A Study of Denitrosation of N-Nitroso Compounds by Irradiation with Long-Wavelength UV Light

Zhenyu J. Wang
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A STUDY OF DENITROSATION OF N-NITROSO COMPOUNDS BY
IRRADIATION WITH LONG-WAVELENGTH UV LIGHT

by

Zhenyu J. Wang

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biochemistry

Approved:

UTAH STATE UNIVERSITY
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1989
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Zhenyu J. Wang
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ABSTRACT

A Study of Denitrosation of N-Nitroso Compounds by Irradiation
with Long-Wavelength UV Light
by
Zhenyu J. Wang, Master of Science
Utah State University, 1989

Major Professor: Dr. L. H. Piette
Department: Chemistry and Biochemistry

N-Nitrosamines and N-nitrosamides have been reported to be mutagenic. N-Nitrosamides are direct mutagens that need no activation to be mutagenic, whereas N-nitrosamines need to be enzymatically activated to exert their mutagenicity. Oxidative demethylation of nitrosamines is a commonly accepted activation mechanism. Another pathway of nitroso compounds, denitrosation, has recently been proposed. The mechanism of denitrosation, however, is still unknown.

The purpose of this study was to use a photo-reaction model to explore the possible denitrosation mechanism of N-nitroso compounds. An N-nitrosamine, N-nitrosomorpholine (NMOR), and an N-nitrosamide, N-methyl-N'-nitrosoguanidine (MNNG), were irradiated with long-wavelength UV light in the presence of phosphate. Denitrosation of these nitroso compounds occurred, and free radicals were generated during the photolysis of N-nitroso compounds. The free radical from NMOR is carbon centered, and the free radical from MNNG is nitrogen centered. They have different responses to phosphate. A stable mutagen is formed during the photolysis of NMOR in the presence of phosphate. The formation of this stable mutagen is through a free-
radical mechanism. MNNG appears to be inactivated in terms of mutagenicity by the photodenitrosation. Free radicals produced during the photolysis of NMOR and MNNG are mutagenic through a direct radical interaction mechanism.

The results of this study indicate that the newly found pathway of N-nitroso compounds, denitrosation, may be related to a free-radical mechanism.
INTRODUCTION

The carcinogenicity of nitrosamines and nitrosamides has been extensively investigated since Magee reported the carcinogenicity of dimethyl nitrosamine in 1956. Thousands of papers dealing with the chemistry, metabolism, mutagenicity, and carcinogenicity of nitroso compounds have been published since then. There are several reasons the study of nitroso compounds has gained so much attention from biochemists, toxicologists, and pharmacologists. First of all, most nitroso compounds are carcinogenic, which makes them a single-chemical group of carcinogens. In fact, noncarcinogens among them may provide important clues as to what makes nitroso compounds carcinogenic. Secondly, nitrosamines are remarkably site specific; essentially all organs are affected by one or another nitrosamine. This makes nitrosamines excellent tools for the study of mechanisms of chemical carcinogenesis. Thirdly, and perhaps most importantly, nitrosamines can be formed in the human environment. The two precursors of nitrosamines, nitrite and amines, are easily found in the environmental mix. Moreover, nitrosamines can be formed in the stomach by ingesting the precursor amines and nitrite.


Nitroso compounds can be readily prepared by reaction of nitrites with secondary and tertiary amines, amides, ureas, carbamates, and guanidines. The components required for this synthesis are ubiquitous in the environment. For example, this nitrosation can take place during the curing, storage, or cooking of certain foods containing nitrites. Studies also show that the nitrosation reaction could occur at a rapid rate in the mammalian stomach.
during digestion. Thus, human beings may well be exposed to nitroso compounds.

Nitroso compounds have been shown to display carcinogenic activity in animal tests. More than 300 nitroso compounds have been tested; most of these have shown carcinogenic activity to a greater or lesser extent. Although many of the experimental studies have been carried out with rats, other animals have also been tested since variations in response frequently occur from species to species. Cancer of the liver was detected in rats, mice, guinea pigs, rabbits, dogs, monkeys, grass parakeets, and pigs dosed daily with 3 mg/kg of nitrosodiethylamine in drinking water. Activities of nitroso compounds are not always restricted to the liver; other sites that have been shown to be susceptible include the kidney, esophagus, bladder, lung, and alimentary system, depending on the particular conditions of the experiment.

Nitrosamides are chemically unstable and consequently display a local irritant action as well as selected cytopathic activity. Nitrosamines, on the other hand, generally produce tumors at sites distant from the point of application. It has been suggested that nitrosamines have to be metabolized before their carcinogenic potential can be expressed. In other words, a metabolite of nitrosamines rather than the intact nitrosamines themselves is the true active species.

The most commonly accepted mechanism for the activation of nitrosamines to mutagens is the $\alpha$-hydroxylation hypothesis. Nitrosamines are enzymatically metabolized by microsomal mixed-function oxidases to alkylating species. Aldehyde and nitrogen are produced as by-products. The mechanism for the activation of nitrosamines can be shown as follows:
The RN$_2^+$ produced in the reaction is the direct active mutagen, which alkylates macro-genetic materials.

This mechanism satisfies much of the metabolic data. However, recently investigators found that the amount of N$_2$ released from the metabolized nitrosamines does not quite agree with the stoichiometry of the above reaction. It has been suspected that multiple enzyme pathways may be involved in the metabolism of nitrosamines; not all of them necessarily release N$_2$. Evidence has been obtained suggesting denitrosation of nitrosamines both in vivo and in vitro. $^{14}$C-Dimethylamine and the formation of nitrite during the microsomal metabolism of nitrosamines have also been detected. The denitrosation of nitrosamines indicates cleavage of the N-N bond in nitrosamines and possibly involves free-radical intermediates. All these studies suggest that denitrosation of nitroso compounds is a possible alternative metabolic pathway.

It is well documented that nitroso compounds undergo light-induced N-N bond cleavage. In the irradiation by UV light with wavelengths corresponding to the absorption of the N-N bond, nitroso compounds are denitrosated. Thus, photolytic denitrosation of nitroso compounds may be a good model to study...
the mechanism of enzymatic denitrosation of nitroso compounds.

Of the modern instruments, Electron Spin Resonance (ESR) spectroscopy is the most powerful tool to explore free radicals. In this study, ESR was utilized through the use of spin-trapping techniques to investigate the involvement of free radicals during the irradiation of nitroso compounds.

As a short-term assay, the Ames test has been widely used as a mutagenesis assay. In this study, the Ames test is employed to examine the mutagenicity of irradiated nitroso compounds and the direct mutagenicity of the free radical itself. The effect of several free-radical inhibitors on the inhibition of mutagen production is also examined.
LITERATURE REVIEW

It was more than 100 years ago when nitrosamines were first made by the simple reaction of secondary amines with nitrous acid. Nitrosamines were not known to have adverse biological effects until 1937, when a case of poisoning by N-nitrosodimethylamine was reported but largely ignored (Freund, 1937). A detailed investigation of the toxicity of nitrosodimethylamine began in England in the 1950s as a result of another accidental poisoning; the first results were reported by Barnes and Magee in 1954. Further tests for possible carcinogenic action showed that malignant tumors developed within less than a year in nearly all the rats fed a diet containing 50 p.p.m. of this compound (Magee & Barnes, 1956). These initial findings stimulated a new field of research. A number of papers dealing with nitroso compounds have been published since then.

It has been shown that nitroso compounds can be readily prepared by nitrosation of secondary and tertiary amines, amides, ureas, carbamates, and guanidines. The components required for this synthesis are ubiquitous in the environment. Most nitroso compounds have been demonstrated carcinogenic in different test animals.

