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THE PRODUCTION OF FORMALDEHYDE FROM THE INTERACTION OF DIMETHYLSULFOXIDE WITH HYDROXYL RADICALS: APPLICATION TO THE DETECTION OF HYDROXYL RADICALS IN BIOLOGICAL SYSTEMS AND ITS USE IN EVALUATING THE MOLECULAR MECHANISM OF THE MICROSOMAL ETHANOL OXIDIZING SYSTEM

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THE PRODUCTION OF FORMALDEHYDE FROM THE INTERACTION OF DIMETHYLSULFOXIDE WITH HYDROXYL RADICALS:

Application to the Detection of Hydroxyl Radicals in Biological Systems and its use in Evaluating the Molecular Mechanism of the Microsomal Ethanol Oxidizing System.

BY

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ABSTRACT

Thesis Advisor: Dr. Arthur I. Cederbaum

Dimethylsulfoxide (Me_2SO) is a potent hydroxyl radical ($^{\bullet}OH$) scavenging agent. The production of methane from Me_2SO has been used to detect the generation of $^{\bullet}OH$ in biological systems. Evidence is presented which demonstrates that formaldehyde is produced during the interaction of Me_2SO with $^{\bullet}OH$ generated by three different systems. These systems were 1) the oxidation of xanthine by xanthine oxidase, (enzymatic system) 2) the iron-catalyzed oxidation of ascorbic acid, (chemical system) and 3) NADPH-dependent electron transfer by rat liver microsomes, (biological membrane system). In all three systems, formaldehyde, rather than methane, represents a major product of this interaction. Formaldehyde production was inhibited by $^{\bullet}OH$ scavenging agents. It appears that $^{\bullet}OH$ is generated by these systems via a Fenton reaction in which the reduction of iron is brought about either by superoxide radicals (xanthine oxidase and microsomes) or by ascorbate (ascorbate system). The mechanism of microsomal $^{\bullet}OH$ generation is dissociated from the classical cytochrome P-450 drug metabolizing pathway.

The activity of the microsomal ethanol oxidizing system is increased after chronic ethanol consumption.

The mechanism responsible for this increase is not known. Rat liver microsomes from ethanol-fed rats oxidized Me_2SO and 2-keto-4-thiomethylbutyric acid (KTBA) at rates which were two-to-three-fold faster than microsomes obtained from pair-fed controls. This increased rate of $^{\bullet}OH$ production correlated with an increased rate of ethanol oxidation. Cross competition among ethanol, Me_2SO and KTBA for oxidation and inhibition by other $^{\bullet}OH$ scavenging agents was observed in both microsomal preparations. The increased rate of $^{\bullet}OH$ production by microsomes obtained from chronic ethanol-fed rats may be responsible, at least in part, for the increased rate of ethanol oxidation.

The production of formaldehyde from Me_2SO may represent a convenient technique to detect and to evaluate the role of $^{\bullet}OH$ in some biological systems such as intact cells or In vivo. In view of the production of formaldehyde, Me_2SO should not be considered to be an inert solvent in biological systems.

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ABBREVIATIONS

$^{\bullet}CH_3$	methyl radical
DETAPAC	diethylenetriaminepentaacetic acid
DMPO	5, 5-dimethyl-1-pyrroline-1-oxide
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance spectroscopy
H_2O_2	hydrogen peroxide
KTBA	2-keto-4-thiomethylbutyric acid
MEOS	microsomal ethanol oxidizing system
Me_2SO	dimethylsulfoxide
$NADP^{\pm}$	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
$^3O_2$	triplet (ground state) oxygen
$O_2^{\cdot-}$	superoxide anion radical
$^{\bullet}OH$	hydroxyl radical
$^{\bullet}OR$	alkoxy radical
S.E.M.	standard error of the mean
SOD	superoxide dismutase

LIST OF RELEVANT EQUATIONS

$3Fe^{3+} + 3\ \text{Ascorbate} \longrightarrow 3\ \text{Dehydroascorbate} + 3Fe^{2+}$

$2Fe^{2+} + O_2 \xrightarrow{2H^+} 2Fe^{3+} + H_2O_2$

$Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$

The Iron-Catalyzed Oxidation of Ascorbic Acid (p. 148)

Net: 3 Ascorbate + $O_2 \longrightarrow {}^{\bullet}OH + OH^- + 3$ Dehydroascorbate

$2CH_3\text{-}O\text{-}O^{\bullet} \longrightarrow CH_2O + CH_3OH + O_2$

The Russell Mechanism (p. 165)

CHAPTER 1

Literature Review and Introduction

1.1. The Evolution of Molecular Oxygen

The primordial gaseous environment of the planet earth was a reducing atmosphere which was composed of hydrogen, ammonia, methane and water vapor. Oxygen was not present in this mixture. It is believed that in the absence of an ozone layer, high levels of irradiation together with high temperatures and abundant water gave rise to simple amino acids which in turn gave rise to primitive anaerobic organisms (1-3). The subsequent evolution of photosynthetic blue-green algae which could harness energy from light to split H_2O into free oxygen (O_2) and a hydrogen pool (e.g. $C_6H_{12}O_6$) resulted in the introduction of O_2 into the earths atmosphere (2, 4). Thereafter, the atmosphere of this planet became an aerobic one.

The subsequent oxygenation of the biosphere was in fact a mixed blessing. It has been proposed that the gradual increase of the atmospheric O_2 concentration was advantageous in that it permitted the further evolution of animal forms dependent on aerobic metabolism. Hence more energy in the form of ATP could be derived from energy reserves such as carbohydrates and lipids.

However, it also applied a severe evolutionary pressure on existing biological organisms which utilized anaerobic photoreduction and fermentation for metabolic energy (5). Moreover, the incomplete biological reduction of molecular oxygen results in the generation of very reactive and toxic species called free radicals. A free radical is defined as any atom, group of atoms or a molecule with one unpaired electron occupying an outer orbital. The lone electron present in the outer orbital of a free radical endows it with very unusual chemical reactivity and physical characteristics. Free radical reactivity and toxicity is accounted for by the strong tendency of the unpaired electron to interact with other electrons to form an electron pair and thus a chemical bond. The survival of life on this planet in an oxygen environment became dependent upon a complicated interplay between the biological generation of these toxic oxygen species and the ability of the various life forms to control these substances and defend themselves against their effects.

