BIOACTIVATION OF DIMETHYLNITROSAMINE FOLLOWING IN VIVO PRETREATMENT WITH ACETONE AND ETHYL ALCOHOL

by

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STATEMENT BY AUTHOR

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To my father, the late Frank Greenberry Slocumb, for the lifelong encouragement and selflessness he extended to all of his children so that they might freely attain the dream of self-fulfillment through education.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

To further investigate the relationship between *in vitro* liver microsomal DMN-N-demethylase enzyme activity and covalent binding of ^{14}C-DMN reactive intermediates to cellular macromolecules, male Sprague-Dawley rats and C57BL/6J mice were administered a single dose of acetone by intraperitoneal injection. Pretreatment with acetone enhanced the *in vitro* liver microsomal activity of DMN-N-demethylase, as measured by formaldehyde production, and the covalent binding of ^{14}C from ^{14}C-DMN to calf thymus DNA and microsomal RNA and protein. All *in vitro* incubations contained a low DMN concentration of 0.15 mM in order to approximate tissue concentrations in animal and human toxicity studies. It was further determined that fasting alone can enhance the level of microsomal DMN-N-demethylase as well as enhance the covalent binding of reactive intermediates to cellular macromolecules above that of controls, but not to the high degree determined following acetone pretreatment with fasting. Intraperitoneal administration of actinomycin D and cycloheximide, inhibitors of protein synthesis, prior to pretreatment with acetone did not block the enhancement of DMN-N-demethylase enzyme activity. Studies using male Sprague-Dawley rats pretreated orally with multiple dose ethyl alcohol similarly displayed an enhancement of *in vitro* microsomal DMN-N-demethylase enzyme activity as well as of the level of covalent binding of ^{14}C from ^{14}C-DMN to DNA, RNA, and protein as compared to control studies.

INTRODUCTION

Human toxicity from accidental exposure to dimethylnitrosamine (DMN) during industrial usage can be documented as early as 1937. At this time an accident occurred during the manufacture of DMN which was to be used in an anti-corrosive. Shortly thereafter, two chemists who had wiped up spills of DMN as well as inhaled large amounts of the chemical became acutely ill. Both showed severe signs of liver damage (Freund 1937). Based on this and other reports, reviewed in part by Hamilton and Hardy (1974), extensive research was initiated by Barnes and Magee (1954) to study the effects of acute and subacute administration of DMN to laboratory animals. It was determined that a single dose (25 mg/kg) of DMN given by varied routes of administration caused centrilobular liver necrosis accompanied by liver and lung hemorrhage within 24 to 48 hours. Either death ensued within three to four days or complete recovery was seen in three to four weeks. Rats, mice, guinea pigs, and dogs all responded with severe liver necrosis when subjected to administration of DMN.

Subacute and chronic administration of various nitrosamines, including DMN, resulted in the formation of tumors in all animals tested (Magee and Barnes 1956). The sites of tumor formation included the kidney, esophagus, bladder, lung, liver, and the alimentary tract. It was determined, however, that the target organ varied with respect to the dose and chemical structure of the compound, the method of administration, and the test animal used. DMN, in particular, has

since been shown to produce tumors in the rat of the nasal sinus by inhalation (Druckery, Steinhoff, et al. 1963) and the liver (Magee and Barnes 1956), kidney (Magee and Barnes 1959), and lung (Zak et al. 1960) by feeding and oral dosing. Concurrent with these studies, it was established that DMN was rapidly metabolized in rats and mice *in vivo*. Magee (1956) determined that the half-life of DMN injected intraperitoneally (50 mg/kg) in rats was four hours based on an analysis of the whole body, and DMN was distributed throughout most of the body tissues and fluids in apparently uniform concentrations. Dutton and Heath (1956) further demonstrated the *in vivo* metabolism of DMN by administering subcutaneously (50 mg/kg) ^{14}C-DMN to rats and mice. A high proportion of the radioactivity was recovered in exhaled carbon dioxide within a few hours of the injection. Based on the evidence of the rapid metabolism of the parent compound, it was assumed that the metabolite was the toxic agent. It also appeared that a direct correlation existed between the specific tissue metabolism and the site of tumor induction. In rats fed chronic low (50 ppm) doses of DMN, liver neoplasms seemed to occur most often (Magee and Barnes 1956). This was followed by a lower frequency of kidney tumors (Magee and Barnes 1959). Since it was established that nitrosamines required metabolic activation to exhibit toxicity and the main site of xenobiotic metabolism occurs in liver tissue, it followed that the highest frequency of tumor induction was found in the liver.

In general, it can be said that nitroso compounds such as DMN can cause a toxic and carcinogenic response in a wide range of animal species, and that activity is present at acute and subacute dosage

levels (Magee and Barnes 1967). As yet, no known case of human cancer has been directly correlated with DMN exposure, but toxic symptoms seen in industrial accidents bear close similarities to those produced in animal experimentation. The entire field of nitrosamines has been reviewed extensively by Magee and Barnes (1967) and others (Montesano and Bartsch 1976, Shank 1975, Heidelberger 1975, Crosby and Sawyer 1976). The possibility that nitrosamines, including DMN in particular, could be a major contributor to human carcinogenesis and, therefore, an environmental hazard to man, prompted research in the direction of their formation, detection, and quantitation in the environment.

Exposure to preformed nitrosamines is usually limited to usage of the chemical as a solvent or as a chemical intermediate in industry. DMN has been detected in ambient air in two United States cities in which there were chemical plants which either used DMN as an intermediate or were industrial users of amines (Fine et al. 1976). However, the potential for the formation of nitrosamines from secondary, tertiary, and quaternary amines undergoing N-nitrosation reactions has greatly widened the scope of the investigations (Fiddler et al. 1972). DMN has been found in cigarette smoke condensates using tobacco grown in high nitrogen fertilizer (McCormick et al. 1973, Rhodes and Johnson 1972) as well as in conventional American brand tobacco (Rhodes and Johnson 1972).

Deserving more concern, however, is the in vitro production of DMN in foodstuffs from precursors widely distributed in nature. Nitrite is thought to be the main nitrosating agent and is present in the environment from plant and microbial enzymatic reduction of nitrate.

In addition, nitrite is used as a food additive primarily in cured meats to prevent the growth of Clostridium botulinum. Nitrogen metabolism in plants and animals results in the production of amines capable of being nitrosated. Crosby and his coworkers (1972) were able to detect low levels (1-4 μgms/kg) of nitrosamines in cured and processed foods that were rich in nitrites. In addition, Lijinsky and Epstein (1970) have postulated that cooking of cured meats such as bacon results in the release of secondary amines due to the pyrolysis of the protein, thereby increasing the detectable nitrosamine level above that of the raw bacon. Prompted by an unusually high incidence of esophageal cancer in an isolated area in Africa, and the reports of Druckery, Preussman, and associates (1963) that nitroso compounds were the only known chemicals to induce esophageal cancer in animals, some investigators (McGlashan, Walters, and McLean 1968) were able to detect 1-3 ppm of DMN in liquor locally distilled and consumed regularly by the native people. In another area of Africa, which showed a similar high incidence of cancer, DMN was detected in one of the plants used as a principal source of sustenance (DuPlessis, Nunn, and Roach 1969). Although there has been no direct cause and effect relationship between the presence of DMN in these foodstuffs and the unusually high incidence of a particular cancer, the specific disease pattern suggests the involvement of environmental etiological agents and warrants further investigation.

Aside from the direct ingestion of preformed nitrosamines, the in vivo formation was then considered. Numerous animal studies using rats showed that the feeding of nitrite and secondary amines resulted in

the formation of nitrosamine (Alam, Saporoschetz, and Epstein 1971a, 1971b). Similarly, *in vitro* testing in the presence of human gastric juices showed that the reaction could possibly occur in human stomachs under optimal conditions of pH, temperature, and substrate concentration (Sen, Smith, and Schwinghamer 1969). Recently, Fine and coworkers (1977) were able to show *in vivo* formation of DMN, as measured in blood samples, after the ingestion of conventional foods such as cooked bacon and spinach which are high in nitrite-nitrate content. These findings suggest that DMN is an environmental contaminant that could possibly contribute to human carcinogenicity due to the widespread occurrence of nitrosamine precursors, nitrite and secondary amines, and the possible *in vivo* formation of nitrosamines.

The metabolism of DMN *in vivo* and *in vitro* has been studied extensively. Magee and Vandekar (1958) proposed that *in vitro* DMN metabolism required oxygen, necessitated the presence of microsomes plus the cell sap fraction, and was dependent on the presence of an enzyme necessary to catalyze the chemical breakdown. Brouwers and Emmelot (1960) confirmed these findings and showed the production of formaldehyde in the *in vitro* system. Formaldehyde production was then used as a measure of the decomposition of DMN as well as an indicator of the activity of the demethylating enzyme believed responsible for the metabolism. Upon further experimentation it was found that incubation of rat liver slices with DMN inhibited amino acid incorporation into proteins (Hultin et al. 1960). Magee and Hultin (1962) pursued this finding and proposed that the metabolite of DMN released was a methylating agent which methylated histidine residues of protein.

Magee and Farber (1962) confirmed the *in vitro* methylation of protein and further showed methylation of DNA and RNA, primarily in the 7-methylguanine position. Swann and Magee (1968) administered ^{14}C-DMN to rats and determined the percentage of 7-methylguanine in nucleic acids in various organs and correlated this with the carcinogenic potency as evidenced by the incidence of tumor production. It was determined that RNA was methylated to a greater extent than DNA and organs such as the testes and the small intestine showed either a low degree of methylation or no methylation and no tumor formation. However, organs such as the liver and the kidney, which maintain a high degree of enzyme activity necessary for metabolism, showed a positive correlation between alkylation of DNA and RNA and subsequent tumor formation. In addition, studies using human liver slices (Montesano and Magee 1970) incubated with labeled DMN showed a similar pattern of nucleic acid methylation. These *in vitro* findings confirmed that man possessed the capability of converting DMN to an alkylating species. The significance of these findings with respect to the toxic and carcinogenic responses will be discussed at length further in this thesis.

The biometabolism of DMN *in vivo* and *in vitro* to yield an active intermediate is generally thought to occur via oxidative demethylation. The enzymes mediating this bioactivation are associated with the endoplasmic reticulum of the cell. The reaction requires molecular oxygen and NADPH suggesting that the enzyme believed to be responsible, dimethylnitrosamine N-demethylase (DMN N-demethylase), is associated with the microsomal mixed-function oxidases, possibly associated with cytochrome P-450, the terminal oxidase in the usual

drug biotransformation system (Magee and Barnes 1967). Studies using rat liver microsomes, NADPH, and exogenous calf thymus DNA *in vitro* (Grilli, Bragaglia, and Prodi 1975) showed that ^{14}C-DMN was metabolized to a reactive species that methylated DNA. When boiled (denatured) microsomes were used, only a low background level of radioactivity was associated with DNA, thus confirming a microsome-dependent system as the site of bioactivation.

The pathway by which nitrosamine metabolism occurs was first postulated to proceed by way of an enzymatic hydroxylation at the alpha carbon of the aliphatic chain yielding an alpha-hydroxy-nitrosamine. Hydrolysis produces an aldehyde (formaldehyde) and a monoalkylnitrosamine. The latter compound is highly unstable and exists only for a fraction of a second at 37°C (Magee and Vandekar 1958, Magee and Hultin 1962). From this point, it was thought that the monoalkylnitrosamine was rearranged to the diazohydroxide, then the diazoalkane, and finally to a methonium ion which could alkylate the cellular macromolecules. However, Lijinsky, Loo, and Ross (1968) have shown that for DMN, diazomethane is not an intermediate and that alkylation may occur by way of a carbonium ion formed directly from the demethylated nitrosamine via a transmethylation. The specific pathway by which the resultant carbonium ion is formed is not yet clear. Since there is no method to detect the presence of the monomethylnitrosamine intermediate, hypotheses must be based on the formation of methylating agents or the effects of inducers or inhibitors on DMN metabolism. *In vivo* rat studies using ^{14}C-DMN have shown $^{14}CO_2$ to be produced. *In vitro* studies using hepatic subcellular fractions in the presence of NADPH,

O_2, and DMN have shown the production of formaldehyde (Magee and Vandekar 1958; Brouwers and Emmelot 1960; Venkatesan, Arcos, and Argus 1968). In addition, Lake et al. (1975) showed *in vitro* and *in vivo* methanol formation from DMN metabolism. Both methyl groups of DMN were accountable in the total production of formaldehyde and methanol. These studies and various hypotheses have become the groundwork for the continuing studies that eventually should elucidate the metabolic pathway(s) of DMN.

