the gap in daughter strand DNA filled in the same way.

3. Post-replication repair, where the normal DNA polymerase cannot copy past the dimer, and so leaves a gap which is filled by a less specific polymerase. The first two types of repair are very accurate, but the third type is not, and may insert an incorrect base even where there is no parent strand dimer to cause an error (Witkin, 1976).

The third type of repair has been named "SOS repair" because the emergency caused by this repair (mutation) is less serious than the emergency which would occur without it (cell death). In addition to being induced by UV damage, SOS repair is also induced by stalled DNA replication forks and DNA degradation products (Gudaş and Pardee, 1976). Das Gupta and Summers (1978) have recently demonstrated that an error-prone repair mechanism is also induced in cultured mammalian cells exposed to UV irradiation. The ramifications of SOS repair will be discussed in the next section.

**Mutagenesis and Cancer**

It is convenient to discuss the metabolism of a carcinogen, and its effects on cellular constituents, but these effects must be related to the end result—cancer.
The only procedure presently accepted for carcinogenesis testing is long-term feeding studies with animals, which can take two to three years and cost $100,000 (Kolati, 1976). As a result, many investigators are developing short-term screening tests, which take a few days to complete and cost a fraction as much as an animal feeding study. They include sister chromatid exchanges, degranulation of the hepatic microsomal membrane, malignant transformation of cultured mammalian cells, and tests for mutation in various eucaryotic and procaryotic systems (Bridges, 1976). One of the most widely used tests is that developed by Dr. Bruce Ames, involving reversion to prototrophy of different histidine auxotrophs of *Salmonella typhimurium* (Ames, McCann, and Yamasaki, 1975).

The relationship of mutagenicity testing to carcinogenesis is based on the theory that cancer is caused by a heritable change in cellular DNA, whether a single base change, or a macromolecular rearrangement. There is circumstantial evidence to support this, including the before-mentioned central nervous system tumors induced by MNU (Margison and Kleihues, 1975), where there is little repair of alkylated DNA. A similar lack of repair is observed in humans suffering from xeroderma pigmentosum, a disease where susceptible individuals develop skin cancer when exposed to sunlight. Skin cells from these individuals show chromosomal aberrations after exposure to UV
light, and the same cells in tissue culture show structural changes in their chromosomes after infection with oncogenic viruses (Rohrborn, 1974). Purchase and co-workers (1976) have shown a high degree of correlation between the ability of a chemical to cause mutagenesis in short term procedures and its carcinogenic potential.

There is also compelling evidence indicating somatic mutation is not the cause of cancer. Mintz and Illmensee (1975) have taken mouse embryonal carcinoma cells from one strain of mouse, and implanted them in blastocysts from a genetically different strain of mice. The result was mice which were fully functional genetic mosaics in a variety of tissues, including hair follicles, red and white blood cells, and liver parenchyma. Similar transplants done with frog carcinoma cells and tobacco crown gall tumor cells resulted in normal development (Rubin, 1976). Sivak (1976) points out a lack of correlation between carcinogenicity, based on percentage of animals with tumors, and mutagenicity, based on revertants per nanomole. The compound with the greatest carcinogenic potential was the least effective mutagen. There is also a lack of sensitivity in bacterial tests to known carcinogens such as hormones, ethionine, plastic films, and cocarcinogens (Sugimura et al., 1976).

Given the qualitative correlation between mutagenicity and carcinogenicity, and the greater convenience
of mutagenicity testing based on cost, time, and personnel required, Bridges (1976) has proposed a hierarchy of screening systems. In her scheme, all chemicals would be subjected to a first level of testing consisting of a battery of \textit{in vitro} tests. According to results of these tests, availability of less toxic substitutes, and expected exposure, a chemical would then progress to a second tier of short and long term tests on mammals. The third tier would evaluate risk versus benefit to man in terms of quantitative toxicity, economic benefits, and proportion of the population exposed.

The Ames \textit{Salmonella} mutagenesis test is one of the sub-mammalian systems proposed for preliminary screening of xenobiotics. The basis for the Ames test is the somatic theory of carcinogenesis discussed earlier. While cytological techniques will detect gross chromosomal aberrations, such as polysomy and sister chromatid exchanges, the complexity of the eukaryotic cell makes it difficult to cause and detect mutations in single genes \textit{in vitro}. Bacteria, with their single chromosome and well defined regulatory mechanisms, are easily applied to the examination of xenobiotic effects on DNA.

Mutations in bacteria can be divided into two basic groups: (1) frame-shift mutations, which include insertions and deletions, and (2) substitution mutations. Since the genetic code is read three bases at a time from
specific starting points (Strickberger, 1968), the "reading frame" can be dislocated by the addition or subtraction of bases. When this happens, the polypeptide being formed may be inactivated by the insertion of an amino acid which alters its tertiary structure, or terminated by a new stop instruction generated by the shift in reading frame. Such a difficulty may be corrected by the addition or subtraction of the proper number of DNA bases needed to restore the triplet reading frame. The efficiency of the resulting polypeptide may vary, depending on whether the second change identically restored the original DNA sequence, or allowed the insertion of an amino acid which would permit just enough of the original peptide function for the cell to survive. In substitution mutations, a base is caused to pair incorrectly (e.g., adenine paired with cytosine instead of thymine) through alkylation, deamination, hydroxylation, or depurination (Strickberger, 1968). The subsequent difficulties and solutions are similar to those found with frameshift mutations.