1.2. The Chemistry of Molecular Oxygen

Molecular oxygen in its ground state (which is also called the triplet state of oxygen ($^3O_2$)) is a biradical which has two unpaired electrons with parallel electron

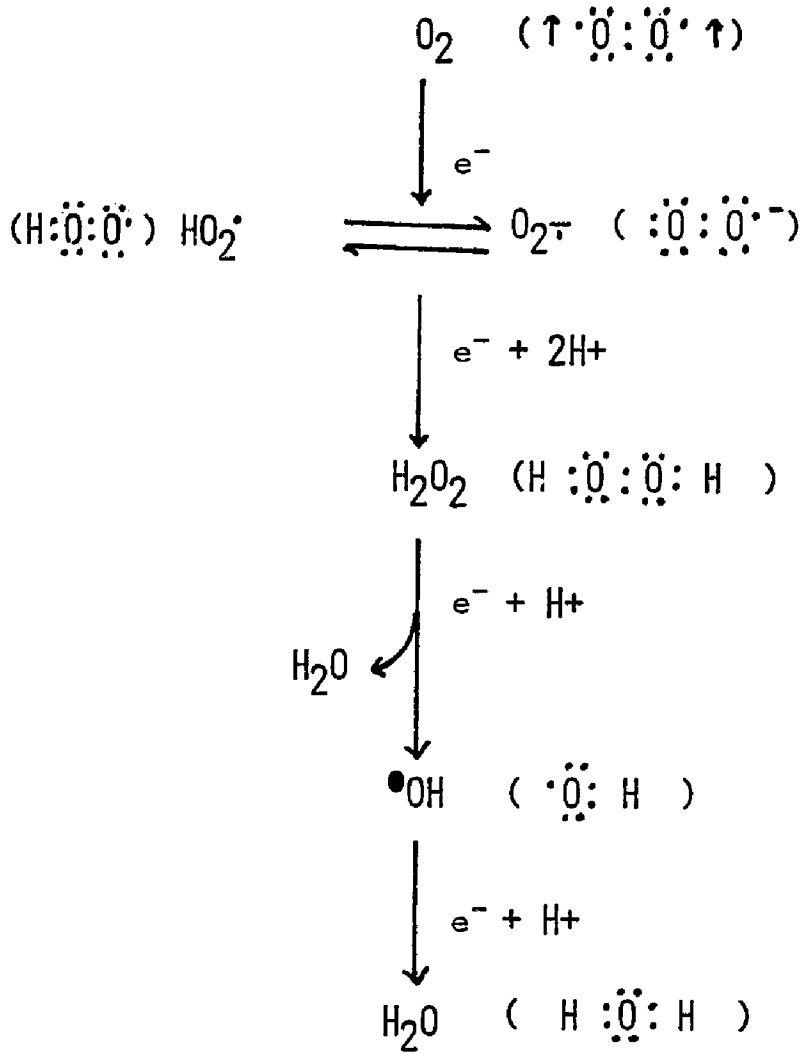
spins in its two outermost molecular orbitals (6, 7). This electronic configuration is responsible for the fact that ground state oxygen is relatively inert and unreactive. Since most other elements have parallel and antiparallel electron spins in their outermost orbitals, the parallel electron spin arrangement of molecular oxygen restricts bond formation with these elements. Therefore, the formation of a bond between molecular oxygen and other molecules would necessitate an electron spin inversion. Since the spin inversion process is slow compared with the lifetime of collision complexes, between these molecules, oxygen in its ground state is a relatively weak oxidant. A variety of mechanisms can accomplish the univalent, divalent or tetravalent reduction of oxygen to water. In aerobic cells, the majority of the oxygen is reduced via cytochrome oxidase. This enzyme catalyzes the tetravalent reduction of molecular oxygen without the release of univalent, divalent and trivalent reduction intermediates into the cellular milieu (8, 9). In contrast, the contribution of the univalent pathway for the reduction of molecular oxygen is rather small, although not unimportant, since it is this pathway which results in the release of oxygen radicals and reactive intermediates such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hy-

droxyl radical ($^{\bullet}OH$) into the cellular environment (10-16). The univalent pathway for the reduction of molecular oxygen is presented in Figure 1. The addition of one electron to molecular oxygen results in the formation of the superoxide anion radical. Addition of a second electron and two protons produces hydrogen peroxide. Reduction of hydrogen peroxide by one electron and one proton results in the formation of the hydroxyl radical. Finally, the addition of a fourth electron and a proton produces water. It has been generally accepted that the mechanism of oxygen toxicity at the molecular level is due to the interaction of oxygen-free radicals, especially $^{\bullet}OH$, with cellular components (17). In order to permit survival in an aerobic environment, a variety of defense mechanisms have evolved to scavenge and destroy these toxic intermediates. The primary defense is provided by enzymes that catalytically scavenge either $O_2^{\bullet-}$ or H_2O_2. A secondary "back-up" defense system also exists in the forms of certain cellular constituents which can interact with free radicals and ameliorate some of their toxic effects. In the subsequent sections, each intermediate of the univalent pathway of oxygen reduction will be discussed in turn, together with the specific cellular defensive mechanisms which prevent their toxicity.

Figure 1.

The Univalent Pathway for the Reduction of Molecular Oxygen.

The addition of one electron at a time to molecular oxygen results in the formation of toxic oxygen species, namely, the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$).


O2
e−
HO2
O2−
e− + 2H+
H2O2
e− + H+
H2O
OH
e− + H+
H2O

1.3. Biological Generation of Superoxide Radicals, Hydrogen Peroxide and Hydroxyl Radicals and Pathways for Their Removal

1.3.1 The Superoxide Anion Radical

1.3.1.1. Biological Sources of Superoxide Radicals

The superoxide anion radical was formerly of concern only to radiation chemists and radiobiologists since this radical was not known to be a normal product of biological oxidations. The discovery of superoxide dismutase in the late 1960's provided a tool to study the generation of $O_2^{\cdot-}$ in biological systems (18). Superoxide dismutase was originally isolated (19), crystallized (20) and extensively studied (21, 22) as a copper-containing protein of unknown function for thirty years prior to the discovery of its superoxide dismutase activity. Since then, a number of reactions of interest to biochemists have been shown to produce $O_2^{\cdot-}$. These include the autoxidations of hydroquinones, leukoflavins, catecholamines, thiols and tetrahydropterins (23-28). Reduced ferrodoxins also produce $O_2^{\cdot-}$ upon spontaneous autoxidation (29). Hemoglobin and myoglobin slowly liberate $O_2^{\cdot-}$ as they are converted to methemoglobin and metmyoglobin (30,