Toxicity, carcinogenicity, and mutagenicity of DMN with respect to the *in vivo* and *in vitro* biometabolism have been extensively studied (Magee and Barnes 1967). Mutagenicity of DMN requires bioactivation by a mammalian system as shown by Czygan and coworkers (1973) using a rat liver microsomal-activated microbial system. Both the activation of DMN to a mutagen and the *in vitro* metabolism were inhibited by carbon monoxide which is known to form a complex with cytochrome P-450, thereby inhibiting cytochrome P-450 dependent reactions (Rosenthal and Cooper 1967). These results confirmed the evidence that *in vitro* N-demethylation of DMN and its activation to a mutagen were related events dependent on cytochrome P-450. Mutagenicity testing using mouse liver microsomes (Umeda and Saito 1975, Malling 1971) contributed to these findings with the additional discovery that the variation in mutagenic potential was dependent on the strain of animal from which the microsomes were isolated.

The role of DMN bioactivation and subsequent methylation of DNA, RNA, and protein in the toxic and oncogenic properties of DMN are a major focus of research. Covalent binding of chemical carcinogens to

cellular macromolecules has been the basis for current theories of cancer induction (Brookes and Lawley 1964). DMN appears to bind primarily to the N-7 and O-6 positions of guanine in DNA (O'Connor, Capps, and Craig 1973). The N-7 position is methylated to a greater extent than the O-6 position, but it appears that base pairing of a 7-methylguanine can occur normally (Ludlum 1970). The O-6 position seems to be most critical in genetic alteration because the O-6 position, when methylated, could result in miscoding and be base-paired with thymine rather than cytosine. Alkylation of nucleic acid bases followed by DNA replication can fix the lesion in the genetic material. This has been referred to as the somatic mutation hypothesis and may be the possible mechanism of DMN-induced carcinogenesis and mutagenesis. Craddock (1975) administered ^{14}C-DMN to partially hepatectomized rats which were expected to undergo a wave of DNA synthesis within 16 hours of the operation. It appeared that DMN administration caused a significant decrease in the rate of DNA synthesis possibly due to the fact that the replication template contained alkylated bases. It was previously shown by the same investigator that a single treatment with DMN to partially hepatectomized rats could induce liver cell cancer when administered during the most active phase of DNA synthesis (Craddock 1971). Therefore, patterns of damage seem to be dependent on the in vivo mechanisms of repair of alkylated DNA bases present in the target organ. It has been shown that the excision rate of O-6 methylguanine proceeds rapidly as compared to the excision rate for other methylated bases (O'Connor et al. 1973). This is in agreement with Loveless (1969) who concluded that: DMN-induced methylation of DNA

bases may be the potential mutagenic site; that organisms have evolved a protective DNA repair mechanism for such a methylation; and that a period of DNA synthesis prior to repair is essential to fix the base alteration through miscoding. Nicoll, Swann, and Pegg (1975) have further shown that the incidence of tumor production in rat liver as compared to rat kidney is highly dependent on the rate of repair which appears to be much slower in kidney tissue than in the liver tissue. A single large dose of DMN given to an adult rat will produce kidney tumors but not liver tumors, unless given after partial hepatectomy (Craddock 1971) or by repeated small doses or prolonged feeding (Swann and Magee 1968, Magee and Barnes 1967). Therefore, the differing susceptibilities of organs toward the carcinogenic stimuli could be dependent on intrinsic rates of repair. Further studies (Swann et al. 1976) showed that tumor production was cumulative when two doses of DMN were administered four days apart. When separated by 16 days, tumor production was not cumulative; it appeared that recovery as seen by excision and repair had taken place before the second dose was administered.

It has also been proposed that toxicity and carcinogenicity of DMN might be due to a change in RNA or protein, thereby affecting expression of genetic information. Methylation of histidine residues were found by Magee and Hultin (1962), and it was presumed that other amino acids might be similarly methylated. The significance of this appears to be related to a "protein deletion" hypothesis of carcinogenesis developed by Potter (1964). This theory held that the primary event of carcinogenesis was the interaction of the carcinogen with

cellular protein. Inhibition of protein synthesis has been seen within a few hours after DMN administration and precedes the development of necrosis (Magee 1962). It has been suggested that methylation of messenger RNA (mRNA) might be responsible for the inhibition of protein synthesis by an inhibition of translation (Villa-Trevino 1967). In vivo experiments by Pegg and Jackson (1976) have shown ^{14}C-DMN induced alkylation of mRNA, but to a significantly lesser extent than that found in ribosomal RNA. It has been suggested that methylation of mRNA in the nucleus may interfere with its transfer to the cytoplasm. This would result in a deficiency in the amount of active mRNA in the cytoplasm of the cells with a subsequent decrease in protein synthesis (Pegg and Jackson 1976).

Recently, information as to the identification and characterization of the enzyme responsible for the bioactivation of DMN, DMN N-demethylase, has become available. In general, enzyme structure is determined genetically; however, the amount of enzyme is determined by both genetic and epigenetic factors, the latter of which can be of internal or external origin. Two general factors include the nutritional status and the age of the animal. Venkatesan, Arcos, and Argus (1970) first reported the effects of 24 hour starvation of rats with respect to DMN N-demethylase and found an enhanced enzyme activity as exhibited by an increase in the V_{max} of DMN N-demethylation with a relatively stable K_m. The increased V_{max} suggests either an accelerated synthesis of enzyme or a lowered rate of enzyme degradation. Subsequent feeding studies showed that dietary glucose appeared to repress DMN N-demethylase and a high protein diet appeared to

induce the enzyme level above that of starvation. McLean and Verschuuren (1969) have reported that nutritional factors also influence the toxicity of DMN. A carbohydrate diet free of protein protected rats against the lethal and hepatotoxic effects of DMN. These findings indicate that the microsomal drug metabolizing enzymes are controlled by multiple factors and nutritional deficits must be considered when animals are subjected to unusually harsh or uncomfortable pretreatment regiments which might cause them to stop eating. Such a pretreatment induced starvation would add another variable to the analysis which would make data interpretation difficult.

The age of the animal used in experimentation has an effect on drug-metabolizing enzymes. Davies and associates (1976) studied the effect of age versus DMN N-demethylase activity and determined that the maximum activity of DMN N-demethylase appears at about one month of age or approximately 85 grams of body weight. This gradually tapers off to a low at 60 days or about 250 grams. This appears to correlate with the development of the microsomal electron transport components. MacLeod, Renton, and Eade (1972) showed that cytochrome P-450, NADPH-cytochrome P-450 reductase, and NADPH-cytochrome c reductase in rat liver gradually increases in activity with age and level off at about one month. Therefore, the age of the animals used is an important factor in experimentation and interpretation of experimental results.

Perhaps one of the most experimentally useful factors affecting drug-metabolizing enzymes is the exposure of the animal to specific foreign compounds which can alter the level of microsomal enzyme activity. This can occur by inducing or repressing enzyme synthesis or

degradation, or by an allosteric or isosteric inhibition or activation of the enzyme molecule. Any of these mechanisms may play a role in the drug-induced changes in microsomal drug metabolizing enzymes. The enzyme DMN N-demethylase is unusual in that inducers of the typical microsomal drug biotransformation system such as phenobarbital (PB) (Arcos et al. 1975; Venkatesan, Arcos, and Argus 1971), 3-methylcholanthrene (MC) (Hoch-Ligeti, Argus, and Arcos 1968; Arcos et al. 1975; Arcos, Valle, et al. 1976; Venkatesan et al. 1968; Venkatesan, Argus, and Arcos 1970; Venkatesan et al. 1971), and Aroclor 1254, a polychlorinated biphenyl (PCB) (Arcos et al. 1975; Argus et al. 1975; Arcos, Valle, et al. 1976), when injected intraperitoneally in rats, have shown a decrease in DMN N-demethylase activity as measured by the production of formaldehyde in vitro in liver microsomal preparations. The animals all displayed liver hypertrophy as measured by an increase of liver to body weight ratio, increased liver cytochrome P-450, induced N-demethylases other than DMN N-demethylase, and decreased toxicity and carcinogenicity to subsequent DMN administration. PB and MC showed an increase in microsomal protein concentration while Aroclor 1254 showed a decrease. Aminoacetonitrile (AAN) (Fiume et al. 1970; Arcos et al. 1975; Bartsch, Malaveille, and Montesano 1975), pregnenolone-16α-carbonitrile (PCN) (Somogyi et al. 1972; Grandjean and Somogyi 1976; Bartsch et al. 1975; Arcos et al. 1975; Arcos, Valle, et al. 1976), and β-Naphthoflavone (Arcos et al. 1975; Arcos, Valle, et al. 1976) were also found to decrease the metabolism of DMN as measured by formaldehyde production. AAN caused a decreased labeled CO_2 production in rats in vivo and in rat liver slices in vitro when

labeled DMN was used. Also, a decreased binding of ^{14}C from ^{14}C-DMN to liver macromolecules was seen. Both AAN and PCN protected against the toxicity from DMN administration *in vivo*, although only AAN decreased the rate of clearance of DMN from the blood stream.

There has been controversy, however, with respect to the effects of the agents PB, MC, and Aroclor 1254 on the level of microsomal DMN biometabolism. Increased rates of demethylation have been found *in vitro* in rat and mouse liver preparations using Aroclor 1254 (Czygan et al. 1973) and MC and PB (Lake et al. 1974b, Frantz and Malling 1975). In accordance with this controversy it has been suggested that there are multiple DMN N-demethylases present in hepatic microsomes (Lake et al. 1974a; Arcos, Davies, et al. 1976; Arcos, Valle, et al. 1976). Arcos, Davies, et al. (1976) and Arcos, Valle, et al. (1976) have proposed that there are two N-demethylases in rats, DMN N-demethylase I and DMN N-demethylase II. It appears that at low DMN concentrations (0-4 mM) typical enzyme inducers such as PB, MC, and Aroclor 1254 repress DMN N-demethylase I. At high DMN concentrations (50-250 mM), the isoenzyme DMN N-demethylase II appears to be induced by the same compounds. Lake et al. (1974a) used DMN concentrations of 0.5-100 mM in microsomal incubations and found three distinct K_m values indicating possibly three kinetically different enzymes.

Cytochrome P-450 involvement in the N-demethylation of DMN has not been a major issue in the literature since it is generally accepted that the biometabolism of DMN involves an NADPH-cytochrome P-450 coupled enzyme. Both the oxidative demethylation of DMN (Argus et al. 1976) and its activation to a mutagen (Czygan et al. 1973) are dependent

on active cytochrome P-450 as shown by carbon monoxide inhibition studies. However, conflicting aspects with reference to the exact level of participation of this element in the metabolism have been documented. Lake et al. (1974a) established that DMN N-demethylase has a significantly greater storage stability than cytochrome P-450 or ethylmorphine N-demethylase, a cytochrome P-450 dependent microsomal N-demethylase. In addition, studies showing DMN N-demethylase repression with PB and MC (Fujita, Shoeman, and Mannering 1973; Argus et al. 1976) show enhanced levels of cytochrome P-450. Evidence for multiple cytochrome P-450 compounds (Argus et al. 1976, Welton and Aust 1974) does not account, however, for the observed repression of DMN N-demethylase since these same proposed cytochrome P-450's are selectively induced by MC and PB.

To pursue the effects of inducers, Sipes and coworkers (1973) investigated the effects of acetone and isopropanol administration on liver microsomal enzymes. Isopropanol and other aliphatic alcohols have been shown to potentiate the hepatic and renal toxicity due to carbon tetrachloride exposure (Cornish and Adefuin 1967, Traiger and Plaa 1971, Folland et al. 1976). Acetone, a metabolite of isopropanol, can be found *in vivo* in high concentrations in poorly controlled diabetics, persons undergoing strenuous weight loss, and those generally in ketosis. Also, both isopropanol and acetone are widely used in industry. When these agents were given orally to rats, it was found that after an 18 hour period, N-demethylation of DMN was induced, but N-demethylation of ethylmorphine was unaffected. Neither agent caused an increase in microsomal protein, cytochrome P-450, nor NADPH

cytochrome-c-reductase. The lack of effect of acetone pretreatment intraperitoneally in rats on cytochrome P-450 was confirmed by Clark and Powis (1974). Maling and associates (1975) studied the effects of single dose isopropanol and multi-dose ethanol in rats administered by oral gavage and found that both the alcohols induced the N-demethylation of DMN but had no effect on the N-demethylation of ethylmorphine. Also there was no change in the amount of microsomal protein, cytochrome P-450, or the NADPH c-reductase activity. The specific mechanism of induction resulting from these three pretreatment regimens is unclear.