The Salmonella used in the Ames test consist of a collection of histidine requiring mutants of Salmonella typhimurium. Of the five most commonly used strains, TA 1537, TA 1538, and TA 98, have deletion mutations, and two of the strains, TA 1535 and TA 100, have substitution mutations. Ames has introduced a plasmid into two of the strains, TA 98 and TA 100, which increases their sensitivity
to mutagenesis through an increase in error prone repair. The plasmid codes for an endonuclease, which may cause gaps in DNA capable of being filled by error-prone repair mechanisms, but the exact mode of action is not clear (McCann et al., 1975).

These strains also have a deletion through one of the genes (uvr B) responsible for excision repair, leaving only the error prone mechanism for repair of non-UV damage. The deletion through uvr B also removes part of the biotin gene, so that minimal media must be supplemented with trace amounts of biotin (Ames et al., 1975). This biotin is supplied in the top agar, along with a trace amount of histidine. The low level of histidine permits several rounds of replication, which are often necessary for the histidine reversion to be expressed. It also permits the growth of a background haze of bacteria which serves as an indicator of the toxicity of the compound tested.

A portion of the lipopolysaccharide coat has also been eliminated by a deletion in a wall gene, rfa. This increases permeability to large molecules and renders the strains nonpathogenic (Ames, Lee, and Durston, 1973).

Bacteria have some metabolizing capabilities of their own (Kada, 1976), but the general sensitivity of the Ames test is greatly enhanced by the addition of a mammalian microsomal system (Ames, Durston, et al., 1973). It is customary to do the assay by combining the chemical, enzyme fraction, and
an NADPH generating system in the top agar overlay. When using DMN, however, the agar seems to trap the reactive species before it can interact with the *Salmonella* DNA. As a result, Malling (1971) developed a liquid incubation system in which DMN and the mammalian activating system are incubated for a given time before being plated with the top agar overlay. This system has been further modified for use in this study.
STATEMENT OF PURPOSE

The somatic mutation theory of chemical carcinogenesis states that an inheritable change in the genetic material of somatic cells results from exposure to chemical carcinogens or their metabolites. However, there is a long delay between the time of exposure and the actual production of neoplasms. This relates, at least in part, to the slow turnover of most mammalian cells. Recently, several quick tests to predict the carcinogenic potential of chemicals have been developed. One of these, the Ames test, couples the incidence of bacterial mutagenesis with the ability of cell subfractions to convert procarcinogens to ultimate carcinogens. The rationale for this approach is that mutations induced in bacteria may predict mutations in mammalian somatic cells. Bacteria also have a very rapid turnover which permits the assessment of mutations within 48 hours after exposure to the putative chemical carcinogen. The Ames test is now widely used, even though its validity is the subject of much debate.

The purpose of this research project is threefold: (1) to develop the Ames test for use by the Toxicology Program at The University of Arizona; (2) to employ the test to determine the role of microsomal metabolism in the mutagenicity of DMN, a potent chemical carcinogen; and (3)
to examine the effects that pretreatment of mice with acetone, isopropyl alcohol, or Aroclor 1254 have on the ability of hepatic microsomal enzymes to convert DMN to a mutagen. These compounds were chosen because it is known that they influence the activity of microsomal DMN-N-demethylase, an enzyme thought to be involved in the bioactivation of DMN to a mutagen/carcinogen.
METHODS

Chemicals and supplies were obtained from the following sources: bulk 95% ethanol was obtained from Scientific Stores, The University of Arizona; NADP, monosodium salt, NADPH, tetrasodium salt, D-glucose-6-phosphate, monosodium salt, L-histidine, and D-biotin from Sigma Chemical Co., St. Louis. Tris and MgCl₂·6H₂O were obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey. Nutrient Broth (NB), agar, and crystal violet were supplied by Difco Laboratories, Detroit Michigan, and KCl by Mallinckrodt, Inc., St. Louis. Falcon 1029 disposable petri dishes were obtained from Falcon Plastics, Oxnard, Ca. The nitrosoguanidine (NTG), dimethylnitrosamine, and diethylnitrosamine were purchased from Aldrich Chemical Co., Milwaukee, Wis. Salmonella strain TA 1535 was a gift from Dr. Bruce Ames, University of California, Berkeley, Ca. Male C57BL/6J mice were obtained from Jackson Laboratories, Bar Harbor, Me.

Salmonella cultures were maintained on NB plates (see Table 1A) in the following manner: every one or two months a fresh NB plate was streaked from the thin part of the streak on the old plate and stored at 37°. On the morning of the second day, a heavy inoculum was taken from the thick part of the streak and added to 5 ml NB (see
Table 1. Methods and Materials Formulae