31). A number of enzymes, including xanthine oxidase, aldehyde oxidase and dihydro-orotic dehydrogenase produce $O_2^{\cdot-}$ as do several flavin-containing dehydrogenases (16, 32). Many of the experiments which will be described in this dissertation utilized xanthine oxidase as an oxygen radical generating system. The superoxide radical has also been demonstrated as an intermediate in the mechanisms of action of galactose oxidase (33), indoleamine dioxygenase (34) and 2-nitropropane dioxygenase (35). Subcellular organelles such as mitochondria (36-40), chloroplasts (41-44), microsomes (45) and nuclei (46), produce $O_2^{\cdot-}$. Phagocytic cells such as polymorphonuclear leukocytes or granulocytes have also been shown to liberate large amounts of $O_2^{\cdot-}$ during the respiratory burst that accompanies active phagocytosis (47-53). The release of $O_2^{\cdot-}$ in the phagosome which contains ingested foreign material is an important component of the bactericidal mechanism of those cells. Patients who have a genetic defect in the ability of their polymorphonuclear leukocytes to produce $O_2^{\cdot-}$ (chronic granulomatous disease) suffer from recurrent bacterial infections to which they often succumb (54, 55). Thus, there is now general agreement among biological scientists that $O_2^{\cdot-}$ is made during biological oxygen reduction.

1.3.1.2. The Superoxide Radical as a Cellular Toxin and Protection by Superoxide Dismutase

In aqueous media, $O_2^{\cdot-}$ is a strong reducing agent and a weak oxidant (9). Superoxide radicals have been shown to inactivate viruses (56), induce lipid peroxidation (57), damage membranes (58, 59) and kill cells in culture (60). There is also evidence that $O_2^{\cdot-}$ is involved in the radiation-induced damage and death of eukaryotic cells (61). It has been suggested that superoxide plays a role in cellular differentiation, aging and cancer (62-65) and in the toxicity of many drugs including 6-hydroxydopamine (26), alloxan (66, 67), paraquat (68, 69), 2-methyldopa (70), nifurtimox (71), and of quinone anti-cancer drugs such as adriamycin, (72, 73) daunorubicin and mitomycin C (72, 73). Many of these drugs are reduced by NADPH-dependent flavoprotein dehydrogenases. Autoxidation of the reduced drug or reduced flavin results in the production of $O_2^{\cdot-}$. Superoxide also appears to play a role in the inflammatory response (74, 75) and in the toxicity of hyperbaric oxygen (17). It appears that in most of these cases $O_2^{\cdot-}$ itself is not directly responsible for these effects but is the immediate precursor of a more potent and more toxic oxidant, namely $^{\bullet}OH$.

In recent years, much evidence has been accumulated

to suggest that superoxide dismutase provides the primary defense system against the toxicity of $O_2^{\cdot-}$. Several lines of evidence indicate that this enzyme is essential for the survival of all respiring cells. Surveys of a variety of microorganisms demonstrated that aerobic and aerotolerant species contained superoxide dismutase whereas the obligate anaerobes did not (76, 77). More evidence was provided by the observation that exposure of facultative anaerobes to oxygen resulted in increased intracellular levels of superoxide dismutase and that elevated levels of this enzyme were correlated with enhanced resistance to the lethal effects of hyperbaric oxygen (78). Interference with the synthesis of the enzyme rendered the cells susceptible to oxygen toxicity. Mutants of Escherichia Coli with a temperature-sensitive defect in their ability to maintain normal intracellular levels of superoxide dismutase showed a parallel temperature-sensitive defect in oxygen tolerance (79). Revertants were found to have regained the ability to maintain normal levels of the enzyme at the restrictive temperature and were also found to have regained oxygen tolerance at the restrictive temperature. Finally, the antibiotic streptonigrin kills bacteria by increasing intracellular $O_2^{\cdot-}$ generation. Strains of bacteria with high superoxide dismutase activity are

resistant to streptonigrin (80).

These observations with microorganisms established that in these lower forms of life, superoxide dismutase serves as an essential defense mechanism against oxygen and superoxide toxicity. It appears that this enzyme serves an identical role in higher organisms. It has been shown that rats are able to tolerate an otherwise lethal atmosphere of 100% oxygen if they are first given a period of adaptation to an atmosphere of 85% oxygen. This period of adaptation is associated with an increase in levels of superoxide dismutase in the lungs of these rats (81). In addition, injection of superoxide dismutase into rats appear to diminish the superoxide mediated toxicity of paraquat (82). Added superoxide dismutase protects the isolated pancreatic islets against damage by alloxan (83).

1.3.1.3. Isoenzymes of Superoxide Dismutase

Three different forms of superoxide dismutase have been described. All three enzymes catalyze the same reaction which is described in equation (1):

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{2H+} H_2O_2 + O_2 \qquad (1)$$

The various enzymes catalyze this reaction with comparable efficiency. The iron-containing and manganese-containing

enzymes are characteristic of prokaryotes and are closely related in terms of their amino acid sequences. The enzyme that contains both copper and zinc is characteristic of eukaryotes and appears to have evolved independently since they have no sequences of amino acids which are homologous to those of the iron- or the manganese-containing enzyme (84). Actually, eukaryotes generally contain both the copper-zinc and the manganese-containing enzyme. The copper-zinc containing enzyme is inhibitted by cyanide and is stable to treatment by a mixture of chloroform and ethanol whereas the manganese-containing enzyme is resistant to cyanide but is denatured by chloroform and ethanol. Yeast (85), plants (86), chicken liver (87) pigheart (87), and rat liver (88) have been shown to contain the manganese form of the enzyme in the mitochondrial matrix and the copper-zinc containing enzyme in the cytosol. Human and baboon liver also have both the manganese- and the copper-zinc-containing enzyme (89). In these species, only the manganese-containing enzyme is found in the mitochondrial matrix whereas the cytosol contains both enzymes.