Several possible mechanisms by which nitroso compounds are activated to carcinogens have been proposed after extensive investigations. Questions of mechanisms, however, still remain to be investigated.

Chemistry of Nitroso Compounds

All N-nitroso compounds have the -N-N=O functional group in common and can be sorted into two categories: nitrosamines and nitrosamides.
In nitroso compounds, nitrosodimethylamine is an oily liquid, miscible with water in all proportions; whereas others are liquids or solids. The compounds are partially soluble in water, the degree of solubility varying according to molecular weight, and they are readily soluble in organic solvents. The simple aliphatic nitrosamines are yellow or yellow-green nonhydroscopic liquids that boil without decomposition, the boiling points lying between 150°C (nitrosodimethylamine) and 220°C.

Nitroso compounds show great versatility in chemical reaction, owing to the n-\(\pi\) conjugation between the lone electron pair on the amino nitrogen and the nitroso group.

**Photochemistry.** Photosensitivity is a characteristic of N-nitroso compounds. The nitroso group is split by exposure to ultraviolet light. N-Alkyl-N-nitrosourethanes undergo photodecomposition in aqueous alcoholic solution to give crystalline compounds among the reaction products. N-methyl-N-nitrosourethane \(Y=\text{OC}_2\text{H}_5\) undergoes partial reduction to yield the diethyl
ester of dimethyl tetrazodicarboxylic acid, \( \text{CH}_3\text{-N(COOC}_2\text{H}_5\text{-N=NN-COOC}_2\text{H}_5\text{-CH}_3 \), and the N-ethyl derivative behaves in a similar manner. When photolytic decomposition of alkyl nitrosamines was carried out in aqueous or methanolic solutions in the presence of hydrochloric acid, the formation of amidoximes and alkylidenimines was postulated (Schoental, 1963). The formation of aldehydes and ketones was further recognized. Numerous other photoreactions involving reduction, addition, rupture, and elimination have been reported by Chow (1967). In aqueous solution in the presence of spin trap, free radicals were found to be produced in the photolysis of several N-nitrosamines and N-nitrosamides by long-wavelength UV light (Grover et al., 1987).

**Transnitrosation.** The nitroso group of a nitroso compound can be transferred to other amines in the absence of nitrous acid.

\[
R_2\text{NNO} + R'_2\text{NH} \rightarrow R_2\text{NH} + R'_2\text{NNO}
\]

The substituents present on the amino nitrogen of nitroso compounds have a strong effect on the rate of transnitrosation. Transnitrosations are usually more rapid with aryl nitrosamines than with dialkyl nitrosamines. The difference in reactivity parallels the N-N bond strength and the stability of the amino fragment (Buglass et al., 1974).

Transnitrosations are of biological importance because they may play a role in metabolic pathways in which certain N-nitroso compounds (which may themselves not be carcinogenic or weakly carcinogenic) act as proximate carcinogens by transferring their nitroso groups to other compounds to generate carcinogens.

**Reduction.** Nitroso compounds can be reduced to yield either the corresponding N,N-substituted hydrazine or the corresponding secondary
amine. They can be reduced by metal zinc dust in acetic acid (Leicester & Vogel, 1950), tin in hydrochloric acid, lithium aluminum hydride (Gleason & Paulin, 1973), and electrochemical methods (English, 1951; Heath & Jarvis, 1955).

Oxidation. Oxidation of nitroso compounds can occur to yield N-nitramines (R₂NNO₂) in the presence of a variety of oxidizing agents: hydrogen peroxide with nitric acid (Brockman et al., 1949), ammonium persulfate with nitric acid (Chute et al., 1948), and trifluoroperacetic acid (Emmons, 1954).

Hydrolysis. Nitrosodialkylamines are stable to strong alkali but are denitrosated to the corresponding secondary amines under acidic conditions. Hydrogen chloride gas (Loffler, 1910; Forlander & Wallis, 1906), bromine and sulfuric acid (Rohde, 1869), and hydrogen bromide in glacial acetic acid (Eisenbrand & Preussmann, 1970) have been used as hydrolytic denitrosating reagents.

Formation of Nitroso Compounds. Under varying environmental conditions, many types of N-containing compounds may undergo N-nitrosation to yield N-nitroso derivatives. The possibility exists for the endogenous formation of nitrosamines from normal dietary supplies of nitrite and amino compounds (Sen et al., 1969; Lijinsky & Epstein, 1970). Of extreme interest is the potential nitrosation of drugs, which are amine or amide derivatives or drug metabolic conversion products containing an amino group. A number of commonly used drugs contain amine groups, or they may be converted into alkylamino structures that may react with nitrite to form N-nitroso compounds as well as those nitroso compounds formed from nitrosatable amino substances.

Studies with nitrosatable precursors, model compounds, or drugs have shown that reaction of precursors with nitrite occurs in vitro, under conditions similar to those found in the mammalian stomach or the environment, as well
as *in vivo*, with the formation of nitroso compounds (Sander et al., 1968; Lijinsky & Greenblatt, 1972). The *in vitro* nitrosation of various drugs of widely diverse chemical structure in the presence of excess $\text{NO}_2^-$ and at physiological pH and temperature was studied by Lijinsky and Greenblatt (1972). Measurable yields of nitrosamines were detected; dimethylnitrosamine was formed from aminopyrine and oxytetracycline, diethylnitrosamine from disulfiram and nikethamide, and N-nitrosohexamethyleneimine from tolzamide. The *in vitro* nitrosation of 12 widely used drugs that are tertiary amines was investigated by Lijinsky and Singer (1974). The above nitrosations were done using somewhat high concentrations of drug and nitrite. Subsequent experimental studies have examined nitrosamine formation under conditions where the levels of precursor drug amines reflected the levels likely to be found *in situ* in the human stomach.

Scheunig and Ziebarth (1976) studied the formation of N-nitroso derivatives in human gastric juice from drugs and nitrite. The quantity of drug in the nitrosation reactions was equal to the maximally tolerated single dose and a nitrite level corresponding to the level most likely to be ingested in a single day. A definite measurable yield of nitrosamine was produced on nitrosation of aminopyrine and analgine. It was clearly demonstrated that under conditions of very low levels of amine drug, nondetectable yields of N-nitroso derivatives are possible. Thus, man may be exposed to naturally occurring N-nitroso compounds as well as those N-nitroso compounds formed from nitrosatable amino substances. For man there is a potential carcinogenic risk in view of the large number of precursor amino compounds that are available for nitrosation *in vivo*.

Biological Properties of Nitroso Compounds

If it were not for the biological activity of N-nitroso compounds, they would
be of little scientific interest. More than 90% of the 300 individual N-nitroso compounds assayed in animals have proven to be capable of producing carcinomas. Although little direct evidence exists showing that they are human carcinogens, much indirect evidence indicates that N-nitroso compounds may be involved in the etiology of certain human cancers.

**Acute Toxicity.** In 1954 Barnes and Magee reported acute poisoning by dimethylnitrosamine in a human following an industrial accident. Experimental N-nitroso compound poisoning in animals was also reported in the same paper; a single fatal dose of dimethylnitrosamine to rats, mice, rabbits, and dogs produced severe liver damage in all species, and death occurred 1 to several days after administration of the poison. The liver damage caused by dimethylnitrosamine was proven later to be typical of most dialkynitrosamines and can be explained by the dependency upon the cytochrome P-450 monooxygenase for the metabolic activation of nitrosamines to form the ultimate toxin (McLean & Verschuuren, 1969; Arcos et al., 1977).

N-nitrosamides do not require metabolic activation to an ultimately toxic form; these compounds are unstable in aqueous solution and rapidly decompose to chemically reactive alkylating agents at physiological pH and temperature and damage cells at the site of administration. The nitrosamides are also quite toxic to tissues with a high rate of cell proliferation and turnover.