<table>
<thead>
<tr>
<th>(A) Nutrient Broth</th>
<th>(B) 10X Spizizen's</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 g Nutrient Broth</td>
<td>20 g (NH₄)₂SO₄</td>
</tr>
<tr>
<td>5 g NaCl</td>
<td>184 g K₂HPO₄·3H₂O</td>
</tr>
<tr>
<td>1.1 distilled H₂O</td>
<td>60 g KH₂PO₄</td>
</tr>
<tr>
<td>before autoclaving</td>
<td>10 g Na-Citrate</td>
</tr>
<tr>
<td>15 g agar for NB plates</td>
<td>2 g MgSO₄·7H₂O</td>
</tr>
<tr>
<td></td>
<td>1.1 distilled H₂O</td>
</tr>
</tbody>
</table>

autoclave in 150 ml quantities

<table>
<thead>
<tr>
<th>(C) Minimal Plates</th>
<th>(D) Tris KCl Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>autoclave:</td>
<td>2.60 g MgCl₂·6H₂O</td>
</tr>
<tr>
<td>1350 ml distilled H₂O</td>
<td>6.05 g Tris</td>
</tr>
<tr>
<td>22.5 g agar</td>
<td>1.15 g KCl</td>
</tr>
<tr>
<td>aseptically add:</td>
<td>1.1 H₂O</td>
</tr>
<tr>
<td>150 ml 10X Spizizen's</td>
<td>autoclave</td>
</tr>
<tr>
<td>6 ml 50% glucose</td>
<td></td>
</tr>
<tr>
<td>1.5 l makes three stacks</td>
<td></td>
</tr>
<tr>
<td>of 20 plates</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(E) Top Agar</th>
<th>(F) His-Biotin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 g agar</td>
<td>12.2 mg biotin</td>
</tr>
<tr>
<td>1.0 g NaCl</td>
<td>4.0 ml histidine stock</td>
</tr>
<tr>
<td>180 ml distilled H₂O</td>
<td>(2 mg/ml)</td>
</tr>
<tr>
<td>autoclave</td>
<td>96.0 ml distilled H₂O</td>
</tr>
</tbody>
</table>

Pour tubes for minimal plates are made by steaming the top agar and adding 10 ml His-Biotin. Three ml quantities are then put in 13 x 100 mm test tubes, covered with a plastic cap, and stored in a 48° water bath until use.

<table>
<thead>
<tr>
<th>(G) Mutagenesis Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S-9</strong></td>
</tr>
<tr>
<td>5.0 mg NADP</td>
</tr>
<tr>
<td>1.0 ml Tris KCl</td>
</tr>
<tr>
<td>0.2 ml Salmonella culture</td>
</tr>
<tr>
<td>0.8 ml S-9</td>
</tr>
<tr>
<td>10 µl DMN solution</td>
</tr>
</tbody>
</table>
Table 1A) in a 50 ml Erlenmeyer flask. The flask was put on a shaking water bath at 37°. The plate was examined for single colony types, indicating a lack of contamination, and stored at 4°. By 4 p.m. the broth culture was turbid, and 0.1 ml spread on each of two minimal plates (see Table 1C) with an L-shaped glass rod flamed in ethanol, along with 0.1 ml his-biotin (see Table 1F). Two NB plates were also spread with 0.1 ml of broth culture each. One of the minimal plates was streaked once across the middle with histidine (2 mg/ml). The bacteria grew on the minimal plate only where cross-streaked with histidine. For the second minimal plate, a toothpick was inserted into a bottle of NTG crystals and then stabbed into the surface of the plate. The hole from the toothpick was surrounded by a clear area about two centimeters wide, then a halo of revertant colonies. This was a test for the substitution mutation in the DNA. The uvrB deletion was tested by exposing half of one of the NB plates to six seconds of UV irradiation at a distance of 33 cm, followed by immediate sequestering in a light tight box to prevent photoreactivation. Growth only occurred on the side of the plate not exposed to UV light. The rfa mutation was tested by sprinkling a small amount of crystal violet onto the surface of the second NB plate. There was a clear area of growth inhibition in the center of the dye spot at the end of the 48 hours. If results of the four plates were not satisfactory, three NB cultures
were inoculated from nine individual colonies on the original plate in groups of three, and the procedure repeated. A NB plate was also streaked from the broth culture. The plate from the broth culture giving the best results was then stored at 4° and used as the stock plate.

Bacteria were stored as a 36 hour culture on NB plates at 4°. At about 4 p.m. on the day before an experiment, a flamed loop was used to transfer bacteria from the stock NB plate to 20 ml NB in a 250 ml Erlenmeyer flask. The flask was then placed in a 37° water bath with shaking until 9:00 the next morning, giving a 17 hour culture. The flask was then taken out of the water bath and left at room temperature until used.

To induce liver microsomal enzymes, mice were pretreated with acetone, isopropanol, or Aroclor 1254 according to the following protocol:

1. Acetone was made 50% v/v in deionized water and administered p.o. or i.p. The total volume administered was 6.6 ml/kg, which represents a dose of 3.3 ml/kg acetone or 45 nmoles/kg. Control animals were administered deionized water by the appropriate route. All animals were then maintained in stainless steel cages (13 cm x 20 cm x 30 cm) containing autoclaved pine chips as bedding. Five mice were housed in each cage, and provided with
water and food until sacrifice 16 hours after acetone administration.

2. Isopropanol was made 25% v/v in deionized water and administered p.o. Total volume was 10 ml/kg, representing a dose of 2.5 ml isopropanol or 33 mmoles/kg. Animals were maintained as described for acetone, except they were sacrificed 18 hours after administration of isopropanol.

3. Aroclor 1254 was prepared in corn oil at a concentration of 50 mg/ml. A single i.p. injection was administered to mice (10 ml/kg) for a dose of 500 mg/kg. Animals were maintained as above, except that they were sacrificed five days after Aroclor 1254 administration.

To remove livers for microsomal isolation, mice were killed by cervical dislocation, placed on their back in a wax-filled dissecting pan and the four legs pinned down with 21 ga hypodermic needles. A wash bottle filled with 95% ethanol was used to wet the entire mouse. Tissue forceps and 4-1/2" blunt scissors were flamed with 95% ethanol and held until the flame went out. Care was taken to prevent the alcohol in the dissection pan and the mouse from igniting.