The superoxide radical is capable of spontaneous dismutation to H_2O_2 and O_2 in the absence of superoxide dismutase. Since the superoxide anion radical is

actually the conjugate base of a weak acid, $HO_2^{\bullet}$, whose pKa is 4.8, one must consider the possibility of three dismutation reactions. These reactions are given in equations (2) through (4)(9).

$$HO_2^{\bullet} + HO_2^{\bullet} \longrightarrow H_2O_2 + O_2 \qquad (2)$$

$$HO_2^{\bullet} + O_2^{\cdot-} + H^+ \longrightarrow H_2O_2 + O_2 \qquad (3)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (4)$$

The spontaneous dismutation is most rapid at pH 4.8 (equation (3)) where the concentrations of the conjugate acid and the conjugate base are equal. The dismutation between $O_2^{\cdot-}$ and $O_2^{\cdot-}$ (equation (4)) is slow because of electrostatic repulsion between the anions. In order to overcome this electrostatic repulsion, superoxide dismutase catalyzes the reduction of one $O_2^{\cdot-}$ and the oxidation of the other $O_2^{\cdot-}$ by alternate reduction and oxidation of the metal catalyst in the active site of the enzyme. The enzyme thereby accomplishes the transfer of an electron from one $O_2^{\cdot-}$ to another without the necessity for close approach of the anions. This appears to be the actual mechanism of action of all of the superoxide dismutases that have been examined (90-95).

The mechanism is described in equations (5) and (6)

$$E\text{-}Me^{n} + O_2^{\cdot-} \longrightarrow E\text{-}Me^{n-1} + O_2 \qquad (5)$$

$$E\text{-}Me^{n-1} + O_2^{\cdot-} + 2H^{+} \longrightarrow E\text{-}Me^{n} + H_2O_2 \qquad (6)$$

where E denotes enzyme and Me denotes the metal. In the case of the copper-zinc enzyme, it is the copper that participates in the catalytic cycle and oscillates from the cupric to the cuprous state. The zinc appears to play primarily a structural role. In the manganese- and iron-containing enzymes, the trivalent and divalent states of the metals are involved in the catalytic cycle. The superoxide dismutases are extraordinarily efficient catalysts whose rate of interaction with $O_2^{\cdot-}$ is approximately $2x10^{9}M^{-1}sec^{-1}$.

1.3.1.4 <u>The Spontaneous versus The Catalytic Dismutation of Superoxide</u>

If $O_2^{\cdot-}$ is capable of spontaneous dismutation, then why should it be necessary for respiring cells to contain an enzyme with superoxide dismutase activity to protect them against the toxic effects of oxygen? Actually, at pH 7.4 in an aqueous environment, $O_2^{\cdot-}$ dismutes spontaneously at a rate of $8x10^{4}M^{-1}sec^{-1}$ (9).

Although this is a large rate constant, the reaction is second order with respect to $O_2^{\cdot-}$ and is a function of the steady-state level of $O_2^{\cdot-}$. Thus at $1x10^{-10}$M $O_2^{\cdot-}$, the spontaneous dismutation would be slow. In contrast, the reaction between $O_2^{\cdot-}$ and superoxide dismutase is first order in $O_2^{\cdot-}$ and first order in enzyme which is present in most tissues at a concentration of approximately $1x10^{-5}$M. At $1x10^{-10}$M $O_2^{\cdot-}$, the enzyme-catalyzed dismutation would thus be 10^5-fold faster than the spontaneous reaction even if the rate constant for the enzymatic reaction were equal to that of the spontaneous reaction. Since at physiological pH of 7.4 the rate constant for the enzymatic reaction is 10^4-fold greater than that for the spontaneous reaction, the net increase in the rate of dismutation of $O_2^{\cdot-}$ caused by superoxide dismutase is 10^9-fold greater than the spontaneous dismutation rate.

1.3.2 Hydrogen Peroxide

1.3.2.1. Biological Sources of Hydrogen Peroxide

Hydrogen peroxide may be formed in the cell as a result of two types of reactions. The first is divalent reduction by cellular oxidases which transfer two electrons to each oxygen molecule as described in equation (7).

$$O_2 + RH_2 + 2e^- \longrightarrow H_2O_2 + R \qquad (7)$$

Examples of these oxidases are urate oxidase, D-amino acid oxidase and glycollate oxidase. These divalent oxidizing enzymes are usually found within specialized organelles called peroxisomes (96). The second type of reaction which can produce H_2O_2 is the spontaneous or enzymatic dismutation of $O_2^{\cdot-}$. Therefore, systems which produce $O_2^{\cdot-}$ will also produce H_2O_2 (97, 98).

1.3.2.2 Hydrogen Peroxide as a Cellular Toxin and Protection by Catalase and Peroxidases

At high concentrations, hydrogen peroxide will kill most cells. Some species of bacteria are highly sensitive to H_2O_2 (99) and H_2O_2 at low concentrations completely inhibits CO_2 fixation by chloroplasts (100). Hydrogen peroxide is also the substrate of the bactericidal myeloperoxidase enzyem of phagocytic cells which generates potent oxidative products such as hypochlorite and possibly singlet oxygen (55).

However, in most aerobes it seems that H_2O_2 is not the primary mechanism of oxygen toxicity. Although H_2O_2 may be toxic at high concentrations, these are orders of magnitude greater than the intracellular flux of H_2O_2 (101). Hydrogen peroxide may not be damaging

to the cell itself, but it is capable of reacting in either a Fenton reaction with metal chelates or in a Haber-Weiss reaction with $O_2^{\cdot-}$ to produce $^{\bullet}OH$ which is a potent cell toxin. These reactions will be discussed in a subsequent section.

Hydrogen peroxide is present at low steady-state concentrations in animal tissues (10^{-8}M to 10^{-6}M) (102). Although these concentrations of H_2O_2 can be tolerated by most aerobic cells, cellular H_2O_2 levels must not be allowed to become excessive. Aerobic cells have evolved two enzyme systems which destroy H_2O_2. Catalase, which is located in peroxisomes, decomposes high concentration of H_2O_2 very rapidly (equation 8).

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (8)$$

However, catalase is almost ineffective at decomposing low concentrations of H_2O_2 because of its high Km for the substrate (103). In addition, since most of the catalase in plant and animal cells is located in the peroxisomes, catalase cannot have any significant effect on the H_2O_2 which is produced outside the peroxisomes by enzyme systems which are located in the cytosol, mitochondria or microsomes (103). Both mitochondria and chloroplasts produce H_2O_2, yet

they are devoid of catalase activity (103). Therefore, as a result of its localization and of its kinetics, catalase is not highly effective in dealing with non-peroxisomal, biologically-generated H_2O_2. In fact, there is some evidence that catalase may not be essential to the life of cells or in the protection against oxygen toxicity. Some aerobic cells lack catalase yet they grow well (104, 105). Conversely, when cells of B. subtilis (106) and E. coli (107) contain higher than normal levels of catalase, these microorganisms do not tolerate exposure to hyperbaric oxygen better than cells with normal levels of catalase. Humans, who have an inborn error of metabolism which results in abnormally low catalase activities in their red blood cells, survive very well in an oxygen environment (103).