In general, increased enzyme activities produced by pretreatments are usually thought to be the result of either increased enzyme synthesis due to de novo protein synthesis, decreased enzyme degradation, or increased affinity of the enzyme for the substrate. Inhibitors of protein synthesis are commonly used to determine if increased de novo protein synthesis is involved. It is currently accepted that mRNA is synthesized by a DNA directed RNA polymerase and that the mRNA specifies the amino acid sequence on a microsomal incorporation site during protein synthesis. Actinomycin D is an inhibitor of DNA directed RNA synthesis and has been used to determine whether DMN N-demethylase induction is due to de novo protein synthesis. When actinomycin D has been administered to rats throughout a period of a 24 hour starvation, the starvation-induced DMN N-demethylase activity was blocked. This indicates that the starvation had resulted in an enhanced synthesis of enzyme possibly due to an increase in the transcription of DNA into mRNA or by a greater stabilization of the

messenger template (Venkatesan, Argus, and Arcos 1970). Similar results have been found for the more general induction of the mixed function oxidases produced by the classical inducers MC (McLean and Verschuuren 1969; Gelboin and Blackburn 1963, 1964; Vainio, Aitio, and Hanninen 1974) and PB (Orrenius, Ericsson, Ernster 1965; Vainio et al. 1974), the effects of both being blocked by administration of actinomycin D. Cycloheximide, another protein synthesis inhibitor, inhibits translation by inhibiting the chain initiation and elongation at the ribosomal level. Studies have shown that cycloheximide will lower induction phenomenon seen with MC and PB (Vainio et al. 1974) as well as reduce the microsomal metabolism of some foreign compounds as aminopyrine and mono-methyl-4-amino antipyrine (Jondorf, Simon, and Avnimelech 1966). Studies pertaining to DMN N-demethylase activity as affected by cycloheximide administration are lacking at this point.

The field of research in nitrosamine metabolism, with special emphasis on DMN metabolism, is vast and contradictory. Induction and repression phenomena have become a popular means to study in vivo and in vitro DMN metabolism in an attempt to elucidate the specific pathway(s) involved in the potentially toxic, carcinogenic, and mutagenic nitrosamine, DMN.

Statement of the Problem

The in vivo biometabolism and subcellular alkylation of macromolecules has been proposed as a mechanism in chemical toxicity and carcinogenesis. Although the specific pathway involved in DMN metabolism is unknown, there is evidence of microsomal enzymatic

metabolism of DMN to a reactive intermediate which can bind to cellular DNA, RNA, and protein. To further investigate this pathway and to determine if there is a direct correlation between DMN N-demethylase activity and alkylation of macromolecules, studies will be performed in an *in vitro* microsomal system.

Pretreatment of rats with acetone or ethyl alcohol is known to enhance microsomal DMN N-demethylase and to potentiate DMN induced toxicity. Liver microsomes will be isolated from animals pretreated with these compounds. The activity of DMN N-demethylase will be estimated by the formation of formaldehyde, a breakdown product of DMN, and correlated with the alkylation of DNA, RNA, and protein as determined by radioactive tracing of ^{14}C from ^{14}C-DMN. In these studies major emphasis will be placed on the effects of acetone pretreatment on rat liver microsomal metabolism of DMN with some reference to mouse liver metabolism. An investigation will also be made to determine the possible mechanism of action of acetone to potentiate the metabolism of DMN. A preliminary investigation will be made with respect to the correlation between multi-dose ethyl alcohol pretreatment of rats and *in vitro* DMN metabolism.

In general, the majority of published DMN research *in vitro* had used high (100 mM) concentrations of DMN. In these studies a low (0.15 mM) concentration of DMN will be used. Such a concentration would better approximate the chronic *in vivo* carcinogenic conditions of DMN exposure.

METHODS AND MATERIALS

Experimental Animals

Male Sprague-Dawley rats (Hilltop Laboratories, Chatsworth, California) weighing 125 to 200 grams and male C57BL/6J mice (Jackson Memorial Laboratory, Bar Harbor, Maine) weighing 20 to 25 grams were used in the experiments. Prior to experimentation, the animals were housed in wire cages in a temperature controlled room (22°C) on a cycle of 14 hours of dark and 10 hours of light. They were fed laboratory chow (Wayne Lab-Blox) and water ad libitum.

Chemicals

N,N-di(C^{14})methylnitrosamine (4.5 Ci/mole) was purchased from New England Nuclear Corporation. Unlabeled dimethylnitrosamine (DMN) (gold label, 99.9% pure) was obtained from Aldrich Company. RNA and calf thymus DNA were obtained from Calbiochem. Ethylmorphine hydrochloride was supplied by Mallinckrodt. Bovine albumin (Fraction V) was obtained from Metrix.

Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Calbiochem and nicotinamide adenine diphosphate and nicotinamide adenine diphosphate, reduced form, were obtained from Sigma Chemical Company. Actinomycin D (Grade III) and cycloheximide were also purchased from Sigma.

Aquasol® Universal Liquid Scintillation Cocktail, obtained from New England Nuclear, and Kimble borosilicate disposable scintillation

vials were used routinely in all studies. A Beckman LS-100C scintillation counter was used for all radiolabel determinations. The external standard ratios method using a ^{14}C-toluene quench curve was used to convert counts per minute to disintegrations per minute. All determinations were corrected for background using a blank consisting of Aquasol and the specific diluent used.

Pretreatment of Animals

Redistilled pesticide grade acetone diluted with an equal volume of 0.9% NaCl was administered to rats and mice by intraperitoneal (ip) injection (40 mmoles/kg body weight). The volume of injection solution was equal to 6 ml/kg body weight. Controls were injected ip with 6 ml/kg body weight 0.9% NaCl. During the period before sacrifice (18 hours), the animals were either fed or fasted according to the experimental design. All were given free access to water.

Ethyl alcohol (4 gm/kg body weight) was administered to rats by oral intubation in four equal doses 48, 42, 24, and 18 hours before sacrifice. Each dose consisted of ethyl alcohol diluted in an equivalent amount of water to give a final volume of 10 ml/kg body weight. Controls were administered water (10 ml/kg body weight). During the induction period, all animals were given food and water ad libitum.

Actinomycin D (0.6 mg/ml in 0.9% NaCl) was injected ip to rats one hour prior to acetone administration in a volume equivalent to 1 ml/kg body weight. Controls were administered ip 1 ml/kg 0.9% NaCl. The rats were fasted before sacrifice, but were allowed water ad libitum.

Cycloheximide (1 mg/ml in 0.9% NaCl) was injected ip to rats immediately preceding acetone pretreatment. The dose was equivalent to 1 ml/kg body weight. Controls were administered an equivalent amount of 0.9% NaCl. The rats were fasted, but water was allowed ad libitum.

Liver Isolation and Microsomal Preparation

The animals were sacrificed between 8 a.m. and 9 a.m. to preclude rhythmic diurnal variation. Rats were decapitated and mice were subjected to cervical dislocation. Livers were quickly removed and placed in cold 50 mM phosphate buffer (pH 7.4) which contained 0.64 mM $MgCl_2$ and 34.7 mM EDTA according to Chin and Bosmann (1976). This buffer will be referred to as 50 mM phosphate buffer throughout the text. The livers were then freed of extraneous tissue debris and blood, and weighed in preparation for microsomal isolation.

To isolate microsomes, the livers were individually homogenized in three volumes of cold 50 mM phosphate buffer using a drill-motor driven teflon pestle and glass homogenizing tube until the livers were homogeneous, usually less than one minute. Care was taken to keep the samples cold throughout this process. The homogenate was centrifuged at 9,000 x g for 30 minutes in a refrigerated (5°C) Sorval Superspeed RC2-B centrifuge. The top floating lipid layer was carefully aspirated before the tubes were removed from the rotor. Recentrifugation of the resulting supernatant followed using an IEC/B-60 ultracentrifuge at 105,000 x g for 60 minutes at 5°C. The resultant microsomal pellet was rinsed several times with cold 50 mM phosphate buffer and either overlayed with the buffer and frozen at -70°C

overnight, or resuspended directly into a small amount of the buffer using the previously mentioned homogenizing equipment for 10 seconds or less. Upon repeated experimentation, no loss of activity was noted due to the low temperature freezing. The initial protein concentration of the microsomal suspension was determined using the Biuret method (Gornall, Bardawill, and Davis 1949) with Bovine albumin (Fraction V) as the standard. All samples were adjusted to a final protein concentration of 6 mg/ml or less as indicated in the specific experimental design.

^{14}C-DMN Alkylation and Isolation of Macromolecules

Microsomal incubation experiments were performed using N,N-di(^{14}C)methylnitrosamine (specific activity 4.5 Ci/mole). A 3 ml incubation volume contained 1 ml (6 mg) of microsomes, 2 mg calf thymus DNA suspended in 1 ml of 50 mM phosphate buffer, and 1 ml of a complete NADPH regenerating system composed of 37.6 mM $MgCl_2$, 0.48 mM NADPH, 0.47 mM NADP, 9.3 mM Glucose-6-phosphate, 1 unit of Glucose-6-phosphate dehydrogenase, and 50 mM phosphate buffer. The complete media was made just prior to addition and the start of the incubation to ensure full activity. Microsomes which had been denatured by heating at 60°C for 30 minutes were used in the procedure as blanks to represent non-enzymatic binding.

Incubations were conducted in a Dubnoff metabolic shaking incubator maintained at 37°C with gentle shaking in a vented hood. All samples were preincubated for 5 minutes followed by the addition of 20 µl of ^{14}C-DMN giving a final concentration of 0.15 mM, unless otherwise

specified. The protein concentration, time of incubation, and mM concentration of ^{14}C-DMN were experimentally determined. A 30 minute incubation was followed by the addition of a mixture of 6% p-aminosalicylic acid-1% NaCl in a volume equivalent to 15 ml per gram of liver according to the method of Kirby and Cook (1967). The volume routinely used was 3 ml of PASA:NaCl. A volume of 3 ml of phenol-m-cresol (454 gms phenol crystals, 50 ml water, 63 meta-cresol, 0.5 gm 8-hydroxyquinoline) was then added and the samples were mixed for 20 minutes using a magnetic stir bar with a magnetic stir plate. The phenol acted to deproteinize the sample and was then separated from the aqueous phase containing the nucleic acids by centrifugation for 10 minutes at 9,000 x g at 5°C.

The resultant aqueous supernatant was carefully aspirated off the phenol layer and overlaying suspended protein pellet using a pasteur pipet. The supernatant was then subjected to two additional phenol extractions using one-half the original volume of phenol. To ensure phase separation and adequate deproteinization, the aspirated supernatant was first made 3% (w/v) with respect to NaCl before the addition of the phenol-m-cresol mixture. This step was omitted for the third extraction to prevent phase reversal upon centrifugation. The final aqueous supernatant contained the calf thymus DNA and the microsomal RNA with less than 3% residual protein. A common pool of the three phenol layers was made for each sample and saved for protein isolation.

Precipitation of DNA and RNA from the aqueous supernatant was facilitated by the addition of ethanol-m-cresol (9:1) equal to two

times the volume of the supernatant. This mixture was gently mixed, allowed to stand at least one hour in the cold, and spun at 12,000 x g for 15 minutes to sediment the precipitated nucleic acids. The nucleic acids were then resuspended in 4 ml of 0.5 M NaCl-0.3 M Na Acetate in a cold room (0-5°C) with gentle shaking on an Eberbach rotator for 12 hours. Reprecipitation with 95% alcohol was then performed using a volume equivalent to two times the suspension. The mixture was gently shaken and allowed to stand on ice for one hour. This was then centrifuged at 10,000 x g for 10 minutes. The alcohol extraction was repeated two times. After the final centrifugation, the pelleted DNA and RNA were suspended in 4 ml of 0.3 N NaOH.