Removal of the liver was accomplished by lifting up the belly skin with the tissue forceps until the skin was
taught. The scissors were used to make a five millimeter wide slit about five millimeters cranial to the pubic symphysis, penetrating the skin only. The scissors were then closed and inserted through the cut, tips pointed slightly up, and pushed between the skin and the muscle layer to the shoulder girdle. By opening the scissors and withdrawing them back out of the body, the skin was separated from the body wall. Two more cuts along the waistline allowed the skin to be folded back. After applying more ethanol to the body wall and flaming a small forceps and 3" pointed scissors, the body wall was lifted with the forceps and a small cut made close to the position of the first transverse cut. This allowed air into the abdominal cavity, and the intestines fell away from the abdominal wall. If they did not, they were gently pushed down with the tip of the closed scissors. A strip of body wall about a centimeter wide was then removed, from the transverse cut to the diaphragm.

The liver was lifted gently with the forceps and connective tissue cut at the diaphragm, below the liver, and a small strip going to the stomach. The whole liver was then lifted out, and the last band of tissue connecting the liver to the mouse's right kidney cut. Removing the liver in one piece eliminated searching for small pieces of liver in the blood-filled body cavity. It also minimized disturbance of the stomach if the esophagus had been broken
during the killing procedure. There was no bacterial contamination due to broken esophagi, probably as a result of taking care with this part of the procedure.

Livers were placed in sterile petri dishes which had been tared and kept on ice. After obtaining the net liver weight, enough ice-cold sterile Tris KCl buffer (see Table 1D) was added aseptically to cover the livers. A flamed forceps was used to agitate the livers and wash off excess blood. The livers were then removed to a sterile glass homogenizing tube and three volumes Tris KCl buffer added per gram of tissue. The sterile Teflon pestle was connected to an electric drill mounted vertically and four passes made from the top of the buffer solution to the bottom of the homogenizing tube. The homogenate was then poured into autoclaved 30 ml Corex tubes and centrifuged twenty minutes at 9500 rpm (9000 x g). The supernatant was pipetted off and used (S-9 fraction).

When microsomes were to be used, the S-9 was put into cold plastic centrifuge tubes which had been sterilized by washing with 95% ethanol and allowed to drain upside down overnight. The caps were filled with 95% ethanol and covered with an ethanol-soaked paper towel and allowed to dry overnight. After noting the volume of S-9, the tubes were filled with ice-cold Tris KCl. The tubes were centrifuged ninety minutes at 29,500 rpm (105,000 x g) in a Beckman L5-50 ultracentrifuge. The supernatant was
poured off, and a volume of cold buffer equal to one-fourth the original volume of S-9 added to each tube. After allowing the tubes to sit for about five minutes, the pellets were gently resuspended with a vortex mixer, leaving the glycogen pellet adhering to the tube. The microsomal pellet was then poured into a sterile homogenizing tube and resuspended with four passes of the Teflon pestle. The resuspended pellet (microsomes) was poured into a sterile glass screw cap tube, taking care not to allow ice from the outside of the homogenizing tube to fall into the tube containing microsomes. The tubes were then placed in ice to prevent loss of enzymatic activity.

Microsomal protein concentrations were determined by Lowry et al.'s (1951) method. A typical standard protein curve is shown in Appendix A. The volumes used in resuspending microsomes gave concentrations between 20 and 30 mg/ml of protein. Microsomal protein concentrations were then adjusted to 10 mg/ml.

Mutagenesis mixtures with microsomes of S-9 were made up in 50 ml screw cap flasks as shown in Table 1G. For convenience, NADP or NADPH was suspended in Tris KCl buffer and filter sterilized. If protein concentration was to be varied, the volume of Tris KCl-NADPH solution was changed to 0.6 ml, and Tris KCl added to each flask to bring the final volume to 2.0 ml. DMN has a specific gravity of 1.0048 which was rounded off to 1.0, and DEN a specific...
gravity of 0.9422, giving a final concentration of 70 mM when 10 µl DMN or 15 µl DEN was added to the incubation mixture. Since the volume of DMN or DEN never exceeded 0.05 ml, no effort was made to correct for the volume change produced by substrate addition. Flasks were kept on ice to prevent substrate metabolism until placed in the water bath. An E-type cylinder fitted with regulator, Tygon tubing, and a Pasteur pipet was used to blow a light stream of oxygen into the flasks, which were then placed in a 37° water bath (operating at 100 rpm) at thirty second intervals. The reaction was stopped at the appropriate time by replacing the flasks in the ice bucket.

Plating was done after making appropriate dilutions in Tris KCl buffer. One-tenth ml of this dilution was added to three ml of top agar (see Table 1E) and vortexed at low speed. The mixture was then poured on a minimal plate and the plate tipped so that the entire surface was covered with top agar. Five plates were poured for each incubation flask. One flask was diluted out to $10^{-6}$ and top agar poured onto a NB plate for a total cell count. Sterility controls were done on minimal plates for each protein solution, top agar, NADP or NADPH, and Tris KCl. To examine the mutagenic responsiveness of the Salmonella apart from the microsomally activated mutagen, ten microliters of a 0.1 mg/ml solution of NTG in 0.1 M sodium acetate buffer, pH 5.0, was added to 0.1 ml of Salmonella culture in a top
agar tube. This mixture was plated on minimal media as a positive control. After all platings were finished, plates were stored upside down at 37° for 48 hours. Colonies were counted on a colony counter (New Brunswick Scientific Co., New Brunswick, N. J.).