Since catalase may not be sufficient, other enzyme systems which remove H_2O_2 must exist. Cells can also dispose of H_2O_2 by using peroxidase enzymes that catalyze the general reaction which is shown in equation (9).

$$RH_2 + H_2O_2 \longrightarrow R + 2H_2O \qquad (9)$$

Catalase can act in a peroxidatic fashion with certain substrates (RH_2) e.g., methanol, ethanol or formate (98).

Glutathione (GSH) peroxidase, which is located in the cytosol and mitochondria of the animal cell, may be the major intracellular system for the decomposition of H_2O_2 (102) (equation (10)).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \qquad (10)$$

Glutathione peroxidase works in conjunction with glutathione reductase which reduces oxidized glutathione (GSSG) back to GSH (equation (11)).

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+ \qquad (11)$$

The equilibrium position of glutathione reductases greatly favors GSSG reduction so that cellular GSH/GSSG ratios are kept very high unless the cells are exposed to increasing oxidative stress (102).

1.3.3. The Hydroxyl Radical

1.3.3.1. Biological Sources of the Hydroxyl Radical

The hydroxyl radical is generated indirectly by many biological reaction systems. For example, $^{\bullet}OH$ is generated during the iron-catalyzed oxidation of ascorbic acid (108, 109), during the oxidation of xanthine by

xanthine oxidase (110), during NADPH-dependent microsomal electron transfer (111) and by phagocytosing polymorphonuclear leukocytes during the respiratory burst (112, 113).

Two H_2O_2-dependent pathways are often invoked as sources of $^{\bullet}OH$ in biological systems. These reactions are outlined in equations (12) through (14).

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + {}^{\bullet}OH \qquad (12)$$

$$Fe^{3+}\text{-chelate} + O_2^{\cdot-} \longrightarrow Fe^{2+}\text{-chelate} + O_2 \qquad (13)$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \longrightarrow Fe^{3+}\text{-chelate} + OH^- + {}^{\bullet}OH \qquad (14)$$

Reaction (12) is known as the Haber-Weiss reaction (114) and reaction (14) is known as the Fenton reaction (115). Taken together, the net result of reaction (13), the reduction of Fe^{3+} by $O_2^{\cdot-}$ and reaction (14), the Fenton reaction, is the Haber-Weiss reaction. It is now generally accpeted that the rate constant for the uncatalyzed Haber-Weiss reaction is too slow to account for the formation of $^{\bullet}OH$ in biological systems (116-119). However, the Haber-Weiss reaction can be catalyzed by transition metals, especially iron. Therefore, in biological systems $^{\bullet}OH$ may be generated by an iron-catalyzed

Haber-Weiss reaction (equations (13) and (14)). The feasibility of reactions (13) and (14) was demonstrated by the ability of iron-EDTA to stimulate the generation of $^{\bullet}OH$ during the oxidation of xanthine by xanthine oxidase (120-123) and during NADPH-dependent microsomal electron transfer (124). In addition to its feasibility, the iron-catalyzed Haber-Weiss reaction is an attractive mechanism since it accounts for the inhibition of $^{\bullet}OH$ formation either by superoxide dismutase or by catalase or by iron-chelating agents in many of the reaction systems that were mentioned above.

1.3.3.2. The Hydroxyl Radical as a Cellular Toxin

The hydroxyl radical is a powerful oxidizing agent which is highly toxic in biological systems, since it reacts readily with many biological compounds (125, 126). Hydroxyl radicals have been implicated in a) the cytotoxic action of alloxan on the Beta cells of the pancreas; (26, 66); b) the toxicity of 6-hydroxy- and 6-aminodopamine on sympathetic nerve cells (26, 127); c) some of the x-ray induced free radical damage to nucleic acids (128); d) the oxygen enhancement effect of radiation induced damage to T4 bacteriophage (129, 130); e) the free radical induced damage to DNA (131-134), f) the increased susceptibility of S. aureus to H_2O_2 (135),

g) the degradation of hyaluronic acid in joints (122); h) rheumatoid arthritis and inflammatory joint disease (136); i) the peroxidation and lysis of lysosomal membrane (137) and j) the premature lysis of phagocytosing leukocytes (112). In a recent review, the possibility that $^{\bullet}OH$ may be implicated in causing some of the properties of the cancer cells is described (138). Indeed, the anti-inflammatory activity of dimethylsulfoxide has been ascribed to the ability of this compound to scavenge $^{\bullet}OH$ (113).

1.3.3.3. Protection Against Cellular Damage by the Hydroxyl Radical

Cells do not contain a specific enzymatic scavenging system for $^{\bullet}OH$. Living systems appear to depend upon superoxide dismutase, catalase and peroxides to destroy cellular $O_2^{\cdot-}$ and H_2O_2, the precursor of $^{\bullet}OH$ in biological systems. By their combined actions, these enzymes keep the steady state concentrations of $O_2^{\cdot-}$ and H_2O_2 small and minimize $^{\bullet}OH$ production so that aerobic life is possible (9). Fortunately, most of the iron in biological systems is stored in forms that do not appear to catalyze the Haber-Weiss reaction (e.g., chelated by ferritin). However, it should be apparent that changes in the production of $O_2^{\cdot-}$ such as those caused by

administration of agents such as paraquat, alloxan, or increases in cellular iron, may overcome the cellular protective mechanisms.