Not much work has been done on structure-activity relationships for the acute toxicity of N-nitroso compounds. For the limited data available, however, it appears that acute toxicity decreases with chain length of dialkynitrosamines. Methylbenzynitrosamine and dimethylnitrosamine appear to be among the most acutely toxic nitroso compounds.

**Carcinogenicity.** Hundreds of N-nitroso compounds have been tested for carcinogenicity since Magee and Barnes (1956) first demonstrated the production of hepatocellular carcinomas in rats fed dimethylnitrosamine; 90%
of these have been shown to be strong carcinogens. These agents have some remarkable properties for tumor production. As a chemical class, they are capable of producing tumors in virtually every vital tissue, although no one agent can induce cancer in all tissue targets. No other chemical class has so broad a scope of tissues for carcinogenic attack. Descriptions of the tumor types are available in the reviews of Magee and Barnes (1967) and Preussmann and Stewart (1984); the carcinogenicity of these compounds is based not on shortening the induction time of spontaneous benign tumors but on the production of malignant and metastatic tumor in animals for which the spontaneous occurrence of such tumors is rare or completely absent.

**Mutagenicity.** Chemically-induced mutagenesis was first reported by Auerbach and Robson (1946). A variety of mutagenic agents are now known and have been classified into different groups according to mode of action as alkylators, intercalators, etc. As alkylating agents, N-nitroso compounds are extremely potent point mutagens in addition to producing chromosome breaks and aberrations. Both N-nitrosamines and nitrosamides have been widely used as model compounds in mutagenesis.

One of the earliest observations of the mutagenic action of N-nitroso compounds reported by Mandell and Greenberg (1960) was that nitrosoguanidine induces mutations in bacteria. Since then, the mutagenicity of nitrosamides in various indicator organisms (bacteria, fungi, and fruit flies) has been demonstrated by many investigators. Marquardt et al. (1964), using various nitrosamides, demonstrated the induction of mutations in Saccharomyces cerevisiae. Adelberg et al. (1965) have claimed that N-methyl-N'-nitro-N-nitrosoguanidine is the most potent chemical mutagen for bacteria yet discovered. The nitroso derivatives Carbaryl (1-naphthyl-N-methylcarbamate), Propoxur (2-isopropoxyphenyl-N-methyl carbamate), and Benzthiozuron (N-(2-benzothiazolyl)-N-methylurea) are mutagenic for Saccharomyces cerevisiae (Zimmerman & Schwaier, 1967; Siebert &
Eisenbrand, 1974). It is generally accepted that nitrosamides require no metabolic activation to be mutagenic in microbial systems (Mandell & Greenberg, 1960; Pasternak, 1964).

The absence of a mutagenic effect by the nitrosamines in microorganisms is attributed to the inability of microbes to transform these compounds to active mutagenic forms due to the lack of an activation system. However, induction of mutagenesis by nitrosamines in the presence of an activation system has been reported for various dialkylnitrosamines, substituted dialkylnitrosamines, and various cyclic nitrosamines.

The most extensively studied N-nitroso compound in the area of chemically induced mutagenesis is dimethylnitrosamine (DMNA). In the absence of metabolic conversion, DMNA has been shown to be nonmutagenic in bacteria, yeasts, and molds (Geissler, 1962; Marquardt et al., 1963, 1964).

Dialkylnitrosamines require enzymatic activation to induce mutagenicity. Combined with a mammalian activation system, dialkylnitrosamines can be shown to induce mutagenesis in microorganisms. The ability of NADPH-dependent microsomal enzymes to form mutagenic compounds from dialkylnitrosamines has been demonstrated by numerous investigators. Gabridge and Legator (1969) have shown that DMNA induces mutation in (his) G46 strain of *S. typhimurium* after activation in the host-mediated assay. DMNA was also shown to be mutagenic to *S. typhimurium* following *in vitro* activation in the liquid culture assay (Malling, 1971). The successful activation of polycyclic hydrocarbons in the presence of liver fraction and co-factor system after incubation on a petri plate prompted Ames and co-workers to study the mutagenic action of DMNA on *S. typhimurium* (Ames et al., 1973). They were unable to demonstrate the mutagenicity of dimethylnitrosamine using their standard plate assay. These findings can be attributed to the absence of oxygen, which is necessary for the activation of dialkylnitrosamines (Malling, 1971), in the incubation system. Mutagenicity of DMNA was also reported by Nakajima and Iwahara (1973) in a streptomycin-dependent strain of *E. coli*.
following in vitro conversion by rat liver microsomes.

The mutagenicity of diethyl, dipropyl, and dibutyl nitrosamines, along with various cyclic nitrosamines in E. coli, following microsomal activation was reported by Nakajima et al. (1974). Reverse mutation was not induced by methyl-phenyl nitrosamine, and diphenyl nitrosamine showed very little mutagenic activity. Hsieh et al. (1976) tested N-nitroso-2-(ethylamino-ethanol) and N-nitroso-4-piperidone, oxidation products of diethylnitrosamine (DENA) and nitrosopiperidine, respectively. These compounds were nonmutagenic in the absence of liver microsomes for S. typhimurium (TA 1535). On the other hand, studies (Olajos & Cornish, 1976; Nagao et al., 1977; Olajos et al., 1978) have shown that selected hydroxy derivatives of dibutyl nitrosamine are mutagenic for S. typhimurium in the absence of a liver activation system.

Activation of Nitroso Compounds

α-Hydroxylated. All available data indicate that N-nitrosamides are activated to alkylating agents by direct chemical hydrolysis of the labile nitrosamide bond at physiological pH. On the other hand, N-nitrosamines require enzymatic activation before they can exert their carcinogenicity or mutagenicity.

The metabolism of nitrosamines was first reported in 1956 by Dutton and Heath, who showed that 14C-labeled DMNA was rapidly metabolized in rats and mice to expired 14CO2, presumably via a formaldehyde intermediate. This finding suggests that, like the carcinogenic N-methyl-substituted aminoazo dyes, DMNA and other alkyl nitrosamines might undergo oxidative dealkylation in vivo to their corresponding aldehydes. By the early 1960s, this hypothesis was confirmed in vitro and in vivo with several carcinogenic nitrosamines; and the involvement of an oxygen-requiring, reduced pyridine nucleotide-dependent, microsomal monooxygenase was indicated (Magee & Barnes, 1967). Furthermore, DMNA was found to bind covalently to cellular
protein upon incubation with rat liver slices, (Magee & Hultin, 1962) and perhaps more importantly, DMNA became the first chemical carcinogen to be shown to bind covalently to DNA (Magee & Farber, 1962; Druckrey et al., 1961). With the identification of 7-methylguanine in the nucleic acids of rats injected with DMNA it was evident that the nature of the reactive species was an alkylation agent that is believed to arise by spontaneous decomposition of the demethylated metabolite, an unstable monoalkyl nitrosamine. Both methylidiazonium hydroxide and diazomethane were proposed as the actual electrophilic intermediate (Lijinsky et al., 1968), and more recent studies have provided direct evidence for their existence (Smith et al., 1985).

In addition, several experimental approaches have strongly correlated oxidative demethylation of DMNA, methylation of DNA, and DMNA mutagenesis as a single metabolic process (Preussmann & Stewart, 1984; Chin & Bosmann, 1980; Jensen et al., 1981; Yoo & Yang, 1985). Thus, over the last two decades, the metabolic activation of a large number of carcinogenic nitrosamines to electrophilic alkylating agents has been extensively investigated in carcinogen-target tissues of several species, and the formation of some 15 different types of DNA alkylation products are now known. Of these, the 7-alkylguanines are the major DNA adducts, but it is the O6-substituted guanine and O4-substituted thymine adducts that have been strongly implicated as critical lesions responsible for both mutations and tumor induction (Singer & Grunberger, 1983).