Dilutions were chosen to give less than 800 his\(^+\) revertants per plate for ease of counting. When this was not possible, colonies in 8 to 12 square centimeters were counted and total count estimated using 56.7 cm\(^2\) as the surface area of a plate. Colonies were counted if touching the right or upper edge of the square, and not counted if touching the left or bottom edge of the square.

Microsomal DMN-N-demethylase activity was determined by incubating for thirty minutes at 37°: 5.0 mg NADPH (3.0 mM), 6 mg of microsomal protein, and the appropriate concentration of DMN (1, 3, or 70 mM) in a final volume of 2 ml. All components were made up in Tris KCl buffer, and incubations done in open scintillation vials in a hood. Reactions were started by adding DMN at 15 second intervals and stopped thirty minutes later by adding 1 ml of 15% ZnSO\(_4\). Controls consisted of omitting DMN from one incubation vial and microsomes from three others containing 1, 3, or 70 mM DMN. After the reactions were stopped, 1 ml of saturated Ba(OH)\(_2\) was added and vials carefully mixed by swirling. Contents of vials were then poured into 15 x 100 mm test tubes and centrifuged 15 minutes at 1900 rpm on a
Sorvall GLC-2 bench top centrifuge. Two ml of the supernatant was put in another tube and 1.0 ml of Nash reagent (30 g ammonium acetate, 0.4 ml acetyl acetone, distilled water to 100 ml) added, followed by incubation at 60° for 30 minutes. After cooling, absorbance was read at 412 nm. A standard formaldehyde curve was then used to calculate micromoles HCHO/mg protein/30 minutes, an example of which appears in Appendix B.
RESULTS AND DISCUSSION

As mentioned above, mutagenesis in the Ames test is normally carried out on plates, but short-chain nitrosamines require a preincubation in liquid before plating or there will be no mutagenesis (Malling, 1971; Bartsch, Malaveille, and Montesano, 1975; Gletten, Weekes, and Brusick, 1975). In developing the Ames test as an analytical procedure, it was also necessary to standardize the component parts of the reaction: amounts of NADP/NADPH per incubation, amount of microsomal protein to be used, final volume of incubation mixtures, and time of incubation. In addition, several problems were encountered. These included lack of microsomal activity, difficulties in storing the bacteria, and enzyme fractions contaminated with bacteria from the mouse. The solutions to these problems will be described as part of the procedure for developing the Ames test for the Toxicology Program.

Solutions to Technical Problems

The first incubations were done at a volume of 2 ml in 18 x 150 mm culture tubes. When no mutagenesis was observed, aeration was attempted by bubbling sterile air through the solution. The absence of mutagenesis suggested that the activating enzymes were denatured by the bubbles.
This problem was solved by performing incubations in 50 ml flasks which were gently flushed with 100 per cent oxygen for thirty seconds before closing with a tight-fitting screw cap.

The mutagenesis obtained using screw cap flasks was inconsistent, possibly due to an unnoticed mutation in the bacteria. To check this possibility, the original stocks frozen at -80°C were streaked out. Only 10-15 colonies grew up on the plates. Subsequent contact with Dr. Ames' laboratory indicated the need for adding 3.5 per cent DMSO to broth cultures before freezing to prevent formation of ice crystals. Even with DMSO, the bacteria did not remain viable in large numbers, so strains were carried on NB plates as described in the Methods section.

It was noted that there were two colony types on revertant plates, even when microsome controls (0.1 ml microsomes + top agar on a minimal plate) showed no contamination. One type was flat and spreading and the other was small and raised. Tests of both types for UV and crystal violet sensitivity suggested that both were Salmonella. Further testing showed the two colony types to be an effect of growth in top agar, and that if cells were applied to a plate without top agar, the flat colony type was the only one observed.

Liver preparations were initially done without regard to sterile technique and then filter-sterilized.
The filtration procedure worked for a while, but the Millipore filters began to break or get clogged. This resulted in insufficient enzyme preparation to complete the experiment, or contaminated revertant plates which could not be read. Therefore, it was decided to use a sterile excision of the liver which I learned in Dr. E. J. Lazear's laboratory at the National Center for Toxicological Research in Jefferson, Arkansas. Dr. Lazear's laboratory works with the Ames test routinely and has refined the methodology to eliminate wasted effort.

The necropsy procedure is one example of their technique. Instead of opening the scissors to separate the skin from the body wall as described in the Methods section, the mouse was opened with an "I" shaped incision through the skin and body wall and connective tissue cut with the scissors. Any intestines in the way were delicately moved aside. However, necropsy procedures need not be so tedious since the intestines are not fragile if handled with closed scissors. This procedure reduced the time for removal of a liver from ten minutes to about four minutes. Since it is known that liver microsomal enzymes deteriorate rapidly at room temperature, the 1-1/2 to 2 hours previously needed to remove the livers may have resulted in partial destruction of liver microsomes. The plastic petri dishes used in Dr. Lazear's laboratory to hold livers until homogenization have also proven more convenient than the
50 ml Erlenmeyer flasks initially used. With the new techniques, the next six months produced more uniform results and those data are presented here.