Alkaline hydrolysis separation of the RNA from the DNA followed according to a modified method of Schmidt and Thannhauser (1945). The mixture of DNA and RNA in 0.3 N NaOH was heated for 30 minutes at 70°C. The DNA was reprecipitated by the addition of 1.6 ml of 5 N perchloric acid (PCA) to give a final concentration of 1 N and separated from the RNA by centrifugation at 20,000 x G for 10 minutes at 5°C. The supernatant containing the RNA was saved for further analysis. The sedimented DNA was washed two times with cold 0.2 N PCA. The sedimented DNA was then hydrolyzed in 6 ml of 1 N PCA for 20 minutes at 70°C. The mixture was chilled and centrifuged at 12,000 x g for 10 minutes yielding a supernatant containing the DNA. Aliquots were removed to determine the quantity of bound ^{14}C radiolabel by scintillation counting and to determine the amount of DNA present using the diphenylamine reaction of Burton (1968) with calf thymus DNA as the standard. Aliquots were similarly removed for quantitation of RNA

present as a contaminant. Values were calculated as picomoles ^{14}C-DMN equivalent bound per mg DNA.

Aliquots of the RNA fraction were similarly analyzed for ^{14}C from ^{14}C-DMN and RNA quantitation using the orcinol reaction (Schneider 1957) with RNA dissolved in 0.1 N NaOH as the standard. Values were expressed as picomoles ^{14}C-DMN equivalent bound per mg RNA. Cross contamination of RNA with DNA was determined by performing both an orcinol and a diphenylamine analysis on each sample. When contamination was found, corrections were made using the specific activity of the sample and the amount of the contaminant present. Protein analyses of the DNA and RNA samples showed less than 3% contamination.

Binding of ^{14}C from ^{14}C-DMN to protein was determined by adding two volumes of methanol to the pooled phenol extracts to precipitate the protein. This mixture was allowed to stand overnight and then centrifuged using a table top Sorval centrifuge at 2,500 rpm until the supernatant was clear (about 20 min). The phenol supernatant was aspirated from the protein pellet and discarded. The pellet was washed with chloroform:methanol (2:1) (2 times), 5% TCA at 60°C for 5 minutes (5 times), methanol:ether (3:1) at 60°C for 5 minutes (5 times), and acetone (2 times). The pellet was dried with a gentle stream of nitrogen and resuspended in 4 ml of 1 N NaOH. An aliquot was removed and diluted with an equal volume of water for scintillation counting. This was done to prevent gel formation upon the addition of the scintillation cocktail. A similar aliquot was removed for protein analysis using the method of Lowry et al. (1951) with Bovine albumin

(Fraction V) as the standard. Experimental values were calculated as picomoles ^{14}C-DMN equivalent bound per mg protein.

DMN N-Demethylase Enzyme Activity

The analysis of DMN N-demethylase activity as measured by the formation of formaldehyde was performed in a separate incubation mixture following the procedure described for the ^{14}C-DMN alkylation experiments. Calf thymus DNA was omitted after preliminary experimentation showed no significant difference between incubations with DNA and those without with respect to N-demethylase levels. Heat denatured microsomes (60°C--30 min) were used as tissue blanks to correct for the non-enzymatic breakdown of the substrate as well as to correct for the slight yellow color of the substrate, DMN. The samples were preincubated for 5 minutes at 37°C before the addition of 0.1 ml of DMN to give a final concentration of 0.15 mM DMN, unless otherwise specified. The incubation time and substrate concentration were experimentally determined by both time course and substrate concentration studies. The reaction was found to be linear up to 30 minutes.

After a 30 minute incubation, the reaction was stopped by the addition of 1 ml $ZnSO_4$ (15%), followed by the addition of 1 ml of saturated $BaOH_2$ five minutes later. The mixture was thoroughly mixed between each addition and then centrifuged at 2,500 rpm for 20 minutes to sediment the precipitated protein. Formaldehyde production as a measure of N-demethylase activity was assayed by the procedure of Nash (1953), as modified by Cochin and Axelrod (1959). Nanomoles of

formaldehyde formed per mg microsomal protein per 30 minutes were calculated.

Ethylmorphine N-Demethylase Activity

Determination of the ethylmorphine N-demethylase activity was determined as described for DMN N-demethylase with ethylmorphine hydrochloride as the substrate. A volume of 0.1 ml of a 300 mM solution of ethylmorphine in water was added to give a final concentration of 10 mM. All experimental variables (time and temperature) as well as the analytical determination of formaldehyde were identical to those employed for DMN N-demethylase studies previously described.

Statistics

The student's t-test was used to analyze the significance of the difference between the means of two groups containing greater than two animals. When a $p < .05$ was obtained, the differences in the means were considered significant. A standard deviation determination was obtained for groups with one or two animals.

When three groups were compared (Table 3, p. 40) an F test was performed to determine if there was a statistically significant difference between the values followed by the Newman-Keuls test using a 0.05 confidence level (Myers 1972).

RESULTS

Standardization of In Vitro Assays

To establish a procedure adequate to measure in vitro DMN metabolism using microsomes isolated from rat liver, an incubation time course was conducted. Liver microsomes from acetone pretreated and control rats were incubated in the presence of calf thymus DNA (2 mg) with ^{14}C-DMN (0.15 mM; specific activity 4.5 Ci/mole). Incubations were performed at 37°C and terminated at 10, 30, 60, and 120 minutes; covalent binding of ^{14}C from ^{14}C-DMN to calf thymus DNA and microsomal protein was determined. Binding of ^{14}C to heat denatured microsomes representing non-enzymatic binding was subtracted from the experimental samples in each case. As shown in Figures 1 and 2, the covalent binding of ^{14}C to DNA and protein was greater when microsomes from acetone pretreated rats were used to bioactivate ^{14}C-DMN as compared to microsomes from control animals. Depending on the time point, the increases produced by acetone pretreatment were 133% to 197% for binding to DNA and 63% to 184% for binding to protein. Values representing the degree of binding were linear between 10 to 60 minutes for both DNA and protein. A standard incubation length of 30 minutes was chosen for subsequent experiments for the following reasons. An incubation of 30 minutes was in the linear portion of the reaction and resulted in greater binding with less variability than 10 minutes. The metabolism of DMN is dependent on NADPH and O_2 and an incubation of 30 minutes

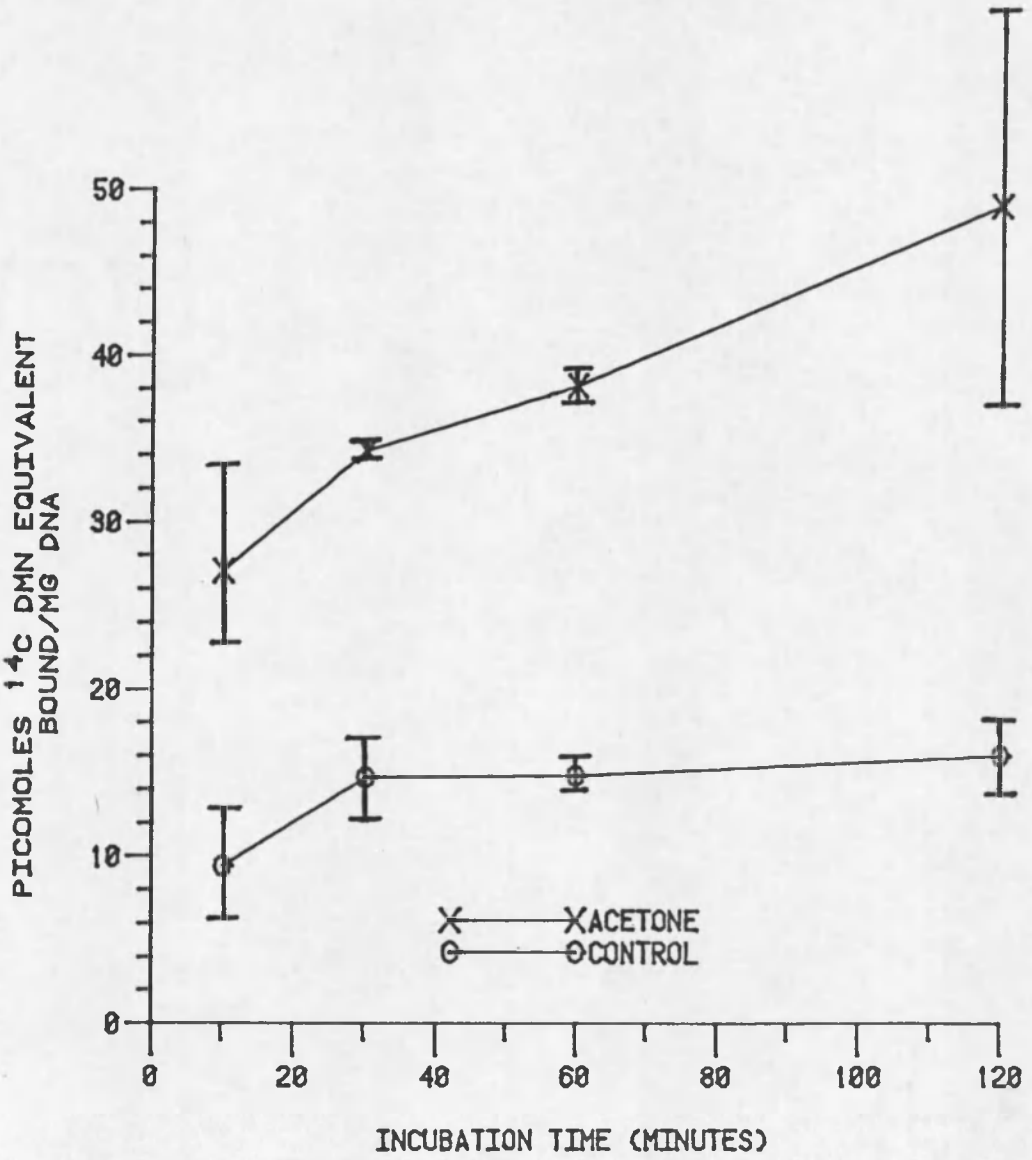

Figure 1. Effect of Incubation Time on the In Vitro Microsomal Catalyzed Binding of ^{14}C from ^{14}C-DMN to Calf Thymus DNA: Comparison of Microsomes from Control and Acetone Pretreated Animals -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of liver microsomes. Incubations were performed at 37°C and binding of ^{14}C from ^{14}C-DMN (0.15 mM) to calf thymus DNA was determined. Each point represents the mean $\pm$ SD for 2 animals.

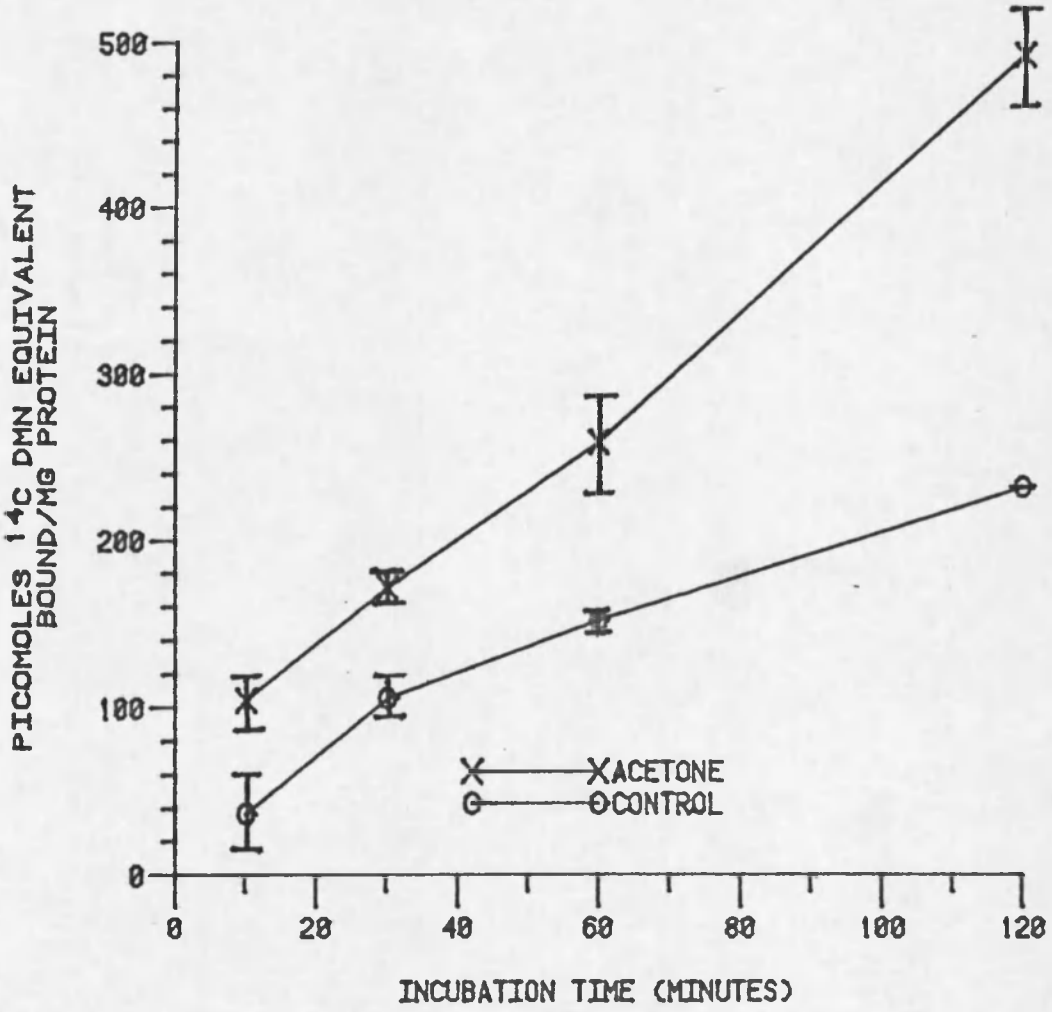

Figure 2. Effect of Incubation Time on the In Vitro Microsomal Catalyzed Binding of ^{14}C from ^{14}C-DMN to Microsomal Protein: Comparison of Microsomes from Control and Acetone Pretreated Animals -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of liver microsomes. Incubations were performed at 37°C and binding of ^{14}C from ^{14}C-DMN (0.15 mM) to microsomal protein was determined. Each point represents the mean ± SD for 2 animals.

would allow reasonable assurance that the NADPH regenerating system and O_2 levels were adequate.