In vivo and in vitro studies with inducers and inhibitors of cytochrome P-450 provided strong evidence that the activation of several nitrosamines via demethylation is cytochrome P-450 dependent.

The nature of the activated metabolite leading to nitrosamine dealkylation and DNA binding has long been considered to be an hydroxylated intermediate as discussed above. This is strongly supported by the synthesis of several α-hydroxynitrosamines and their acetoxy esters and by recent studies of their chemical properties and potent biological activities
The mechanism of \(\alpha\)-hydroxylation has generally been presumed to be similar to that for oxidative dealkylation of N-methyl-substituted drugs and carcinogenic aminoazo dyes (Miwa et al., 1983; Ketterer et al., 1982). In this regard, the measurement of intramolecular deuterium isotope effects suggests that demethylation involves an initial electron transfer in the oxygenated P-450-substrate complex to yield a radical cation that subsequently undergoes \(\alpha\)-deprotonation to a neutral \(\alpha\)-carbon radical (Miwa et al., 1983). The latter then collapses by radical recombination with Fe-OH to the \(\alpha\)-hydroxy metabolite. However, recent studies with dialkylnitrosamines containing fluorine at several positions except the \(\alpha\)-carbons indicate that the negative inductive effect decreases oxidative dealkylation, which is interpreted to be more consistent with an initial step involving a direct H- abstraction by homolytic cleavage of the \(\alpha\)-(C-H) by the perferryl oxygen (Janzowski et al., 1985), as proposed by Loew et al. (1983). However, both the low deuterium isotope effects and the negative inductive effect of fluorine substitution are consistent with an overall two-electron oxidation mechanism as shown in Scheme I. (a). The process, which would be analogous to that proposed for aromatic amine N- and ring-oxidation, would involve an initial one-electron abstraction and \(\alpha\)-carbon deprotonation to form neutral radical. A second electron transfer, which would be inhibited by neighboring fluorine due to increased electronegativity of the \(\alpha\)-carbon radical, would result in formation of cationic intermediate, shown as carbenium and iminium ion resonance forms. This caged complex would then decompose to the \(\alpha\)-hydroxylated product.

**Denitrosation.** Total metabolism of nitrosamines also appears to involve reductive pathways Scheme I. (b) and (c). Limited evidence has been provided for the formation of 1,2-dimethylhydrazine from DMNA and of an intermediate diazene from N,N-dibenzylnitrosamine upon incubation with liver cytosolic enzymes or homogenates, which suggests the existence of enzymatic
Scheme I. Proposed mechanisms for activation of carcinogenic nitrosamines:
(a) oxidative demethylation mechanism, (b) and (c) denitrosation mechanisms.
four- and two-electron reductions, respectively (Grilli & Prodi, 1975; Gal et al., 1978). By comparison, the apparent one-electron reduction of nitrosamines to nitric oxide (measured as nitrite) and their corresponding secondary amines has been investigated by several laboratories, and cytochrome P-450s have been strongly implicated in catalysis (Lorr et al., 1982; Appel et al., 1984, 1985). This denitrosation of nitrosamines by cytochrome P-450 was first indicated by Appel and co-workers (1979, 1982) and subsequently by Lorr et al. (1982), who showed inhibition of the reaction by carbon monoxide, alcohols, and other P-450 inhibitors, a requirement for NADPH or O2 that could be replaced by cumene hydroperoxide and the induction of this activity by ethanol and pyrazole. Some of the best quantitative evidence for denitrosation is the work of Appel et al. (1987a). Kinetic analyses suggest that denitrosation and oxidative demethylation are separate metabolic pathways, while the Lorr's data (Lorr et al., 1982) indicate that reductive and oxidative pathways must be closely linked. That denitrosation of N-nitrosamines is an important pathway of detoxification or another pathway of activation is of interest.

**Photoactivation.** As mentioned previously, N-nitrosamines can be photolitically decomposed. N-Nitrosamines may undergo denitrosation in the photolysis. Hayatsu et al. (1984) and Shimada and Hayatsu (1985) reported N-nitrosamines, such as N-nitrosomorpholine and N-nitrosopyrrolidine, can be converted *in vitro*, in the absence of S-9, into unknown mutagenic compounds by irradiation with near-ultraviolet light. Stable mutagens were formed by irradiation of N-nitrosamines in the presence of phosphate at neutral pH. The mutagenic activity was detected by Ames in *Salmonella Typhimurium* Strain TA 100. Webman et al. (1986) further discussed the possibility of free-radical involvement in the photoactivation of N-nitrosamines to mutagens. They were able to trap free radicals by a spin-trap technique during the photolysis of N-nitrosamines. It was shown that the formation of mutagenic compounds from
the photolysis of N-nitrosamines is inhibited in the presence of spin trap.
MATERIALS AND METHODS

Materials

Nitrosomorpholine (NMOR) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were obtained from Sigma Chemical Co. (St. Louis, MO). N-tert-Butyl-α-phenylnitron (PBN) and α-(4-pyridyl 1-oxide)-N-tert-butylnitron (POBN) were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade and are commercially available. Salmonella typhimurium strain TA 100 was kindly provided by Dr. B.N. Ames, (U.C. Berkeley, CA, USA).

Methods

UV Irradiation. Photolysis of reaction mixtures was affected by means of a black light bulb (20 W) with an emission range of 300 nm to 400 nm. Unless otherwise indicated, reaction mixtures (1 ml) containing 25 mM NMOR in 12.5 mM phosphate buffer (PH 7.4) were irradiated in 12-well plates (Corning Glass Works) with well diameters of 22 mm using long-wavelength UV light (300 ~ 400 nm) at room temperature (22~25°C). The plates were covered with a piece of 3-mm thick glass at a distance of 5 cm from the lamp center. The intensity of irradiation at the sample was 1.5 mW/cm². The irradiated samples then served as the input to the mutagenesis assay or ESR studies.

Denitrosation. The wavelength for maximal absorption of the N-N bond of N-nitroso compounds in the far UV was determined by scanning solutions of N-nitroso compounds on a Beckman DU-7 spectrophotometer (Fullerton, CA) from 250 nm to 450 nm. Denitrosation of N-nitroso compounds was followed in two ways: (a) by monitoring the decrease in maximal absorbance at a corresponding wavelength and (b) by monitoring the formation of nitrite. Nitrite
was determined by a colorimetric method in which an azo dye is formed by reaction of nitrite with sulfanilic acid and N-1-naphthylethyleneamine (Oda et al., 1981). In this determination 0.5 ml of 1:10 diluted reaction mixture of irradiated N-nitroso compound was added to 1 ml of azo-dye reagent. The azo-dye reagent was prepared by mixing equal volumes of 0.5% sulfanilic acid in 30% acetic acid and 0.1% N-1-naphthylethyleneamine in 30% acetic acid immediately before use. The amount of azo dye developed was photometrically determined at 542 nm on a Beckman DU-7 spectrophotometer (Fullerton, CA) after standing for 10 min at room temperature. Standard solutions were prepared by using known amounts of sodium nitrite. The calibration curve was linear in the measured concentration range of 2.5 µM to 100 µM sodium nitrite.