Another time-saving step was the elimination of total cell counts for each incubation. Gletten et al. (1975) and Malling (1971) expressed their results as revertants per $10^8$ or $10^6$ survivors, respectively. However, a 100 per cent error is easily accumulated in a long series of dilutions. Since bacteria have a constant titer at stationary phase, it is possible to assume a constant number of bacteria from one experiment to the next which can be controlled for by background reversion and NTG revertant colonies. Revertant counts alone are then more accurate, and more revertant plates can be poured per sample while using the same total number of plates. An odd plate can then be discarded without affecting the results, and by leaving out total cell counts, about ten minutes per sample is saved. Between the change in plating procedure and the modification of necropsy technique, about three hours was taken off the time required to do an experiment.

**DMN-Induced Mutagenesis Under Standard Conditions**

Almost all reports on DMN-mediated mutagenesis in the literature involve DMN concentrations of 40 mM or greater. This is a good concentration range to work at from the viewpoint of detectable levels of mutagenesis, but
a poor choice for comparison with cancer-feeding studies or environmentally pertinent exposure levels of DMN which would be much lower. Therefore, the initial efforts were to develop a reproducible assay capable of detecting DMN-induced mutagenesis at low (< 5 mM) concentrations of DMN. In addition, the effects of acetone pretreatment on this mutagenesis was also studied.

As can be seen from Figure 1, there is a 45-minute lag period before mutagenesis is observed with control microsomes at 1 mM DMN. A lag is also present with microsomes from acetone-pretreated mice, but mutagenesis is apparent at thirty minutes. In comparison, Gletten et al. (1975) demonstrated a similar ten-minute lag period in mutagenesis using untreated micromes from Swiss albino mice and 40 mM DMN. This lag period was absent in their S-9 fraction possibly due to the presence of a more activating enzyme.

The effect of time of incubation on mutagenesis at 5 mM DMN is shown in Figure 2. The lag period was less at 5 mM than at 1 mM, with mutagenicity being obvious at fifteen minutes for induced, and thirty minutes for non-induced microsomes. Since there was detectable mutagenesis with both control and induced microsomes at thirty minutes, this time was chosen as the standard incubation time.

Figure 3 shows the effect of varying microsomal protein concentration on mutagenesis with 1 mM DMN and a
Figure 1. The Effect of Time of Incubation and Acetone Pretreatment on DMN-Induced Mutagenesis of Salmonella typhimurium Strain TA 1535; DMN Concentration of 1 mM, Microsomal Protein Concentration of 3 mg/ml -- Microsomal enzymes were induced by i.p. injection of acetone, 45 mM/kg, 18 hr prior to sacrifice. Points represent the average number of his\(^+\) revertants per plate. The error bars represent the standard error of the mean. \(\Delta\) — \(\Delta\) control microsomes; \(\square\) — \(\square\) acetone pretreated microsomes.
Figure 2. The Effect of Time of Incubation and Acetone Pretreatment on DMN-Induced Mutagenesis of Salmonella typhimurium Strain TA 1535; DMN Concentration of 5 mM, Microsomal Protein Concentration of 3 mg/ml — Microsomal enzymes were induced by i.p. injection of acetone, 45 mM/kg, 18 hr prior to sacrifice. Points represent the average number of his\(^+\) revertants per plate. The error bars represent the standard error of the mean. △—△ control microsomes; □—□ acetone pretreated microsomes.
Figure 3. The Effect of Microsomal Protein Concentration and Acetone Pretreatment on DMN-Induced Mutagenesis of Salmonella typhimurium Strain TA 1535; DMN Concentration of 1 mM, Time of Incubation of 30 Minutes -- Microsomal enzymes were induced by i.p. injection of acetone, 45 mM/kg, 18 hr prior to sacrifice. Points represent the average number of his+ revertants per plate. The error bars represent the standard error of the mean. △—△ control microsomes; □—□ acetone pretreated microsomes.
30-minute incubation period. Three milligrams of microsomal protein per milliliter was chosen because it is in the linear portion of the curve, and because it was the same as that used by Guttenplan et al. (1976).

The graph in Figure 4 shows the result of varying the concentration of DMN in the incubation media. Microsomal protein concentration was 3 mg/ml and incubation times were thirty minutes for each sample. A dose-response effect is apparent. Other experiments were done, but the previously mentioned variations in mutagenesis caused the standard error to be very large. The scale on this graph is also much more compressed than the scales of the other figures, so direct visual comparison is not possible.

The comparative effects of different inducing agents on mutagenesis are tabulated in Table 2. In cases where a multiplicative relationship exists between two groups of data, statistical analysis is often assisted by taking the logarithms of the numbers involved (Schefler, 1969) as applied by Somjen et al. (1973). Such a relationship was found to exist with both acetone and PCB-induced liver preparations when compared to their respective controls.