1.3.4. Singlet Oxygen

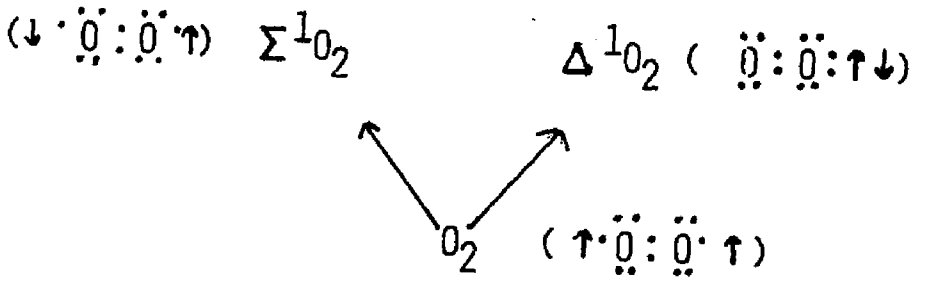
Electrons generally occur in pairs that are stabilized by spins which are in opposite directions. As was indicated in Figure 1, molecular oxygen in its ground state contains two unpaired electrons with spins in the same direction. Singlet oxygen ($^1O_2$) is formed when an absorption of energy shifts one of these electrons to an orbital of higher energy with an inversion of spin as described in Figure 2 (55). There are two forms of singlet oxygen. One of these is delta singlet oxygen (Δ^1O_2) in which the newly paired electrons occupy the same orbital. The other form is sigma singlet oxygen (Σ^1O_2) in which the electrons of opposite spin occupy different orbitals. Δ^1O_2 has a lower energy above ground state (22Kcal) than Σ^1O_2 (38 Kcal) but also has a considerably longer lifetime. The lifetime of Δ^1O_2, however, is only approximately two microseconds in water. Its lifetime in solution is increased by the substitution of deuterium oxide for water with a corresponding increase in chemical reactivity.

It has been suggested that the spontaneous dis-

Figure 2.

The Excitation of Triplet Oxygen to Form Singlet Oxygen.

Singlet Oxygen is formed when one of the unpaired electrons of molecular oxygen (triplet state) is raised to an orbital of higher energy with an inversion of spin.


(↓ · O : O · ↑) Σ^1O_2
Δ^1O_2 (O : O : ↑↓)
O_2
(↑ · O : O · ↑)

mutation of $O_2^{\cdot-}$ (equation 1) releases oxygen in the form of $^1O_2$ (139). The oxygen that is released during the Haber-Weiss reaction (equation 11) may be $^1O_2$ (140). In addition, it has been suggested that the interaction of hypochlorite (which is formed by the myeloperoxidase antibacterial system in neutrophils) with H_2O_2 results in the formation of $^1O_2$ (55). The generation of $^1O_2$ during these reactions has been based on the use of $^1O_2$ scavengers. However, these agents (e.g., bilirubin, histidine, alpha-tocopherol and dimethylfuran) are not specific scavengers for $^1O_2$. Therefore, at the present time, both the generation and role of $^1O_2$ in all three of these reaction systems is open to question (141).

1.4. Non-Enzymatic Defenses Against Oxygen Radicals

Aside from the enzymatic defense mechanisms which were described above and that are available to cells in order to protect themselves against damage by oxygen radicals, non-enzymatic defenses also exist. Alpha-tocopherol (vitamin E) which is intercolated in cellular membranes, has been shown to prevent oxygen radical-dependent chain propagating reactions in lipid membranes and to halt lipid peroxidation (142). Other compounds protect the cell from free radical damage in the hydro-

phillic, or non-membranous regions of the cell. These include ascorbic acid, cysteine and reduced glutathione (142). A recent report described a cooperative interaction between alpha-tocopherol and ascorbic acid in the suppression of peroxidation of membrane phospholipid by ferrous ion (143). A copper-containing plasma glycoprotein called ceruloplasmin has been proposed as a circulating scavenger of oxygen derived free radical (144-146). These non-enzymatic defenses are considered by many to be a "back-up" defense system in cases where the enzymatic defenses may be overcome.

1.5. The Detection of Superoxide, Hydrogen Peroxide and the Hydroxyl Radical

In the preceding sections, the biological sources of the reactive intermediates of oxygen reduction and their potential role in cellular toxicity were described. The ability to detect these intermediates is therefore an important consideration in many areas of biochemistry, cell biology, toxicology, and medicine. In general, assays for the detection of $O_2^{\cdot-}$ utilize a $O_2^{\cdot-}$-dependent reaction with certain chemicals. This reaction is sensitive to the addition of superoxide dismutase. The reduction of cytochrome c, measured at 550 nm, is most often employed (147). Other assays involve spectropho-

tometric monitoring of the irreversible oxidation of epinephrine to adrenachrome (25) or the chemiluminescent reaction of $O_2^{\cdot-}$ with oxidized luminol (148). However, these latter two reactions are considered to be less specific than the reduction of cytochrome C (149). Superoxide radical anions are also detectable by electron paramagnetic resonance spectroscopy (EPR) (32) which will be described in a subsequent section.

The methods for the detection of H_2O_2 in biological systems have recently been reviewed (149). These methods may be divided into two types. The first of these employs direct spectrophotometric measurement of either the catalase - H_2O_2 complex or the peroxidase - H_2O_2 complex. The almost irreversible reaction of catalase or the peroxidases with H_2O_2, combined with the intense absorption bands of hemoproteins makes the enzyme-substrate complex a uniquely sensitive detector of H_2O_2. The second of these methods involves the oxidation of certain compounds by H_2O_2 and the subsequent measurement of the oxidation product. For example, diacetyldichlorofluroscin is oxidized by peroxidase - H_2O_2 to a fluorescent product; H_2O_2 - catalase oxidizes ethanol to acetaldehyde or formate to CO_2; H_2O_2 oxidizes ferrous ammonium sulfate and the resulting Fe^{3+} can be detected colorimetrically with thiocyanate. The sen-

sitivity of these methods that detect the reaction product can be quite substantial since the reaction can be carried out over a sufficient period of time such that large amounts of product accumulate.

Currently, the presence of $^{\bullet}OH$ is detectable by two different techniques. The first of these is electron paramagnetic resonance spectroscopy (EPR). This technique exploits the paramagnetic nature of the unpaired electron of free radicals by directly detecting the magnetic moment that is exerted by the unpaired electron. Since only paramagnetic species are detectable by this technique, EPR is useful in complex biological systems. The hydroxyl radical is a short-lived species since it reacts so readily with most other molecules at diffusion controlled rates (125). Its rate of reaction is limited mainly by the frequency with which it collides with other species. Therefore, in most cases, $^{\bullet}OH$ never reaches a concentration ($10^{-8}M$ to $10^{-6}M$), which is high enough (i.e., a long enough half-life) to be detected by EPR. In order to overcome this limitation, the hydroxyl radical is trapped as an adduct with a "spin-trapping" agent which generates a new stable free radical by forming a covalent bond with the free radical. In this form, $^{\bullet}OH$ gives an EPR signal which is characteristic of the inter-

action of $^{\bullet}OH$ with that spin-trapping agent. Spin-trapping agents such as 5, 5-dimethyl-1-pyroline-1-oxide (DMPO) and phenyltertiarybutylnitrone (PBN) have been used to detect the generation of $^{\bullet}OH$ during the oxidation of xanthine by xanthine oxidase (150), during NADPH - dependent microsomal electron transfer (151, 152), by respiring rat heart mitochondria (153), by the interaction of DNA-ferrous iron with H_2O_2 (154) and by autoxidation of 6-hydroxydopamine (155). There are two major problems with this method to detect $^{\bullet}OH$. Instrumentation is very sophisticated and expensive and therefore often not available in many laboratories. Second, the interpretation of some EPR signals with respect to the detection of $^{\bullet}OH$ in biological systems is the subject of some controversy as the signal which is assumed to be characteristic of $^{\bullet}OH$ may have arisen from the decay of a different spin-trapped radical, e.g., $O_2^{\bullet-}$(156-158).