**Mutagenesis Assay.** Reaction mixtures were assayed for mutagenic activity by the plate incorporation assay as described by Ames (Ames et al., 1973). Overnight Salmonella Typhimurium TA 100 cultures were grown in Oxoid Nutrient Broth No. 2 for 10 hrs to a density of approximately 2~4 X 10^9 cell/ml. The overnight Salmonella Typhimurium TA 100 cultures were then centrifuged and washed with 0.9% NaCl to remove the nutrient broth. The bacteria were resuspended in 0.9% NaCl and 100 µl mixed with 100 µl of the irradiated N-nitroso compounds sample to be assayed, and the mixture was added to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. The spontaneous revertants in plates without sample served as negative controls, and a 5 µg sodium azide/plate was routinely used as a positive control in the assays. Revertant colonies were counted manually after incubation at 37°C in the dark for 48 hrs. The background lawn was present in every experimental plate, and there was no evidence of toxicity to the bacteria in any of the photoreaction mixtures.

**Direct Mutagen Assay.** The overnight *Salmonella typhimurium* TA 100
cultures were centrifuged and washed with 0.9% NaCl to remove the nutrient broth. The bacteria were then resuspended in 0.9% NaCl. 1 ml of the bacteria suspension and 1 ml of N-nitroso compound (specific reaction mixtures are described in the results section) were mixed and irradiated with long-wavelength UV light to examine the possible mutagenic effect of free radicals produced in the irradiation directly on the bacteria. After the irradiation, 200 µl of the mixture was added to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. Controls for the mutagenicity of long-wavelength UV light alone to the bacteria were routinely made.

**Electron Spin Resonance (ESR) Studies.** NMOR or MNNG reaction mixtures were irradiated in the presence of 50-mM spin-trap PBN, unless otherwise indicated. The spectra were obtained at room temperature with a Varian E-line Century series spectrometer (Varian Associates, Palo Alto, CA) using a quartz flat cell. Specific conditions are given in the Results section.

**Attempt to Identify the Chemical Structure of PBN-NMOR Spin Adduct.** PBN-NMOR spin adduct was generated by the method described above. Phosphate and unreacted NMOR were removed by loading the reaction mixture on a C_{18} SEP- PAC column and washing with water. Spin adduct and unreacted PBN were eluted out with MeOH and further separated on a Si 60 preparative plate developed with chloroform : MeOH = 95 : 5. Two bands were found on the TLC plate under UV light. A broad band with heavy UV absorption was identified as unreacted PBN. The other, thinner band with R_f value of 0.25 was extracted with MeOH. The extract had a strong ESR response, which characterizes the spin adduct. This pure spin-adduct extract was analyzed in a mass spectrometry. However, the spectrum was not interpretable. The failure to get an interpretable MS spectrum was probably due to the instability of the nitroxide structure. It was reported that nitroxide spin adduct can be decomposed if it is warmed to above room temperature,
e.g., during heating in GC equipment or while recrystallizing or distilling (Janzen et al., 1985).
RESULTS

Denitrosation

Fig. 1 shows the absorbance spectrum of NMOR over the wavelength range of 250 nm to 450 nm. A maximum absorbance in the far UV occurred at 343 nm. This absorbance is due to the n → π* transition of the N-nitroso bond of NMOR. The same transition for MNNG occurs at 332 nm (Fig. 2).

Irradiation of the N-nitrosamine NMOR and the N-nitrosamidine MNNG results in a time-dependent decrease in the absorbance of NMOR at 343 nm and MNNG at 332 nm, as illustrated in Fig. 3. This decrease in N-nitroso bond absorbance indicates breakage of N-N bonds in these compounds.

Cleavage of the N-nitroso bond produces nitric oxide, which reacts with oxygen to form nitrite. Therefore, measurement of nitrite formation is another indicator of N-N bond cleavage. A colorimetric method, in which an azo dye is formed, was used to determine if nitrite is produced in the irradiation of NMOR. The amounts of nitrite formed during the irradiation are illustrated in Fig. 4. The results from Fig. 3 and Fig. 4 indicate that N-nitroso compounds are denitrosated during irradiation with long-wavelength UV light to form nitrite.

ESR Spectra

Photolysis of NMOR or MNNG in the presence of the spin trap PBN produces spin adducts with very different ESR spectra (Fig. 5). The spin-trapped adduct formed during NMOR irradiated with PBN shows a 6-line pattern, which indicates a carbon-centered radical addition to the spin trap. In contrast, MNNG irradiated with PBN shows a nitrogen-centered radical addition to the spin trap with a spectrum consisting of 18 lines. Similar results have been obtained previously by Grover et al. (1987). Hyperfine splitting constants for the spin adducts are listed in Table I. Computer-simulated ESR
Fig. 1. The absorption spectrum of NMOR at pH 7.4, 10 mM of NMOR, and 5 mM of PO$_4^{3-}$. 
Fig. 2. The absorption spectrum of MNNG at pH 7.4, 5 mM of MNNG, and 2.5 mM of PO$_4^{3-}$. 
Fig. 3. Breakage of N-N bonds of NMOR and MNNG. 10 mM of NMOR and 5 mM of \( \text{PO}_4^{3-} \) was irradiated with long wavelength UV. Absorbance of N-N at 343 nm was measured. 5 mM of MNNG and 2.5 mM of \( \text{PO}_4^{3-} \) was irradiated with long wavelength UV. Absorbance of N-N at 332 nm was measured.
Fig. 4. Formation of nitrite from NMOR. 20 mM of NMOR and 10 mM PO₄³⁻ was irradiated with long wavelength UV at 22°C. Nitrite was determined by a Griess reaction in which an azo-dye is formed with maxima absorbance at 542 nm.
Fig. 5. ESR spectra of the spin-trapped adducts formed during nitrosamine and nitrosamide irradiation with PBN. (A) MNNG-PBN spin-trapped adduct. MNNG was 0.05 mM; PBN was 20 mM, irradiated with long wavelength UV light for 10 min. (B) NMOR-PBN spin-trapped adduct. NMOR was 25 mM; PO$_4$ 12.5 mM; PBN 50 mM, irradiated with long wavelength UV light for 20 min. ESR condition: Microwave power was 10 mW; modulation amplitude, 1.0 G; gain, (A) $1.25 \times 10^4$, (B) $1.25 \times 10^3$; time constant, 0.25 s; scan time, 2 min; and scan range, 200 G.
Table I. Hyperfine-Splitting Constants of PBN Spin Adducts Formed During the Photolysis of NMOR and MNNG.

<table>
<thead>
<tr>
<th>Nitroso compound</th>
<th>PBN Adduct</th>
<th>$a_{\text{NO}}$</th>
<th>$a_{\text{N}}$</th>
<th>$a_{\text{b}}^H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMOR</td>
<td>16.2</td>
<td>-</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>15.2</td>
<td>3.7</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

All splitting constants are reported in Gauss.
spectra generated from the hyperfine splitting constants and a line width of 1.0 Gauss are shown in Fig. 6.

In the photolysis mixture, the presence of \( \text{PO}_4^{3-} \) had different effects on the stability of the different spin adducts. Table II shows that phosphate has little effect on the ESR signal intensity of MNNG. ESR signal intensity remains relatively unchanged at phosphate concentrations from zero to 1 mM, which is 20 times higher than the concentration of MNNG. The effect of phosphate on the ESR signal intensity of the spin adduct from NMOR, however, is quite pronounce, as shown in Table III. The signal intensity increases as the phosphate concentration increases from zero to 100 mM, which is four times higher than the concentration of NMOR. Furthermore, the ESR-spin adduct spectrum from irradiated NMOR with PBN differs when in the presence versus the absence of phosphate (Fig. 7).

Mutagenesis Assay

Irradiation of NMOR in the presence of phosphate produces a stable mutagen that is active in a dose-dependent manner in the *Salmonella typhimurium* TA 100 reversion assay (Fig. 8). MNNG, as expected, demonstrated mutagenic activity in a dose-dependent manner without UV irradiation, as shown in Fig. 9.