When S-9 was obtained from livers of mice pretreated with acetone, no significant difference in mutagenesis was observed at 1 mM or 70 mM DMN, relative to corresponding control S-9. However, at 1 mM DMN, the difference is highly significant with liver microsomes from these acetone
Figure 4. The Effect of DMN Concentration and Acetone Pretreatment on Mutagenesis of Salmonella typhimurium Strain TA 1535; Protein Concentration of 3 mg/ml, Time of Incubation of 30 Minutes -- Microsomal enzymes were induced by i.p. injection of acetone, 45 mM/kg, 18 hr prior to sacrifice. Points represent the average of number of his\textsuperscript{+} revertants per plate. △——△ control microsomes; □——□ acetone pretreated microsomes.
Table 2. Effects of Acetone, Isopropanol, or Aroclor 1254 Pretreatment on the 
In Vitro Microsomal or S-9 Mediated Mutagenesis of S. typhimurium 
Produced by DMN or DEN

<table>
<thead>
<tr>
<th>Liver Preparation</th>
<th>Inducing Agent</th>
<th>Mutagen</th>
<th>1 mM</th>
<th>3 mM</th>
<th>70 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Induced</td>
<td>Control</td>
</tr>
<tr>
<td>S-9</td>
<td>Ac</td>
<td>DMN</td>
<td>417±128</td>
<td>816±191</td>
<td>--</td>
</tr>
<tr>
<td>( \mu )</td>
<td>Ac</td>
<td>DMN</td>
<td>11.5±5.5</td>
<td>204±66.1**</td>
<td>--</td>
</tr>
<tr>
<td>( \mu )</td>
<td>PCB</td>
<td>DMN</td>
<td>17.8±8.5</td>
<td>3.0±2.8*</td>
<td>48.0±25.8</td>
</tr>
<tr>
<td>( \mu )</td>
<td>IP</td>
<td>DMN</td>
<td>8</td>
<td>188</td>
<td>--</td>
</tr>
<tr>
<td>S-9</td>
<td>Ac</td>
<td>DEN</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>S-9</td>
<td>PCB</td>
<td>DEN</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Results are given as mutants per plate, and presented as the mean ± the standard error of the mean. Statistical formulae are given in Appendix C.

* \( p < 0.05 \).

** \( p < 0.001 \).

a Ac = acetone.

b \( \mu \) = microsomes.

c PCB = Aroclor 1254.

d IP = isopropanol.
pretreated mice, suggesting acetone as a specific enhancer of a DMN-demethylase I type enzyme (Arcos et al., 1977). Isopropanol, which is metabolized to acetone, seems to increase mutagenesis at both high and low concentrations of DMN. This would seem puzzling, but little can be concluded from one experiment. The alcohol may act independently of its ketone metabolite, affecting the DMN-demethylase II (Arcos et al., 1977) after the fashion of 3-MC rather than acetone.

Since acetone pretreatment was found to affect DMN-induced mutagenesis in the same manner as DMN-N-demethylase (Sipes, Slocumb, and Holtzman, 1978), it was anticipated that Aroclor 1254 would decrease the activation of DMN to a mutagen, as was reported with N-demethylation (Sipes et al., 1978; Arcos et al., 1977). The 3 mM incubation was then added as a means of raising the mutagenesis baseline, since preliminary experiments already had shown low levels of mutagenesis without pretreatment at 1 mM DMN. Table 2 shows that mutagenesis by Aroclor 1254-induced microsomes is significantly depressed at 1 mM and 3 mM DMN, and significantly increased at 70 mM DMN. Mutagenesis data are paralleled by DMN-N-demethylase data, presented in Table 3, which show repression at 1 mM and 3 mM DMN and an increase in demethylation at 70 mM DMN similar to values presented in the literature (Arcos et al., 1977; Sipes et al., 1978).
Table 3. N-Demethylation of DMN by Aroclor 1254-Induced Microsomes

<table>
<thead>
<tr>
<th></th>
<th>1 mM</th>
<th>3 mM</th>
<th>70 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Induced</td>
<td>Control</td>
</tr>
<tr>
<td>1 mM</td>
<td>34.2±8.4</td>
<td>20.0±5.6</td>
<td>40.8±10.8</td>
</tr>
</tbody>
</table>

Results are given as nanomoles formaldehyde formed per milligram of microsomal protein per 30 minutes, plus or minus the standard error of the mean. Statistical formulae are given in Appendix C.
Mutagenesis by DEN was difficult to detect. It was about half that reported by Frantz and Malling (1975), and similar to that reported by Weekes and Brusick (1975). Since DEN alkylates DNA much less efficiently than DMN (Goth and Rajewsky, 1972), these lower levels of mutagenesis were expected. An increase in mutagenesis would be expected with Aroclor 1254 pretreatment (Frantz and Malling, 1975), but variation from one experiment to the next prevents the drawing of any conclusions here.
CONCLUSION

Ashby and Styles (1978) recently discussed the relationship of the Ames test to carcinogenic potential and outlined instances where diet, strain differences, and co-mutagenic effects markedly influenced the metabolism of a compound. Thus, compounds in the environment, in the diet, and in the workplace, which are not carcinogenic of themselves, should be expected to affect the metabolism of carcinogens in humans. The data presented here indicate that two such compounds (acetone and Aroclor 1254) may be found to exert entirely opposite effects on mutagenesis, and perhaps carcinogenesis. Such data should be considered by OSHA, EPA, and FDA when they issue guidelines for exposure levels to suspected or known carcinogens. Levels are usually set for a particular compound without reference to the co-carcinogenic effects produced by exposure to other agents.

One which may come under closer scrutiny is ethanol, a common social xenobiotic. If the comparison of increased DMN-N-demethylating capacity with increased mutagenic potential and possibly carcinogenic potential were to include ethanol (Maling et al., 1975), people who are exposed to quantities of ethanol might be expected to exhibit a greater incidence of DMN-induced cancer. This
is borne out by OSHA's classification of ethanol as potentially carcinogenic (Smith, 1978). The possibility that it influences the carcinogenic expression of known carcinogens should be considered.