The second major technique for the detection of $^{\bullet}OH$ in biological systems makes use of $^{\bullet}OH$ scavenging agents which form measurable products upon interaction with $^{\bullet}OH$. While there are many compounds which are capable of scavenging $^{\bullet}OH$ in biological systems (125, 159), the products of these interactions are not known in most cases. A list of some $^{\bullet}OH$ scavenging agents

which are relevant to this dissertation and the known products of their interaction with $^{\bullet}OH$ are presented in Figure 3. Alcohols are a class of compounds which are oxidized by $^{\bullet}OH$ to their respective aldehydes. However, alcohols represent non-specific probes for the detection of $^{\bullet}OH$ in biological systems since alcohols can also be oxidized by cellular pathways which do not involve $^{\bullet}OH$ (e.g., alcohol dehydrogenase and catalase). The oxidation of methional (3-thiomethylpropanal) or of 2-keto-4-thiomethylbutyric acid (KTBA) by $^{\bullet}OH$ to form ethylene gas has also been used to detect the presence of $^{\bullet}OH$ in biological systems (110, 111, 160). The detection of ethylene requires gas chromatography techniques. However, it has recently been reported that alkoxy radicals ($^{\bullet}OR$) and peroxy radicals ($^{\bullet}OOR$) also give rise to ethylene upon reaction with methional (161, 162). The hydroxylation of tryptophan by $^{\bullet}OH$ may be used to detect the presence of these radicals (120). The disappearance of tryptophan is measured as a change in optical density at 278 nm. However, changes in the concentration of tryptophan are very small, are not specific for $^{\bullet}OH$ alone and are not easily detectable, especially in turbid biological suspensions. Benzoic acid is known to be decarboxylated to produce carbon dioxide during interaction with $^{\bullet}OH$ (163, 164).

Figure 3.

Chemical equations for the interaction of hydroxyl radical scavenging agents with $^{\bullet}OH$.

The equations represent the interaction of ethanol (1), 1-butanol (2), methional (3), 2-keto-4-thiomethylbutyric acid (4) and dimethylsulfoxide (5) with $^{\bullet}OH$ to produce acetaldehyde (1), butyraldehyde (2), ethylene (3 and 4) and methane (5).

1) $CH_3-CH_2-OH + {}^{\bullet}OH \longrightarrow CH_3{}^{\bullet}CH-OH \longrightarrow CH_3CHO$

2) $CH_3-CH_2-CH_2-CH_2-OH + {}^{\bullet}OH \longrightarrow CH_3CH_2CH_2{}^{\bullet}CH-OH \longrightarrow CH_3CH_2CH_2CHO$

3) $CH_3-S-CH_2CH_2CHO + {}^{\bullet}OH \longrightarrow CH_2=CH_2 + HCOOH + \frac{1}{2}(CH_3S)_2$

4) $CH_3-S-CH_2-CH_2-\underset{\underset{O}{\|}}{C}-COOH + {}^{\bullet}OH \longrightarrow CH_2=CH_2 + 2CO_2 + \frac{1}{2}(CH_3S)_2$

5) $CH_3-\underset{\underset{O}{\|}}{S}-CH_3 + {}^{\bullet}OH \longrightarrow {}^{\bullet}CH_3 + CH_3SOOH$

$\downarrow$

CH_4

Recently, the production of $^{14}CO_2$ from ^{14}C-carboxyl labeled benzoic acid has been used to detect the production of $^{\bullet}OH$ in biological systems (165, and G. Winston and A.I. Cederbaum, manuscript in press). However, in biological systems benzoate may also be decarboxylated by aromatic acid decarboxylases and therefore, CO_2 production may not be specific for $^{\bullet}OH$. In addition, the measurement of $^{14}CO_2$ requires radioactive counting equipment. Consequently, although several $^{\bullet}OH$ scavenging agents have been used to detect $^{\bullet}OH$ in biological system, there are limitations in the use of each of these probes. Clearly, there is a need to develop an inexpensive, sensitive, specific and easy to use chemical assay to detect $^{\bullet}OH$ in biological systems.

Dimethylsulfoxide (Me_2SO) is a potent $^{\bullet}OH$ scavenging agent (125, 159). This compound reacts with $^{\bullet}OH$ to produce methyl radicals ($^{\bullet}CH_3$) (166-168), which can give rise to methane gas by hydrogen abstraction. The production of methane from Me_2SO has been used to detect the generation of $^{\bullet}OH$ by several biological systems including NADPH-dependent microsomal electron transfer (111, 160) phagocytosing polymorphonuclear leukocytes during the respiratory burst (113) and the hemolysis of red blood cells (161). Two model $^{\bullet}OH$-generating systems namely Fenton's reagent and the iron-catalyzed oxidation of ascorbic

acid also produced methane from Me_2SO (160). However, previous investigations with Me_2SO, methional and KTBA as $^{\bullet}OH$ scavengers have demonstrated that the production of methane from Me_2SO is at least an order of magnitude less than the generation of ethylene gas from either methional or KTBA when these scavengers were used as substrates during microsomal electron transfer or for model $^{\bullet}OH$-generating systems (111, 160). Therefore, the detection of methane gas in these systems is limited by the small amounts which are generated.