The effect of irradiation time on NMOR mutagen formation is shown in Fig. 10. NMOR in the absence of UV irradiation did not induce mutations, but NMOR plus UV produced a stable mutagen in a UV dose-dependent manner. The mutagenic activity of irradiated NMOR in the presence of phosphate increased steadily with irradiation time. Samples irradiated for 2 hrs still showed an increase in mutagenic potential. In contrast, photolysis of MNNG decreased its mutagenicity as shown in Fig. 11.

The effect of phosphate on the formation of mutagen during the irradiation of NMOR can be seen in Table IV. In the absence of phosphate, no mutagen
Fig. 6. Computer simulated ESR spectra. (A) MNNG-PBN spin adduct; (B) NMOR-PBN spin adduct. The hyperfine splitting constants are listed in Table 1. Line width of (A) is 0.9 gauss; line width of (B) is 1.0 gauss.
Table II. Effect of Phosphate Concentration on the ESR Signal Intensity of MNNG\textsuperscript{a}

<table>
<thead>
<tr>
<th>PO\textsubscript{4} Conc. (mM)</th>
<th>0</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Height\textsuperscript{b}</td>
<td>4.6</td>
<td>4.92</td>
<td>4.44</td>
<td>4.76</td>
<td>3.49</td>
<td>3.81</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Result of a representative experiment in which MNNG was 0.05 mM, PBN was 20 mM, irradiated with long wavelength UV light for 20 min at room temperature.

\textsuperscript{b}Arbitrary unit.
Table 3. Effect of phosphate concentration on the ESR signal intensity of NMOR\textsuperscript{a}

<table>
<thead>
<tr>
<th>PO\textsubscript{4} Conc. (mM)</th>
<th>0</th>
<th>6.25</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Height\textsuperscript{b}</td>
<td>-</td>
<td>0.55</td>
<td>1.06</td>
<td>2.50</td>
<td>6.00</td>
<td>12.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Result of a representative experiment in which NMOR was 25 mM, PBN was 50 mM, irradiated with long wavelength UV light for 30 min at room temperature.

\textsuperscript{b}Arbitrary unit.
Fig. 7. ESR spectra of the spin-trapped adducts formed during NMOR irradiation with PBN. (A) in the presence of PO$_4$ of 12.5 mM. (B) in the absence of PO$_4$. NMOR was 25 mM, irradiated with long wavelength UV light for 20 min. ESR condition: microwave power was 10 mW; modulation amplitude, 1.0 G; gain, (A) 1.25 X 10$^4$, (B) 1.25 X 10$^3$; time constant, 0.25 s; scan time, 2 min; and scan range, 200 G.
Fig. 8. NMOR dose response in mutagenesis assay. NMOR was irradiated with long wavelength UV for 2 hrs in the presence of PO₄³⁻ (10 mM). 100 µl of the irradiated reaction was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. Revertant numbers are the average of duplicated determinations.
Fig. 9. MNNG dose response in mutagenesis assay. MNNG was directly applied to mutagenesis assay without irradiation. 100 µl of MNNG solution at different concentrations was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. The number of revertants at o time (~180) represent spontaneous mutations. Revertant numbers are the average of duplicated determinations.
Fig. 10. NMOR irradiation time course. In the experiment, NMOR (20 mM) and PO₄³⁻ (10 mM) were irradiated with long wavelength UV. 100 µl of the irradiated reaction was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. Revertant numbers are the average of duplicated determinations.
Fig. 11. MNNG irradiation time course. In the experiment, MNNG (0.10 mM) and PO₄ (0.05 mM) were irradiated with long wavelength UV. 100 µl of the irradiated reaction was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. Revertant numbers are the average of duplicated determinations.
Table IV. Effect of Phosphate on the Formation of Mutagen in the Irradiation of NMOR\textsuperscript{a}

<table>
<thead>
<tr>
<th>PO\textsubscript{4} Conc. (mM)</th>
<th>Revertant Number\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>124</td>
</tr>
<tr>
<td>0.1</td>
<td>161</td>
</tr>
<tr>
<td>0.25</td>
<td>164</td>
</tr>
<tr>
<td>0.5</td>
<td>244</td>
</tr>
<tr>
<td>1</td>
<td>358</td>
</tr>
<tr>
<td>5</td>
<td>1148</td>
</tr>
<tr>
<td>10</td>
<td>1558</td>
</tr>
<tr>
<td>25</td>
<td>1324</td>
</tr>
<tr>
<td>50</td>
<td>922</td>
</tr>
<tr>
<td>100</td>
<td>637</td>
</tr>
<tr>
<td>250</td>
<td>499</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Results of a representative experiment in which 25 mM NMOR was irradiated with long wavelength UV for 2 hrs. 100 µl of the irradiated reaction mixture was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9. Spontaneous mutations were 124.

\textsuperscript{b} Revertant numbers are the average of duplicated determinations.
was produced. The number of revertants peaked at 10 mM PO₄ and declined at high concentrations of phosphate. However, toxicity tests showed that irradiated NMOR plus added phosphate is not toxic to the bacteria (Table V). Phosphate has no effect on the mutagenicity of MNNG, as shown in Table VI.

Since the photolysis of NMOR produces a stable mutagen, and a free radical can be trapped in the presence of a spin trap, it was suspected that the formation of the stable mutagen may be through a free-radical intermediate. If this is the case, spin traps or free-radical scavengers should be able to inhibit the formation of the stable mutagen. The data in Table VII indicate that irradiation of NMOR in the presence of spin-trap PBN or POBN or free-radical scavengers, such as ascorbic acid or thiourea, greatly reduces the amount of mutagen formed during photolysis. The reduction in mutagenesis is dose dependent with inhibitor concentration.

Control experiments were carried out to investigate the possibility that the reduction in mutagen formation in the presence of inhibitors is due to the absorption of light by the inhibitors. Such an effect might screen the amount of light absorbed by NMOR and decrease its transformation. In these experiments, one of the reaction plates was placed on top of another. The upper one contained water or a solution of PBN or POBN. The result is shown in Fig. 12. When water was on top and PBN or POBN was in the reaction mixture, the formation of mutagen was blocked. With a solution of PBN or POBN on top, however, the formation of mutagen was not reduced. Since ascorbic acid and thiourea do not significantly absorb light at 340 nm, they were not tested in this control.

It has been shown that the formation of a stable mutagen during the irradiation of NMOR is blocked in the presence of spin traps or free-radical scavengers. However, the addition of these inhibitors to the pre-irradiated NMOR did not reduce its mutagenicity (Fig. 13). The mutagenicity of the stable mutagen formed during the irradiation of NMOR is not inhibitable by radical inhibitors. Since the stable mutagen has been formed through a free-radical
Table V. Toxicity Test of Irradiated NMOR with Different Phosphate Concentrations.

<table>
<thead>
<tr>
<th>NMOR Concentration (mM)</th>
<th>0</th>
<th>25</th>
<th>25</th>
<th>25</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Concentration (mM)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Number of Colonies</td>
<td>384</td>
<td>359</td>
<td>362</td>
<td>390</td>
<td>362</td>
</tr>
</tbody>
</table>

Overnight TA 100 culture with a density of $2 \sim 4 \times 10^9$ cells/ml was washed with 0.9% NaCl and diluted 1:10^6. 100 μl diluted TA 100 were plated with irradiated NMOR and different concentrations of phosphate on nutrient agar plates. Number of colonies were counted after incubation at 37°C for 48 hrs.
Table VI. Effect of Phosphate Concentration on the Mutagenicity of MNNG$^a$

<table>
<thead>
<tr>
<th>PO$_4$ Conc. (mM)</th>
<th>0</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revertant No$^b$</td>
<td>311</td>
<td>328</td>
<td>325</td>
<td>323</td>
<td>330</td>
<td>338</td>
<td>353</td>
<td>319</td>
<td>316</td>
</tr>
</tbody>
</table>

$^a$Results of a representative experiment in which 0.05 mM MNNG was irradiated with long wavelength UV for 10 min. 100 µl of the irradiated reaction mixture was added with 100 µl of TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9.