To determine the minimum concentration of ^{14}C-DMN that could be used in the microsomal system to give efficient and representative binding data, varying concentrations of ^{14}C-DMN were added to the microsomal samples. All samples contained 2 mg calf thymus DNA and were incubated at 37°C for 30 minutes. Binding to DNA and protein in heat denatured microsomes, representing non-enzymatic binding, was subtracted from each sample point. The amounts of ^{14}C-DMN used were 10, 20, and 50 µl equivalent to a final concentration of 0.07, 0.15, and 0.34 mM, respectively. The data in Figures 3 and 4 demonstrate the relationship between *in vitro* binding of ^{14}C from ^{14}C-DMN to calf thymus DNA and microsomal protein in control and acetone pretreated rat liver microsomes. Acetone pretreatment of rats resulted in enhanced *in vitro* covalent binding of DMN as compared to control microsomes. Binding of ^{14}C to DNA and protein increased with increasing concentrations of ^{14}C-DMN in both control and acetone pretreated microsomal samples. A final concentration of 0.15 mM ^{14}C-DMN was chosen because it gave the greatest difference between control and acetone-treated animals, and allowed the direct use of a convenient volume of undiluted ^{14}C-DMN. This concentration represented approximately 5.4×10^6 dpm per sample. At this concentration binding to calf thymus DNA catalyzed by microsomes from acetone pretreated rats was 259% greater than that from control rats. Binding to protein was increased by 272% in acetone pretreated microsomal samples as compared to binding in control microsomes.

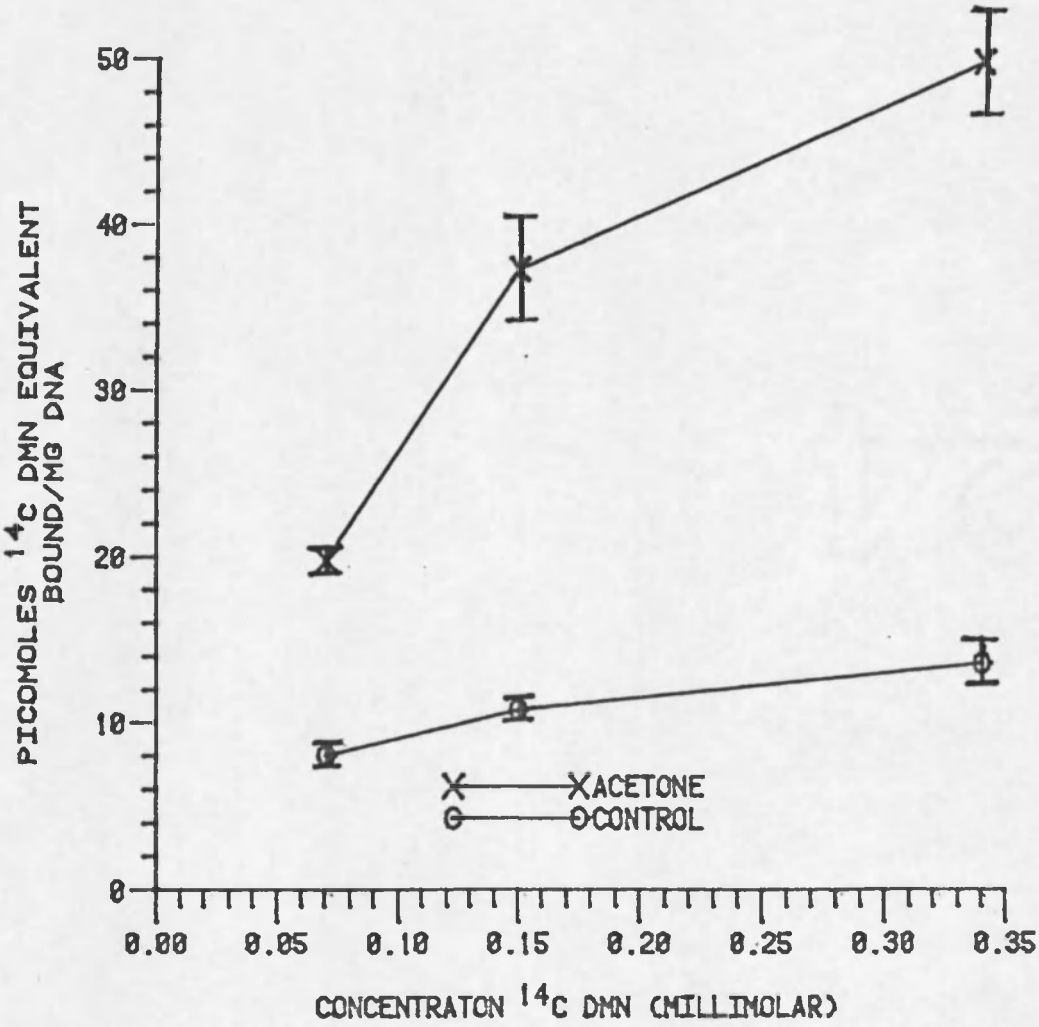

Figure 3. Effect of ^{14}C-DMN Concentration on the In Vitro Microsomal Catalyzed Binding of ^{14}C from ^{14}C-DMN to Calf Thymus DNA: Comparison of Microsomes from Control and Acetone Pretreated Animals -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of liver microsomes. Incubations were performed at 37°C for 30 minutes in duplicate. Each point represents the mean $\pm$ SD for 2 animals.

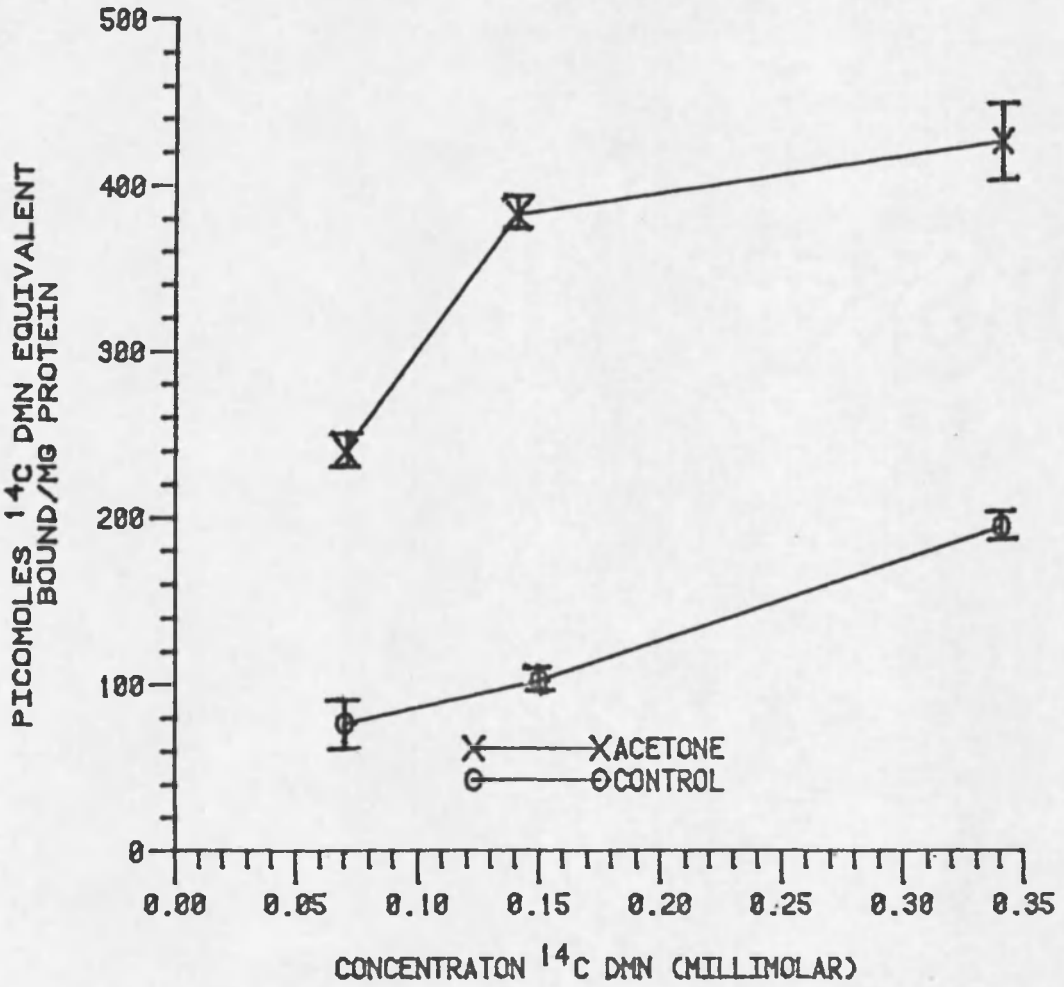

Figure 4. Effect of ^{14}C-DMN Concentration on the In Vitro Microsomal Catalyzed Binding of ^{14}C from ^{14}C-DMN to Microsomal Protein: Comparison of Microsomes from Control and Acetone Pretreated Animals -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of liver microsomes. Incubations were performed at 37°C for 30 minutes in duplicate. Each point represents the mean ± SD for 2 animals.

It was noted that the rats were subject to anesthesia immediately following ip injection of acetone. They were limp and unable to stand or move within 15 minutes of the injection. It was assumed that the rats might be so physically ill that they were undergoing pretreatment-induced fasting resulting in increased toxicity. Quantitation of the amount of food and water consumed during the 18 hours following pretreatment confirmed this assumption and showed that acetone pretreated animals failed to eat and drink. To investigate the effects of fasting and acetone-induced fasting on the liver, the liver weight/100 gm body weight ratio and the liver microsomal protein/gm liver ratio were compared in three experiments. Table 1 shows that fasting for 18 hours and acetone-induced fasting significantly decreased the liver weight and microsomal protein concentration ratios as compared to the control-fed group. Since these results suggested the possible contribution of pretreatment-induced fasting on metabolism of DMN, further experimentation was designed to eliminate fasting as a variable in the metabolic induction phenomenon seen with acetone pretreatment. This was accomplished by the restriction of food from both control and acetone pretreated animals during the 18 hour period following pretreatment and preceding sacrifice. All animals were allowed water ad libitum.

Using parameters established in the standardization of the *in vitro* ^{14}C-DMN microsomal covalent binding assay, a standardization of the N-demethylation reaction as measured by formaldehyde production was investigated. All animals were fasted following pretreatment and DMN N-demethylase activity was measured in control-fasted and acetone-fasted

Table 1. Effect of Acetone and/or Fasting on Rat Liver Weight and Microsomal Protein Concentration -- Male rats were administered ip saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and microsomal isolation. Each value represents the mean ± SEM of number of animals in parentheses. Food was restricted from the experimental animals with water ad libitum. Control animals were fed ad libitum.