The measurement of mutagenesis with Aroclor 1254-induced microsomes was difficult because the system was operating at the lower limit of its sensitivity with the controls. However, the significant decrease in mutagenesis at "environmental concentrations" of DMN indicates a possibility that Aroclor 1254 pretreatment will also decrease carcinogenesis by DMN as is already known to be the case with DEN (Makiura et al., 1974).

The basic procedures for the Ames test have been worked out for use by the Toxicology Program with a few modifications of the procedures used in this study. A flowsheet has been prepared (see Appendix D) with the steps used in the procedure and a checklist of equipment needed included in Appendix E. Ames' methods paper (Ames, McCann, and Yamasaki, 1975) should also prove helpful, particularly with reference to the necessity for dose-response curves of each compound tested. Otherwise, a given compound may be tested at a single dose, and mutagenesis repressed below the levels of detectability by the inducing agent used, as indicated in this work.
APPENDIX A

STANDARD CURVE FOR THE DETERMINATION OF MICROSONAL PROTEIN
BY THE LOWRY PROCEDURE

Abs. at 660 nm

milligrams protein
APPENDIX B

STANDARD CURVE FOR THE DETERMINATION OF FORMALDEHYDE BY THE NASH PROCEDURE

Formaldehyde calculation:

\[
\frac{(\text{µmoles HCHO from curve-tissue blank-DMN blank})}{6 \text{ mg protein}} \times (\text{dilution factor})
\]

\[
= \text{µmoles HCHO/30 minutes/mg protein}
\]
APPENDIX C

FORMULAE FOR STATISTICAL CALCULATIONS

Standard Error of the Mean

\[ S.E. = \frac{s}{\sqrt{n}} \quad \text{where} \quad s^2 = \frac{\sum x_i^2 - (\sum x_i)^2}{n-1} \]

Test for Equality of Variance; \( \sigma_1^2 = \sigma_2^2 \)

\[ F = \frac{SS_1/(n-1)}{SS_2/(m-1)} \quad \text{where} \quad SS = \sum (x_i - \bar{x})^2 \]

Student's "t" variable for \( \sigma_1^2 = \sigma_2^2 \)

\[ t_{n+m-2} = \frac{\bar{x}_1 - \bar{x}_2}{SS_1 + SS_2 \sqrt{\frac{1}{n} + \frac{1}{m}}} \frac{1}{n} \sqrt{\frac{1}{m}} \]

---

APPENDIX D

FLOW SHEET

The following scheme is presented to assist future users of the Ames test. It is designed to incorporate induction of the liver microsomes with Aroclor 1254. Since this induction is usually for 4 days, it is recommended that animals be injected on Fridays.

Inject rats w/Aroclor 1254 (Friday; weekday sequence for illustration only).

Inoculate Salmonella (Monday, 4 p.m.).

Mark plates:
  5 minimal plates per sample
  2 NB plates for total cell count
  2 minimal plates for S-9 mix
  1 minimal plate for buffer
  1 minimal plate for agar

Remove livers (Tuesday).

Start top agar melting in steamer or boiling water bath.

Homogenize and centrifuge livers.

Place melted agar in 48° water bath and aseptically add his-biotin.

Aseptically mix NADP solution and S-9 (S-9 mix).

Aseptically pipet agar into 12 x 75 mm capped tubes in 48° water bath.

Add chemical to 5 agar tubes in water bath.

Add bacteria to same tubes.

Add S-9 mix to same tubes.
Vortex and pour onto minimal plates.

Do serial dilutions ($10^{-7}$) of bacterial culture and plate on NB plates.

Count plates (Thursday)
work area used for carcinogens should be covered with protective paper and lab coat, gloves, sleeves, eye protection, and mask worn.
## APPENDIX E

### CHECK LIST

<table>
<thead>
<tr>
<th>Sterile Equipment</th>
<th>Other Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue grinding tubes</td>
<td>95% ethanol in a squeeze bottle</td>
</tr>
<tr>
<td>30 ml Corex tubes</td>
<td>Dissecting pan or styrofoam sheet</td>
</tr>
<tr>
<td>Petri dishes for livers</td>
<td>21 ga needles</td>
</tr>
<tr>
<td>500 ml buffer</td>
<td>Tissue forceps</td>
</tr>
<tr>
<td>Top agar</td>
<td>Blunt scissors</td>
</tr>
<tr>
<td>His-biotin</td>
<td>Forceps</td>
</tr>
<tr>
<td>12 x 75 mm tubes with caps</td>
<td>Small scissors</td>
</tr>
<tr>
<td>Pipets</td>
<td>Bunsen burner</td>
</tr>
<tr>
<td>NADP/G-6-P/MgCl₂ solution filter-sterilized (stable for 4 weeks)</td>
<td>Water baths at 37°C and 48°C</td>
</tr>
<tr>
<td>NB plates</td>
<td>Eppendorf pipets and tips</td>
</tr>
<tr>
<td>Minimal plates</td>
<td>Solutions of chemical to be tested</td>
</tr>
<tr>
<td></td>
<td>Carcinogen waste receptable</td>
</tr>
<tr>
<td></td>
<td>Cold centrifuge rotor</td>
</tr>
</tbody>
</table>
REFERENCES


Guttenplan, Joseph B., Ferenc Hutterer, and Anthony J. Garro (1976) Effects of Cytochrome P-448 and P-450 Inducers on Dimethylnitrosamine Demethylase Activity and the Capacity of Isolated Microsomes to Activate Dimethylnitrosamine to a Mutagen. Mutation Res. 35:415-422.