$^b$Revertant numbers are the average of duplicated determinations.
Table VII. Inhibition of Mutagen Formation\textsuperscript{a}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% of Rev. No.</th>
<th>Inhibitor</th>
<th>% of Rev. No.</th>
<th>Inhibitor</th>
<th>% of Rev. No.</th>
<th>Inhibitor</th>
<th>% of Rev. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBN (mM)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>74</td>
<td>5</td>
<td>85</td>
<td>0.5</td>
<td>78</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>48</td>
<td>12.5</td>
<td>43</td>
<td>1.25</td>
<td>57</td>
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<td></td>
<td>25</td>
<td>32</td>
<td>25</td>
<td>27</td>
<td>2.5</td>
<td>16</td>
<td>2.5</td>
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<tr>
<td></td>
<td>37.5</td>
<td>21</td>
<td>37.5</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1</td>
<td>50</td>
<td>16</td>
<td>10</td>
<td>4</td>
<td>10</td>
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\textsuperscript{a}Result of representative experiments in which 25 mM NMOR and 12.5 mM PO\textsubscript{4} were irradiated with long wavelength UV for 2 hrs. 100 µl of the irradiated reaction mixture was added with 100 µl of the TA 100 overnight culture to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9.

\textsuperscript{b}Revertant numbers are the average of duplicated determinations. The absence of any inhibitor was taken as a control of 100% revertant number.
Fig. 12. The effect of absorption of PBN or POBN on the inhibition of mutagenicity. NMOR (25 mM) and PO₄ (12.5 mM) were irradiated in the presence of PBN (50 mM) or POBN (50 mM) with H₂O on the top, or were irradiated in the absence of PBN or POBN with PBN (50 mM) or POBN (50 mM) on the top.

- ☒ POBN or PBN on top of the sample (except control).
- ☐ POBN or PBN in the sample.
Fig. 13  Toxicity control of inhibitors. 25 mM NMOR and 12.5 mM PO₄ were irradiated for 2 hrs in the presence of inhibitors or with addition of inhibitors after irradiation. 5 mM thiourea, 5 mM ascorbic acid, 50 mM POBN, and 50 mM PBN were used in the experiment.
intermediate during the irradiation, addition of free-radical inhibitors to the pre-irradiated NMOR will not block the production of the mutagen. Fig. 13 also shows that these inhibitors are not toxic to the bacteria. The addition of inhibitors to the pre-irradiated reaction mixture did not decrease the revertant number.

Another possibility to be considered is the mutagenic potential of the free-radical intermediates produced during irradiation. To examine this possibility some modifications of the experimental techniques were made; N-nitroso compounds and TA 100 bacteria were irradiated together to see if the free radicals produced during photolysis were mutagenic. The results of this experiment are shown in Table VIII. Column 1 is the spontaneous revertant number. As shown in columns 2 and 3, TA 100 bacteria were mixed with NMOR with and without phosphate, no irradiation. Both had revertant numbers about the same as the spontaneous revertant number. As shown in columns 4 and 5, TA 100 bacteria were irradiated alone or with added phosphate. This result demonstrates that UV light at this wavelength and intensity is not mutagenic. As shown in columns 6 and 7, TA 100 bacteria with NMOR were irradiated in the presence and absence of phosphate. Both gave high revertant numbers. The revertant numbers were dramatically reduced when the spin trap PBN was present in the reaction mixtures 6 and 7.

Table IX lists results for control experiments. Some comparisons between the direct mutagen and the stable mutagen formed in the irradiation of NMOR were done. As shown in columns 2 and 3, NMOR alone without irradiation or even with irradiation was not mutagenic. Pre-irradiation of NMOR in the presence of phosphate, however, was mutagenic (column 4). It was, however, less mutagenic than the direct mutagen (Table VIII, column 6 or 7). The formation of this stable mutagen was also inhibitable by the spin trap PBN (column 5).

As shown before, MNNG lost its mutagenic ity in the irradiation to form some compound that is not mutagenic. However, the free radical produced during
Results of experiments in which NMOR was 25 mM, PO$_4$ was 12.5 mM, PBN was 25 mM, and TA 100 was 2~4 $10^9$ cells/ml. Samples were irradiated with long-wavelength UV light for 30 min. After irradiation 200 µl of the reaction mixture was added to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9.

Revertant numbers are the average of duplicated determinations.
Table IX. Direct-Acting Mutagen Control

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Result of a representative experiment in which NMOR was 25 mM, PO₄ was 12.5 mM, PBN was 25 mM. Samples were irradiated with long-wavelength UV light for 30 min. After irradiation 100 µl of the reaction mixture was plated with 100 µl of TA 100 overnight culture with a density of 2~4 \(10^9\) cells/ml for mutagenicity assay.

Revertant numbers are the average of duplicated determinations.
Irradiation of MNNG was mutagenic towards TA 100 (Table X). Column 1 was spontaneous revertant number. In column 2, MNNG without irradiation gave the revertant number of 346. Pre-irradiation of MNNG for 10 min decreased its mutagenicity (column 3). Irradiation of MNNG together with TA 100 increased the revertant number to 809 (column 4). Addition of spin traps POBN or DMPO or the free-radical scavenger thiourea to reaction mixture 4 somewhat reduced the mutagenicity.
Table X  Mutagenicity of Intermediate Formed in the Irradiation of MNNG.

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Revertant No. 124 346 193 809 577 520 562

Result of a representative experiment in which MNNG 50 μM, POBN was 5 mM, DMPO was 5 mM, thiourea was 5 mM, TA 100 2~4 x 10^9 cells/ml. For column 2 and 3, 100 μl of the reaction mixture (containing 5 n mol of MNNG) was added with 100 μl of TA 100 to 2 ml of top agar; for column 4,5,6, and 7, 200 ml of the reaction mixture (containing 5 n mol of MNNG) was added to 2 ml of top agar and plated in a standard Ames plate incorporation assay in the absence of S-9.
DISCUSSION

In this study, there is clear evidence that the N-nitroso compounds can be denitrosated by irradiation with long-wavelength UV light at neutral pH. Free radicals are generated in the photo-denitrosation of nitroso compounds. The N-nitrosamine NMOR is activated in the photo-denitrosation, whereas the N-nitrosamide MNNG is deactivated in terms of mutagenicity. A free-radical mechanism is suggested in the formation of a stable mutagen. Labile free radicals themselves produced during photolysis of N-nitroso compounds are also potent direct mutagens, as described in the Free-Radical Mutagens section.

Denitrosation of N-Nitroso Compounds by Photolysis

In the irradiation of N-nitroso compounds with long-wavelength UV light, the maximal absorbance corresponding to the N-N bonds is decreased (Fig. 3). Also, nitrite is formed (Fig. 4) in the irradiation. It can therefore be concluded that N-nitroso compounds are denitrosated during the long-wavelength UV light irradiation.