Measurement	Pretreatment		
	Control fed (C)	Control Fasted (CF)	Acetone-Fasted (AF)
Liver weight/100 gm body weight			
Experiment I (2)	4.26±.06	3.69±.11	3.79±.06
Experiment II (4)	4.22±.09	3.44±.04	3.93±.12
Experiment III (4)	4.46±.07	3.38±.22	3.93±.26
Mean ± SEM	4.32±.06	3.45±.10[a]	3.85±.11[a,c]
Micrsomal protein/gm liver			
Experiment I (2)	26.3±.3	19.6±.1	19.8±.8
Experiment II (4)	21.0±.9	19.3±1.1	20.0±.4
Experiment III (4)	24.3±.9	20.0±.7	19.2±.3
Mean ± SEM	23.4±.8	19.6±.5[b]	19.7±.2[b,c]

[a] $p < .001$ as compared to C.

[b] $p < .005$ as compared to C.

[c] No significant difference between CF and AF.

rat liver microsomal incubations. The microsomal samples contained 6 mg microsomal protein and 0.15 mM unlabeled DMN. Calf thymus DNA was eliminated from the incubation mixture for several reasons. First, an attempt to use a double incubation volume for each sample which was to be divided into two samples for binding and N-demethylase determinations was not efficient. A double aliquot of labeled DMN was required for each sample and attempts to equally divide the sample failed due to the slight viscosity of the exogenous DNA. A comparison of DMN N-demethylase activity in the presence and absence of exogenous calf thymus DNA was conducted and the results of this investigation are shown in Table 2. At all four time points (10, 20, 30, and 60 minutes) DMN N-demethylase activity was consistently higher in the samples without DNA. The percentage increase ranged from 7 to 32% and was linear up to 30 minutes. Since it was observed that the increase was proportional with time and that DMN N-demethylase activity as measured in these analyses would be a relative number subject to experimental conditions and used only to compare DMN N-demethylation in control and experimental models, exogenous calf thymus DNA was excluded from the N-demethylase determination mixture.

Figure 5 shows the results of N-demethylation of DMN (0.15 mM) at three time points (10, 30, and 60 minutes). Heat denatured microsomes were used to correct for the non-enzymatic breakdown of the substrate, DMN. Acetone pretreated microsomes consistently yielded higher N-demethylase values than control microsomes. These values were 27% at 10 minutes ($p < .005$), 72% at 30 minutes ($p < .001$), and 136% at 60 minutes ($p < .001$), expressed as percentages of control values. In

Table 2. Effect of Exogenous Calf Thymus DNA on *In Vitro* Liver Microsomal N-demethylation of DMN -- Control rat liver microsomes were incubated with 4 mM DMN in the presence and absence of 2 mg calf thymus DNA. Values represent the mean ± SD for two animals.

	Formaldehyde Production (nanomoles/mg microsomal protein)	
Incubation time (minutes)	With DNA	Without DNA
10	7.0 ± .5	7.5 ± .2
20	11.0 ± .9	12.5 ± .7
30	14.2 ± .7	18.7 ± 1.4
60	27.3 ± 1.9	32.2 ± .2

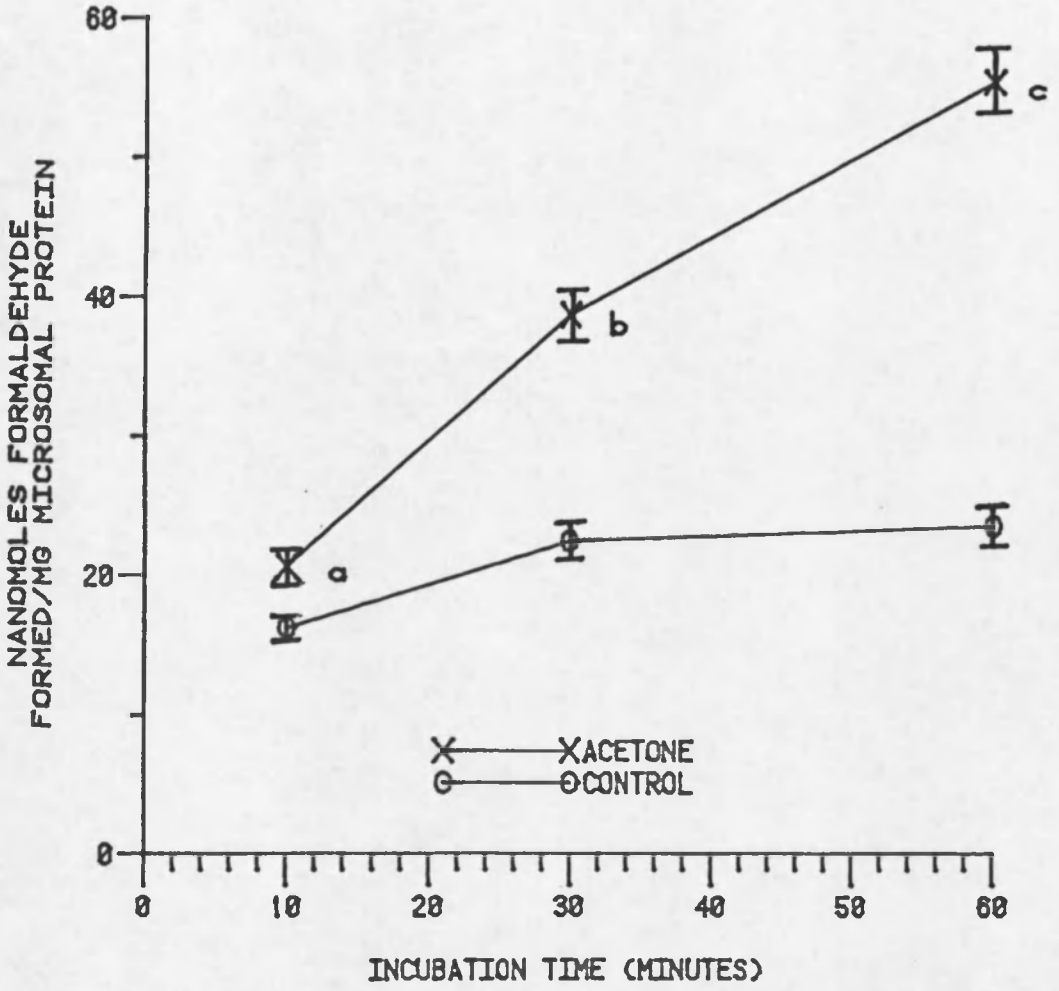

Figure 5. Effect of Incubation Time on In Vitro N-demethylation of DMN Catalyzed by Microsomes from Control and Acetone Pretreated Rat Liver -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of liver microsomes. Incubations were performed at 37°C in duplicate and contained 0.15 mM DMN. Each point represents the mean ± SEM of 3 experiments with 3-4 animals per experiment. a: $p < .005$; b: $p < .001$; c: $p < .001$.

order to compare the degree of binding of ^{14}C from ^{14}C-DMN and the enzymatic production of formaldehyde (i.e., DMN N-demethylase activity) formed during the metabolism, 30 minutes was chosen as the time of incubation in subsequent experiments. Similarly, a 0.15 mM concentration of unlabeled DMN was selected to be used in further N-demethylase analyses to correspond to that used in the covalent binding studies.

In Vitro DMN Microsomal Metabolism

The effects of ip acetone pretreatment of rats and 18 hour fasting on liver microsomal metabolism are shown in Table 3. All procedures were conducted following the standardized rat liver microsomal assay at 37°C for 30 minutes in the presence of 6 mg microsomal protein. Each determination was corrected for non-enzymatic breakdown of the substrate and binding using heat denatured microsomes. N-demethylation of ethylmorphine, a known cytochrome P-450 dependent reaction, was reduced by fasting and acetone pretreatment with fasting. The values determined were not statistically significant when compared to control-fed animals. With respect to the in vitro metabolism of DMN, acetone pretreatment with fasting and 18 hour fasting both showed enhanced formaldehyde formation. N-demethylation of DMN was statistically significantly increased 65% in acetone pretreated animals as compared to control-fed and 38% as compared to control-fasted. Metabolism of DMN as measured by covalent binding showed a similarly greater percentage increase in acetone pretreated animals as compared to the control groups. Binding to DNA in microsomes from acetone pretreated rats as compared to control-fed rats was increased 101% and 48% as

Table 3. Effects of Acetone and/or Fasting on In Vitro Liver Microsomal Metabolism of DMN and Ethylmorphine -- Male rats were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of microsomes. One group of controls was fed ad libitum and all other rats were fasted. All were given water ad libitum. The values represent the mean ± SEM of four animals.

Measurement	Pretreatment of rats		
	Control-Fed (C)	Control-Fasted (CF)	Acetone-Fasted (AF)
N-demethylation of ethylmorphine (nanomoles HCHO/mg microsomal protein/30 minutes)	41.2±2.3	34.7±3.4	37.6±2.3
N-demethylation of DMN (nanomoles HCHO/mg microsomal protein/30 minutes)	20.7±1.7	24.7±1.3	34.1±2.1[a]
Covalent binding of ^{14}C from ^{14}C-DMN (picomoles/mg/30 minutes)			
DNA (calf thymus)	28.0±2.6	38.0±2.0	56.3±4.7[a]
RNA (microsomal)	70.8±2.9	102.0±2.4	153.8±14.4[b]
Protein (microsomal)	196.5±10.4[b]	328.7±14.5[b]	465.5±34.8[b]

[a]Significantly different from C and CF.

[b]Significantly different: C vs. CF, CF vs. AF, C vs. AF.

compared to control-fasted. Binding to microsomal RNA, in general, was greater than that observed to calf thymus DNA. Acetone pretreatment resulted in a 117% greater binding of ^{14}C from ^{14}C-DMN to RNA than that to RNA from control-fed rats and 51% greater than the binding to RNA from control-fasted rats. An even greater level of binding of ^{14}C to protein was observed relative to the levels of binding to other macromolecules. Binding to microsomal protein was increased in acetone-pretreated microsomes 137% over that in microsomes from control-fed rats and 42% greater than that found in microsomes from control-fasted rats.

Pretreatment of mice with ip acetone resulted in an enhancement of the in vitro microsomal metabolism of DMN (Table 4). The N-demethylation of DMN as measured by formaldehyde production was significantly increased ($p < .01$) by 116% in acetone pretreated mouse liver microsomes as compared to control mouse liver microsomes. Covalent binding of ^{14}C from ^{14}C-DMN to subcellular macromolecules showed a similar but greater percentage increase due to pretreatment. Acetone pretreatment resulted in a 183% increase in binding to exogenous calf thymus DNA, 324% to microsomal RNA, and 273% to microsomal protein with a statistical significance of $p < .001$ as compared to control mouse liver preparations. The percentage increase in covalent binding of two to three fold was not reflected to the same degree in the determination of N-demethylase activity which showed slightly more than a one fold increase.

Table 4. Effect of Acetone Pretreatment of Mice on the In Vitro Liver Microsomal Metabolism of DMN -- Male mice were injected ip with either saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice and isolation of microsomes. Each sample contained 4 mg microsomal protein. Values represent the mean ± SEM of 6 animals.

Measurement	Pretreatment of mice		
	Control	Acetone	% Increase
N-demethylation of DMN (0.15 mM) (nanomoles HCHO/mg microsomal protein/30 min)	10.7±0.7	22.5±2.0[a]	116
Covalent binding of ^{14}C from ^{14}C-DMN (0.15 mM) (picomoles/mg/30 min) to:			
DNA (calf thymus)	21.3±1.0	60.2±3.9[b]	183
RNA (microsomal)	102.6±10.6	434.7±33.9[b]	324
Protein (microsomal)	119.2±10.7	444.7±41.2[b]	273

[a] $p < .01$.

[b] $p < .001$.

Effects of Protein Inhibitors on Acetone-Induced DMN Metabolism

Actinomycin D (ACT), an inhibitor of DNA dependent RNA synthesis, was administered to rats one hour prior to acetone pretreatment and DMN N-demethylase activity was determined *in vitro* (Table 5). All animals were fasted from the time of pretreatment to the time of sacrifice and the DMN N-demethylase analysis was conducted according to the standardized protocol using 6 mg microsomal protein per 3 ml and 0.15 mM DMN. The administration of ACT resulted in a 26% repression of DMN N-demethylase in control animals and a 5% repression in acetone pretreated-ACT animals as compared to acetone controls. Acetone pretreatment resulted in a 61% increase in DMN N-demethylase as compared to saline controls ($< .02$) and acetone-ACT pretreated microsomes showed a 101% increase as compared to saline-ACT controls ($p < .02$).

An analysis of the means of the liver weight/100 gm body weight ratio and the content of microsomal protein of the ACT-treated animals as compared to the non-ACT treated animals showed no statistically significant differences between the means of the groups.

Cycloheximide, a powerful inhibitor of protein synthesis at the ribosomal level, was administered to rats one hour prior to acetone or saline pretreatment. The analysis of DMN N-demethylation was performed in the same manner as the ACT experiment according to the standardized protocol. Table 6 shows the results of this experiment. A 25% decrease in DMN N-demethylase activity was observed in control-cycloheximide pretreated rat liver microsomes as compared to saline controls with a statistical significance of $p < .02$.

Table 5. Effect of Actinomycin D on Acetone-induced Rat Liver Metabolism In Vitro -- Male rats were administered ip saline (1 ml/kg) or ACT (0.6 ml/kg as a 1 ml/kg solution in saline) 1 hour prior to ip administration of saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) and fasted 18 hours before sacrifice and isolation of microsomes. Values represent the mean ± SEM of 2 experiments with the number of animals in parentheses. C--Saline; CA--Saline and ACT D; A--Acetone; AA--Acetone + ACT D.

Treatment	DMN N-demethylase (nanomoles HCHO/mg protein/30 min)	Liver Weight (gms/100 gms body weight)	Microsomal Protein (mg/gm liver)
Control (C) (5)	27.9±2.2[a]	3.4±0.1	16.1±0.8
Control + ACT (CA) (5)	20.7±1.4[b]	3.6±0.1	16.7±1.4
% Inhibition	26%		
Acetone (A) (5)	44.8±3.7[c]	3.9±0.1	18.5±0.5
Acetone + ACT (AA) (8)	42.6±3.7	3.8±0.1	16.0±1.3
% Inhibition	5%		

[a] $p < .02$ as compared to A and no significant difference from CA.

[b] $p < .02$ as compared to AA.

[c] No significant difference from AA.

Table 6. Effect of Cycloheximide on Acetone-induced Rat Liver Metabolism *In Vitro* -- Male rats were injected ip with saline (1 ml/kg) or cycloheximide (1 mg/kg as a 1 ml/kg dose in saline) prior to ip pretreatment with saline (6 ml/kg) or acetone (6 ml/kg as a 50% saline solution) 18 hours before sacrifice. All animals were fasted and the values represent the mean ± SEM of 4 experiments with the number of animals in parentheses.

Treatment	DMN N-demethylase (nanomoles HCHO/mg protein/30 min)	Liver Weight (gms/100 gms body weight)	Microsomal protein (mg/gm liver)
Control (C) (10)	28.6±2.0	3.6±0.2	17.0±1.5
Control + Cycloheximide (CC) (10)	21.4±1.0[a]	4.2±0.1	18.5±0.7
% Inhibition	25%		
Acetone (A) (10)	41.7±2.2[b]	4.0±0.1	18.1±0.7
Acetone + Cycloheximide (AC) (10)	27.8±0.9	4.0±0.1	18.7±1.3
% Inhibition	33%		

[a] $p < .02$ as compared to C and $p < .005$ as compared to AC.

[b] $p < .005$ as compared to C and $p < .001$ as compared to AC.

Acetone-cycloheximide pretreated microsomes demonstrated a 33% decrease in N-demethylase activity as compared to acetone pretreated rat liver microsomes with a statistical significance of $p < .001$. A comparison of the acetone-cycloheximide group to the control-cycloheximide group showed a 30% increase due to the acetone pretreatment with a $p < .005$. Analysis of the liver weight and microsomal protein content showed no significant differences between the groups.

Effect of *In Vivo* Ethyl Alcohol on *In Vitro* DMN Metabolism

Ethyl alcohol administered orally in four equal doses over a two day period to rats resulted in an enhancement of DMN metabolism *in vitro* and a repression of ethyl morphine metabolism *in vitro*. All samples were prepared using the established protocol with 6 mg microsomal protein per sample. As shown in Table 7, the metabolism of ethylmorphine (10 mM) catalyzed by liver microsomes obtained from ethyl alcohol pretreated rats showed a decrease of 35% as compared to control values ($p < .02$). However, the *in vitro* metabolism of DMN as measured by N-demethylation of DMN and covalent binding of the active (alkylating) intermediate to liver macromolecules was significantly increased ($p < .01$) when hepatic microsomes from ethyl alcohol pretreated rats were used. DMN N-demethylase activity was enhanced in pretreated rats 85% as compared to controls ($p < .01$); a similar increase in covalent binding of ^{14}C from ^{14}C-DMN was observed to calf thymus DNA (61%) ($p < .01$), to microsomal RNA (104%) ($p < .01$), and to microsomal protein (133%) ($p < .01$). An analysis of the liver weights

Table 7. The Effects of Repeated Oral Ethanol to Rats on the In Vitro Liver Microsomal Metabolism of DMN and Ethylmorphine -- Male rats were orally administered ethanol (10 ml/kg as a 50% water solution) or water (10 ml/kg) four times 48, 42, 24, and 18 hours before sacrifice and isolation of liver microsomes. Values represent the mean ± SEM of 5 animals.

Measurement	Pretreatment of rats	
	Water	Ethanol
N-demethylation of ethylmorphine (10 mM) (nanomoles HCHO/mg microsomal protein/30 min)	35.6±2.2	23.3±1.9[a]
N-demethylation of DMN (0.15 mM) (nanomoles HCHO/mg microsomal protein/30 min)	17.7±0.7	32.8±2.6[b]
Covalent binding of ^{14}C from ^{14}C-DMN (picomoles/mg/30 min) to:		
DNA (calf thymus)	37.7±2.0	60.6±4.0[b]
RNA (microsomal)	53.3±1.6	108.8±10.6[b]
Protein (microsomal)	59.7±1.8	139.0±14.8[b]
Liver weight ratio (gm/100 gm body weight)	4.2±.1	4.4±.3
Microsomal protein concentration (mg/gm liver)	25.6±.9	22.8±1.3

[a] $p < .02$.

[b] $p < .01$.

and microsomal protein content of the treated and nontreated animals showed no significant differences.

DISCUSSION

In vivo biometabolism of DMN to an active alkylating intermediate has been suggested as the initiating factor in nitrosamine-induced carcinogenesis and mutagenesis. The initial step in the bioactivation is an N-demethylation mediated by a DMN N-demethylase enzyme system associated with the microsomes. Based on the findings that this reaction required both NADPH and molecular oxygen (Magee and Vandekar 1958) and was inhibited by carbon monoxide (Czygan et al. 1973, Argus et al. 1976) it was assumed that the enzyme system was dependent on the NADPH-cytochrome P-450 coupled reaction. However, the extent of this association has become increasingly controversial with respect to the usual cytochrome P-450 drug metabolizing enzyme systems. *In vitro* studies using various metabolic inhibitors (Lake et al. 1975) have shown a difference in response between DMN N-demethylase and two known cytochrome P-450 coupled enzymes, ethylmorphine N-demethylase and aniline 4-hydroxylase. For example, SKF525A, an inhibitor of various microsomal drug metabolizing enzymes, significantly inhibited ethylmorphine and aniline metabolism at 0.1 mM, but DMN N-demethylation was inhibited only at 1.0 mM. Pyrazole, a potent inhibitor of alcohol metabolism, inhibited only DMN metabolism. Similar results were obtained with KCN and NaN_3 which again inhibited DMN N-demethylase activity but were ineffective with respect to the other substrates. Lake et al. (1974a) established that DMN N-demethylase has a significantly greater storage stability than either

cytochrome P-450 or two known cytochrome P-450 dependent N-demethylases (ethylmorphine N-demethylase and 4-chloro-4-methylaniline N-demethylase). These studies suggest that the relationship between DMN N-demethylase and the usual cytochrome P-450 dependent mechanisms is atypical.

Studies with microsomal inducing agents to further define the role of cytochrome P-450 in DMN N-demethylation have not been definitive. Based on numerous conflicting reports, the possibility of multiple DMN N-demethylases was suggested. For example, classical including agents that increase microsomal cytochrome P-450 such as phenobarbital, 3-methylcholanthrene, or Aroclor 1254 (Remmer et al. 1967) apparently repress DMN N-demethylation, particularly at low substrate concentrations (< 4 mM) (Venkatesan, Argus, and Arcos 1970; Venkatesan et al. 1968; Argus et al. 1975). At higher substrate concentrations (> 25 mM) DMN N-demethylase activity was enhanced in microsomes isolated from animals pretreated with these inducing agents (Czygan et al. 1973, Lake et al. 1974b, Frantz and Malling 1975). It has been suggested by Arcos et al. (1977) that there are two enzymic forms of DMN N-demethylase which have different kinetic characteristics and respond in opposite ways to in vivo "enzyme inducer" pretreatment. DMN N-demethylase I is repressed at substrate concentrations between 0 and 4 mM and DMN N-demethylase II is induced at substrate concentrations of 50 to 200 mM following pretreatment with the same agents. Pretreatment with Aroclor 1254 resulted in a decreased V_{max} for the DMN N-demethylase reaction at 4 mM and an increased V_{max} at 200 mM with greater responses noted in rats than mice (Arcos et al. 1977). In

addition, the Km was increased in both concentration ranges indicating that the affinity of the enzyme was affected by Aroclor 1254 pretreatment. An increase in DMN concentration beyond the 300 mM level caused a decreased N-demethylation probably due to denaturation of microsomal protein (Argus et al. 1966) as demonstrated in experiments using ovalbumin, a model protein, and such high DMN concentrations.

There are two theories which attempt to explain the apparent multiplicity of DMN N-demethylase. It has been suggested that there are two pre-existent enzymic forms of DMN N-demethylase as suggested by induction and repression of enzyme activity at high and low substrate concentrations following pretreatment (Arcos et al. 1977). This theory relates directly to the apparent multiplicity of cytochrome P-450 species which have widely different substrate specificities (Coon et al. 1975) and physical properties (Welton and Aust 1974). It has been determined that species of cytochrome P-450 which differ only slightly in spectral characteristics are present in different subfractions of liver microsomal preparations indicating different parts of the endoplasmic reticulum (Mailman et al. 1975). To account for the overall increase in cytochrome P-450 seen with inducing agents such as PB, MC, or Aroclor 1254, it has been suggested that the decrease in mixed function oxidase activity in a limited regional area may be masked by the overall increase in mixed function oxidase activity induced by the same agents (Argus et al. 1976). Such a multiplicity of cytochrome P-450 species would further suggest the possibility of multiple DMN N-demethylases.

The second theory is based on the effects of the DMN substrate concentration and the resultant physio-chemical effects. It has been presented that DMN in a high concentration (> 300 mM) is capable of causing conformational changes in ovalbumin. In further studies (Bemis, Argus, and Arcos 1966) it was shown that between 200 mM and 20 mM DMN, the effects of decreasing concentration were that of a tightening of the secondary structure of ovalbumin. Therefore, the enzyme DMN N-demethylase I could be undergoing an activation due to conformational changes giving DMN N-demethylase II which is subject to greater sensitivity upon pretreatment with "inducer agents." This theory allows for one DMN N-demethylase enzyme which is subject to conformational changes resulting in what appears to be two enzyme forms with separate kinetic properties.

In this manuscript a low DMN concentration of 0.15 mM was used to eliminate the multiplicity of results obtained when a wide range of substrate concentrations are used. In addition, a low concentration is more closely related to physiological conditions where it would be highly unlikely that a cellular system would achieve greater than a 5 mM concentration of DMN (Argus and Arcos, in press). The data presented in this study have shown that DMN N-demethylase activity and covalent binding of DMN metabolites can be accurately measured using a low DMN concentration (0.15 mM) present in microsomes from control rats versus those of acetone or ethyl alcohol pretreated rats. In addition, pretreatment of rats with acetone or ethyl alcohol significantly enhances the <u>in vitro</u> DMN N-demethylation and subsequent covalent binding of the reactive intermediates.