Oxidative demethylation is a commonly accepted mechanism for the activation of N-nitrosamines. However, it has been questioned whether or not this oxidative demethylation is the sole pathway for nitrosamine activation. Metabolic denitrosation of N-nitrosamines has been demonstrated by Appel et al. (Appel & Graf, 1982; Appel et al., 1985). They showed that reductive decomposition of the cytochrome P-450 enzyme and N-nitrosamine complex will produce nitrite via NO and the parent amines. This denitrosation pathway of N-nitrosamines has been confirmed by several other investigators. However, the mechanism still remains unclear. Photolytic denitrosation may, therefore, be a good and simple model for the exploration of the denitrosation pathway.
Mutagenesis

N-Nitrosamines are pre-mutagens and have to be enzymatically activated before they are able to express mutagenic activities. The results presented in this study show that the nitrosamine NMOR can be non-enzymatically activated to a stable mutagen by irradiation with long-wavelength UV light in the presence of phosphate. The formation of this stable mutagen is UV-light-dose dependent and parallels the photo-denitrosation of NMOR. This correlation suggests that the stable mutagen is a denitrosation product of NMOR. In contrast to N-nitrosamines, N-nitrosamides are direct mutagens and need no activation to be mutagenic. My results show that photo-denitrosation actually decreases the mutagenicity of the nitrosamide MNNG. A possible explanation of this decrease in mutagenicity of MNNG has been deduced from ESR studies. However, NMOR and MNNG both can produce direct free-radical mutagens in photolysis, which is discussed in the Free-Radical Mutagens section.

ESR Studies

ESR studies show that in the photolysis of the nitrosamine NMOR and the nitrosamide MNNG two different free radicals are formed, carbon-centered and nitrogen centered radicals, respectively. This observation suggests that free radicals are involved in the photo-denitrosation of NMOR and MNNG. In the case of NMOR, the primary radical may be a nitrogen-centered radical \((R_2N^-)\), which results from the photocleavage of the N-N bond with a loss of the \(-\text{NO}\) group. The nitrogen-centered radical is a highly reactive radical that may rapidly rearrange or abstract a hydrogen atom from the parent NMOR to form a carbon-centered radical.
In the case of MNNG, the nitrogen-centered radical is produced during the photo-decomposition, and this initial amidyl radical is trapped by a spin trap. The nitrogen-centered spin adduct is much less stable than the carbon-centered spin adduct. The radical spin adduct obtained with MNNG decays very fast. The ESR signal is completely gone a few minutes after irradiation. The radical adduct obtained with NMOR, however, is stable enough to be detected by ESR several days after irradiation.

These results suggest both carbon-centered radicals from N-nitrosamines and nitrogen-centered radicals from N-nitrosamides are mutagenic. (This will be discussed in more detail in the Free-Radical Mutagens section). The carbon-centered radical can further react with phosphate to form a stable mutagen, but the nitrogen centered radical cannot. The initial nitrogen-centered radical formed on amines rearranges faster than it can react with spin trap. The nitrogen-centered radical of N-nitrosamides cannot rearrange. It is these different types of radicals with different stabilities that may explain the difference in mutagenicities between photo-denitrosated NMOR and MNNG.

The possible involvement of free radicals in the denitrosation of nitroso compounds has been suggested by a number of researchers (Apple et al., 1987 b; Tu & Yang, 1985). The data presented in this study may provide some clue as to the mechanism of denitrosation of N-nitroso compounds.

Phosphate Effect

The presence of phosphate in the NMOR reaction mixture appears to be
important in both the stabilization of free-radical spin adduct and the formation of the stable mutagen. The amount of radical spin adduct produced in the irradiation decreases with the decrease of amount of phosphate present in the NMOR reaction mixture (Table IV). In the absence of phosphate, irradiation of NMOR and spin trap PBN produces an unstable ESR signal. This signal disappears soon at room temperature after irradiation (data not shown). The ESR spectra also appear different with or without phosphate in the NMOR and PBN reaction mixture (Fig. 7). Addition of phosphate to the NMOR and PBN mixture after irradiation does not change the ESR spectrum or the intensity of ESR signal (data not shown). It is suggested that the phosphate effect may be through the direct involvement of phosphate in the stabilization of radical rather than indirect intermolecular interaction. Irradiation of NMOR in the absence of phosphate does not produce a stable mutagen. This also indicates the involvement of phosphate in the formation of the stable mutagen during the irradiation of NMOR. A direct free-radical mutagen, which is different from the stable mutagen discussed above, however, is produced in the irradiation of NMOR without presence of phosphate, which is discussed in the Free-Radical Mutagens section.

At high concentrations of phosphate, the mutagen formation is inhibited (Table IV). There is no simple explanation for this observation, but it may have to do with the actual trapping mechanism. In contrast to the N-nitrosamine NMOR, phosphate does not have an effect in terms of enhancing on either the production of free radical or the mutagen formation from the N-nitrosamide MNNG. It appears that only carbon-centered radicals generated from N-nitrosamines react with phosphate.

Since the formation of the stable mutagen and the production of spin-trapped free radical are consistently correlated, it is suggested that the mutagen formation is free-radical mediated. To support this suggestion I found that free-radical scavengers, such as ascorbic acid and thiourea, or free-radical spin trap, such as PBN and POBN, are capable of inhibiting the
mutagen formation during the irradiation of NMOR. I was able to demonstrate a concentration-dependent inhibition in the presence of ascorbic acid, thiourea, PBN, or POBN in the NMOR reaction mixture before irradiation. The addition of these inhibitors to the reaction mixture after irradiation does not inhibit the mutagenicity of irradiated NMOR with phosphate. Since the highly reactive radical produced in the irradiation rapidly reacts with phosphate to form the stable mutagen, inhibition with free-radical scavengers can only occur if they are present at the start of the reaction. Therefore, it is confirmed that the formation of the stable mutagen during the irradiation of NMOR is free-radical mediated.

Free-Radical Mutagens

As discussed above, carbon- or nitrogen-centered radicals are generated during the irradiation of the N-nitrosamine NMOR or the N-nitrosamide MNNG, respectively. These labile free radicals may also act as alkylating agents. If these radicals are produced in vivo, they may be responsible for cellular damage in general, which can lead to tumorigenesis. My results indicate that these radicals are potent mutagens (Table VIII, IX, X). Irradiation of a mixture of NMOR or MNNG with tester bacteria TA 100 produces higher revertant numbers than those produced by mixing tester bacteria TA 100 with pre-irradiated N-nitroso compounds. The highly reactive free radicals produced during the irradiation of N-nitroso compounds can rapidly react with DNA protein and other cellular components at the time of exposure to the tester bacteria. Cellular damage and incipient mutations are introduced into the bacteria at this time. This higher mutagenicity is also inhibitable by free-radical spin traps and other scavengers. It is therefore concluded that these radicals themselves are direct mutagens.
Some Speculation on the Structure of the Stable Mutagen

Since phosphate has a critical effect on the formation of the stable mutagen and the formation of the spin adduct from nitrosamines, it can be reasonably suspected that the phosphate is directly involved in the photo-reacted intermediate, and the phosphate may structurally be part of the intermediate. Further confirmation needs to be done in future research.

Summary

In conclusion, this study has demonstrated:

1. N-nitroso compounds are denitrosated by irradiation with long-wavelength UV light.
2. Free radicals are generated in the photolysis of N-nitroso compounds.
3. The N-nitrosamine NMOR and the N-nitrosamide MNNG differ upon undergoing photo-denitrosation: (a) by formation of different types of spin adducts with different stabilities and (b) by giving different responses to phosphate.
4. The N-nitrosamine NMOR appears to cause mutagenesis after photo-activation through formation of a stable mutagen. A free-radical mechanism is suggested in the formation of the stable mutagen with phosphate. The N-nitrosamide MNNG appears to be inactivated by the irradiation.
5. Free radicals produced during the irradiation of N-nitroso compounds are mutagenic through a direct radical interaction mechanism.

The proposed denitrosation mechanisms of N-nitroso compounds are summarized in Scheme II.
Scheme II. A summarized scheme of the photo-reaction of N-nitroso compounds.
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