Modification of rat liver microsomal DMN N-demethylation with oral acetone, ethyl alcohol, and isopropyl alcohol resulting in enhancement of such activity has been demonstrated by Sipes et al. (1973) and Maling et al. (1975). The enhancement was observed several hours after in vivo pretreatment. However, there was no change in the N-demethylation of ethyl morphine or in the microsomal content of protein, cytochrome P-450, or NADPH cytochrome-c-reductase. The mechanism of enhancement due to these pretreatment agents appears to differ from agents previously discussed (MC, PB, Aroclor 1254) in that the level of cytochrome P-450 was unaffected. Kinetic analysis of the acetone induction mechanism in particular showed that the induction phenomenon was also present in mouse liver microsomes isolated from in vivo pretreated animals (Sipes, Slocumb, and Holtzman 1978). Pretreatment of these animals resulted in a decreased Km with an increased V_{max} for the N-demethylase reaction as compared to control animals. A decreased Km indicates an increased affinity of the enzyme for the substrate while the increased V_{max} suggests either an increase in enzyme synthesis or a decreased enzyme degradation.

In vivo pretreatment of rats with ip acetone acts as a modifier to enhance in vitro DMN metabolism in rat liver microsomes as measured by both formaldehyde production and covalent binding of the active methylating agents to DNA, RNA, and protein. Concurrent with these studies it was established that a pretreatment-induced fast and an 18 hour fast decreased both liver microsomal protein content and liver weight as compared to controls; however, there was no significant difference between the two fasted states. With respect to DMN

N-demethylase activity, an 18 hour fast and acetone pretreatment-induced fast both showed an enhancement of activity. Acetone pretreatment, however, showed a much greater increase (65%) in activity than the 18 hour fasted controls (19%) when compared to the control-fed group. The explanation for such a short term fasting-induced enhancement of DMN N-demethylase in control animals has been proposed to be the result of either an enhanced enzyme synthesis due to increased transcription or to a greater stabilization of the messenger template (Venkatesan, Arcos, and Argus 1970) as determined using actinomycin D, a de novo protein synthesis enhibitor.

The acetone-induced enhancement of DMN N-demethylase activity was similarly reflected in the in vitro covalent binding analysis of ^{14}C from ^{14}C-DMN to calf thymus DNA and microsomal RNA and protein. Fasting resulted in an enhanced binding with acetone pretreatment producing an even greater level of covalent binding. Similar results were obtained in mouse liver microsomal preparations with respect to an acetone-induced enhancement although the effects of starvation in mice were not pursued.

In an effort to explain the mechanism of acetone enhancement of DMN N-demethylase, experiments reported here were designed to pursue the possibility of enhanced protein synthesis. In these attempts protein synthesis inhibitors failed to eludicate the mechanism of the acetone-induced enhancement. Actinomycin D did not inhibit the in vivo acetone-induced enhancement of in vitro DMN N-demethylase activity. The action of actinomycin D is to inhibit the synthesis of specific messenger RNA which essentially blocks template activity of DNA at the

nuclear level. Therefore, it appeared that the acetone-induced N-demethylase enhancement was not the result of increased enzyme synthesis following increased messenger RNA synthesis. Similar experiments were performed using in vivo cycloheximide to inhibit protein synthesis by inhibiting amino acid uptake or incorporation, thereby inhibiting chain initiation and elongation at the ribosomal level. It was determined that cycloheximide did reduce by 33% the DMN N-demethylase activity in acetone-pretreated animals as compared to acetone-controls. However, the significance of this finding is suspect because cycloheximide administered to control animals reduced DMN N-demethylase activity by 25%. Jondorf et al. (1966) showed that in vivo pretreatment of rats 24 hours before sacrifice with an equivalent amount of cycloheximide (1 mg/kg) as used in these experiments will inhibit N-demethylation of aminopyrine and mono-methyl-4-amino antipyrine by about 50%. The dose was determined to be effective for several days. It therefore seems reasonable to conclude that enhanced DMN N-demethylase activity induced by in vivo acetone pretreatment was not the result of increased protein synthesis and that the decrease observed in acetone-pretreated animals reflected the general inhibition of functional DMN N-demthylase present in the microsomal system rather than inhibiting the enhancement or increased synthesis due to acetone itself. It is possible that the enhancement due to acetone occurs via a mechanism which allows more of the substrate, DMN, to be N-demethylated without a specific increase in enzyme concentration. At present DMN N-demethylase activity is measured by the production of formaldehyde and is not a measure of the quantity of the specific enzyme.

Acetone pretreatment has been reported to increase the affinity of DMN N-demethylase for its substrate, but the mechanism of this change is not known. It is possible that acetone pretreatment could result in a membrane phenomenon altering membrane lipids to allow more DMN to enter and be metabolized by the same amount of enzyme normally present in untreated microsomes. It is also possible that acetone might release enzymes present which are not in use so as to give an effective increase in enzyme activity which would not be due to an increased enzyme synthesis. In general, it is also possible that an increased affinity between an enzyme and a substrate which is present in low cellular concentration would have a greater net effect than a change due to increased enzyme concentration (Cumps, Razzouk, and Roberfroid 1977). These theories appear plausible in that in vivo acetone pretreatment to male rats did not cause a change in the content of liver cytochrome P-450, NADPH cytochrome-c-reductase, and microsomal protein content or the N-demethylation of ethyl morphine (Sipes et al. 1973). Similarly, Clark and Powis (1974) showed no change in liver cytochrome P-450 as a result of acetone pretreatment to female rats; however, N-demethylation of aminopyrine and p-hydroxylation of aniline showed an initial biphasic reaction at 0.5 hours following pretreatment with a decrease and an increase, respectively, returning to control values within four hours. It appears that the metabolism of DMN following acetone pretreatment is atypical in that it does not follow the general trends of other mixed function oxidases. It is different in that the K_m is decreased and there is no modification in components of the microsomal electron transport system.

With respect to the significance of formaldehyde production as a measure of DMN metabolism, it appeared that pretreatment with acetone caused a greater percentage increase in covalent binding of ^{14}C to DNA, RNA, and protein *in vitro* than in formaldehyde production. Such an effect could be inherent in the analytical methodology, but it is more reasonable to assume that N-demethylation of DMN as measured by formaldehyde production may not be as sensitive an indicator of DMN metabolism as initially believed. Such a theory would suggest that there is another mechanism or pathway present in the microsomal system that would allow for the release of methylating intermediates capable of binding to cellular macromolecules. Recent reports that used piperonyl butoxide, an insecticidal synergist and inhibitor of cytochrome P-450, support such a pathway. Pretreatment of male mice with this chemical resulted in a decreased *in vitro* DMN N-demethylase and a decreased mutagenesis as compared to non-treated controls (Friedman and Sanders 1976a, 1976b). However, *in vivo* studies showed no change in the LD_{50} of DMN or in the level of alkylation of nucleic acids and protein in various organs from mice pretreated with piperonyl butoxide. It appears that piperonyl butoxide has an inhibitory effect on *in vitro* metabolism of DMN but has no effect on *in vivo* metabolism and toxicity.

Similar contradictory findings have been obtained with pregnenolone-16α-carbonitrile (PCN) pretreatment. PCN, a hormonally inactive steroid, administered orally to female rats results in decreased *in vitro* DMN N-demethylase levels (Somogyi et al. 1972, Grandjean and Somogyi 1976) with decreased *in vivo* acute toxicity from DMN (Somogyi et al. 1972, Bartsch et al. 1975, Grandjean and Somogyi

1976) and decreased carcinogenicity from DMN administration (Arcos 1978). However, no change in the *in vivo* level of alkylation of liver DNA, RNA, or protein was seen (Bartsch et al. 1975, Grandjean and Somogyi 1976). In addition, PCN pretreatment did not delay the rate of clearance of DMN from the blood or affect the concentration of DMN in the liver as compared to untreated animals (Somogyi et al. 1972). However, β-naphthoflavone enhances the carcinogenicity of DMN with a concurrent decrease in DMN N-demethylase enzyme activity (Arcos 1978). The apparent controversy suggested in these cases is that *in vivo* metabolism cannot always be predicted from *in vitro* analysis. It is possible there are unknown mechanisms of DMN metabolism which account for the decreased DMN N-demethylase activity and have no effect on the *in vivo* metabolism, clearance, or alkylation of macromolecules. Such a mechanism might include enzymes which are present *in vivo* but are not present in the isolated *in vitro* system. Another possibility is that the DMN N-demethylase analysis as measured by formaldehyde production is inadequate or inaccurate. It appears that in the cases of piperonyl butoxide and β-naphthoflavone pretreatment there exists a lack of correlation between DMN N-demethylase activity and acute toxicity or carcinogenicity.

Short-term oral pretreatment of rats with ehtyl alcohol similarly modified the *in vitro* microsomal metabolism of DMN. An enhancement in formaldehyde production and covalent binding of the active alkylating ^{14}C-labeled intermediates to DNA, RNA, and protein was observed. The mechanism of induction in this case is unknown. As discussed in Maling et al. (1975) a similar pretreatment regimen with

oral ethanol caused no changes in the content of microsomal protein and cytochrome P-450 and the activity of NADPH cytochrome-c-reductase. It is possible that the changes seen in DMN metabolism due to alcohol pretreatment might be related to a lipid phenomenon as previously discussed for acetone pretreatment. Lipids are involved in hepatic microsomal drug metabolism (Chaplin and Mannering 1970, Eling and DiAugustine 1971) and the induction by drugs of drug-metabolizing enzymes (Century 1973). Also, phospholipids are essential for in vitro drug metabolism, since phosphatidylcholine is the active component of the lipid fraction in the activation of microsomal drug metabolizing enzymes (Strobel et al. 1970). Chronic intake of ethanol modifies hepatic phospholipid metabolism (Ishii, Joly, and Lieber 1973) and alters the composition of total (Mendenhall, Bradford, and Furman 1969a, 1969b) and microsomal hepatic phospholipids (French et al. 1971). Whether the lipid alteration seen in chronic ingestion of ethanol occurs during the two day acute ethanol pretreatment regimen used in this study is unknown at this time.

CONCLUSION

The significance of the modification of DMN metabolism with agents such as acetone or ethanol should be considered with care. Acetone and similar ketones are common in industrial utilization as solvents in paint, rubber, plastic, and dyeing industries. Isopropyl alcohol is similarly used in these industrial situations and is converted *in vivo* to acetone. It has been shown that *in vivo* pretreatment of rats with isopropyl alcohol and acetone results in an enhanced *in vitro* metabolism of carbon tetrachloride (Sipes et al. 1973), a solvent recognized as an industrial hazard. Similar studies with mice have shown an increased hepatotoxic response to chloroform administration as measured by increased serum glutamic pyruvic transaminase activity (Traiger and Plaa 1974). Human exposure to carbon tetrachloride following long term atmospheric exposure to isopropyl alochol in an industrial situation resulted in severe toxicity to 14 of 43 workers within 12 to 48 hours after carbon tetrachloride exposure (Folland et al. 1976). These workers had high levels of acetone in samples of expired alveolar gas which medically predisposed them to sustain injury when accidentally exposed to carbon tetrachloride. Studies conducted to determine the effects of inhaled methyl ethyl ketone (MEK) and methyl n-butyl ketone (MBK) solvent vapors on hepatic microsomal biotransformation activities in rats showed a significant enhancement (two to three fold over control) in aniline hydroxylase, aminopyrine demethylase, Neoprontosil reductase, and p-nitrobenzoate reductase

(Couri et al. 1977). In the same sense chronic exposure to ethyl alcohol has been reported to potentiate the effects of carbon tetrachloride hepatotoxicity in rats (Maling et al. 1975) and humans (Guild, Young, and Merrill 1958; New et al. 1962). Such findings indicate that chronic exposure of humans to solvent vapors and oral ethyl alcohol could have important pharmacological and toxicological implications resulting from the stimulatory effects of these agents on the metabolism of a wide variety of foreign chemicals and drugs.

Included in the category of exogenous agents is DMN which is not only present in industrial situations but is an environmental contaminant formed via a multitude of pathways both in vivo and in vitro. By itself DMN is a well known toxin, carcinogen, and mutagen. Concomitant with this is the evidence presented in this study for enhanced in vitro liver metabolism and bioactivation of DMN following pretreatment with acetone and ethyl alcohol. Although specific pathways and mechanisms of DMN metabolism and toxicity are in debate, the evidence for enhancement of DMN metabolism due to exposure to acetone and ethyl alcohol warrants further investigation to determine not only the hazards of DMN but the increased hazards due to potentiation by such agents.

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