One of the goals of this dissertation research is to demonstrate and to characterize the production of formaldehyde from Me_2SO by $^{\bullet}OH$-generating systems. The initial observation of formaldehyde production from Me_2SO was made during studies of the microsomal oxidation of methanol (169). It is known that alcohols can be oxidized to their respective aldehydes as a result of their interaction of $^{\bullet}OH$ (125). It has been suggested that $^{\bullet}OH$ plays a role in the oxidation of alcohols by liver microsomes (169-173). During a study of the effect of $^{\bullet}OH$ scavengers on methanol oxidation by microsomes, it was observed that Me_2SO produced formaldehyde even in the absence of methanol. Therefore, in the current study the ability of Me_2SO to be oxidized to formaldehyde by three different $^{\bullet}OH$-generating

systems was characterized. These systems were: 1) the oxidation of xanthine by xanthine oxidase, an enzymatic $^{\bullet}OH$-generating system (174,175); 2) the iron-catalyzed oxidation of ascorbic acid, a chemical $^{\bullet}OH$-generating system (175) and 3) NADPH-dependent rat liver microsomal electron transfer, a biological membrane $^{\bullet}OH$-generating system (175 and Klein, S.M., G. Cohen and A.I. Cederbaum, manuscript in preparation). In all three systems the production of formaldehyde from Me_2SO was shown to be dependent upon interaction with $^{\bullet}OH$. An important observation was that in all three of these $^{\bullet}OH$-generating systems, the yield of formaldehyde was much greater than the yield of methane. Therefore, the production of formaldehyde from the interaction of Me_2SO with $^{\bullet}OH$ may be used as a simple and convenient tool by which to detect the presence of $^{\bullet}OH$ in biological systems.

1.6. The Microsomal Ethanol Oxidizing System

1.6.1 Background

The liver represents the main site for the oxidation of ethanol. The activity of the major enzyme systems which are associated with ethanol metabolism

are highest in the liver. Extrahepatic ethanol oxidation usually does not exceed 15% (176). The main pathway of ethanol metabolism in the liver is via the cytosolic enzyme, alcohol dehydrogenase (177). However, a minor microsomal pathway for ethanol metabolism was also found to exist. In 1965, Orme-Johnson and Ziegler described a microsomal system which was capable of the oxidation of methanol and ethanol (178). Subsequent work by Lieber and DeCarli led to the characterization of the microsomal ethanol oxidizing system (MEOS) (179, 180). This system was found to require molecular oxygen and NADPH for its activity and was partially inhibited by carbon monoxide. In these respects, MEOS resembles the mixed function oxidase system which is responsible for the metabolism of many drugs. The stoichiometry of the overall reaction of MEOS is represented in the following equation:

$$CH_3CH_2OH+NADPH+H^+ + O_2 \longrightarrow CH_3CHO + NADP^+ + 2H_2O. \quad (15)$$

MEOS was differentiated from alcohol dehydrogenase on the basis of subcellular compartmentation, cofactor requirements, pH optimum, effect of pyrazole and Km value for ethanol (179, 180). However, differentiation of MEOS from the peroxidatic activity of catalase has

been the subject of much controversy. Catalase is found as a contaminant in isolated microsomes. Since microsomes are capable of generating H_2O_2 during NADPH-dependent microsomal electron transfer (98, 181), it has been suggested that the oxidation of ethanol by microsomes represents the combined activities of microsomal NADPH oxidation and of the peroxidatic activity of contaminating catalase (182, 183). MEOS has now been differentiated from catalase by various studies utilizing inhibitors of catalase (179, 180), by physical separation of MEOS from catalase via column chromatography (184, 185), and by studies involving alcohols which do not serve as substrates for the peroxidatic activity of catalase e.g. 1-butanol (184, 185) and isopropanol (171). Moreover, the oxidation of ethanol has been observed in reconstituted systems containing purified cytochrome P-450, NADPH-cytochrome c reductase and phospholipids. These systems contain neither alcohol dehydrogenase nor catalase (186, 187).

At low concentrations of ethanol, the contribution of MEOS to ethanol oxidation is approximately 10% (188). However, as the concentration of ethanol is elevated, the rate of ethanol oxidation is increased and the contribution of MEOS towards ethanol oxidation increases (188). It has been estimated that at 50 mM ethanol,

MEOS, because of its high Km for ethanol, may become responsible for up to 50% of the total ethanol metabolism (188). In addition to playing an increasingly important role in ethanol metabolism at higher concentrations of ethanol, the activity of MEOS was found to increase after chronic ethanol consumption (179, 180). It has been demonstrated that chronic administration of ethanol results in an increased *in vivo* and *in vitro* rate of ethanol metabolism (189-192). Part of this metabolic tolerance has been attributed to an adaptive increase in the activity of MEOS (189). Chronic ethanol feeding has been found to result in an induction of a specific cytochrome P-450 hemoprotein which is distinct from that induced by phenobarbital or by 3-methylcholanthrene(187, 188). This partially purified cytochrome P-450 was more active for ethanol oxidation than cytochrome P-450 obtained from control animals (187).

The significance of MEOS assumes additional importance with respect to its affect on and interaction with microsomal drug metabolism. The NADPH-mediated microsomal oxidation of ethanol is believed to involve, at least in part, some of the components which participate in the mixed function oxidase pathway of microsomal drug oxidation, namely, NADPH-cytochrome P-450 reductase and cytochrome P-450 (179, 180). In the presence of ethanol,

drug metabolism is depressed because ethanol competes with drugs for oxidation by cytochrome P-450 (193). However, after chronic ethanol consumption, proliferation of the endoplasmic reticulum occurs and there is an increase in the microsomal content of cytochrome P-450 (193). Consequently, the rate of drug metabolism in chronic alcoholics is increased when ethanol is not present to compete with the drug for oxidation.

1.6.2 Role of Oxygen Radicals in MEOS

In the preceding section it was pointed out that MEOS, which is a normal, although minor pathway of ethanol metabolism, becomes especially important with respect to the metabolism of high concentrations of ethanol, after chronic ethanol consumption and in relation to microsomal drug metabolism. However, the molecular mechanism of MEOS remains to be elucidated.

Recent studies in this laboratory have indicated that microsomes are capable of generating $^{\bullet}OH$ during NADPH-dependent electron transfer. Typical scavengers of $^{\bullet}OH$ such as methional, KTBA, Me_2SO, tertiary-butyl alcohol or benzoate were oxidized by liver microsomes to products which are known to result from the interaction of these scavengers with $^{\bullet}OH$ (111, 160, 173, G. Winston and A.I. Cederbaum, manuscript in press). These ob-

servations suggested the possibility that the molecular mechanism which is responsible for microsomal oxidation of alcohols may be due, at least in part, to the ability of alcohols to scavenge $^{\bullet}OH$ radicals that are generated during microsomal electron transfer. Microsomal oxidation of alcohols was inhibited by a variety of $^{\bullet}OH$ scavenging agents including mannitol, benzoate,dimethylsulfoxide and DMPO, (160, 170, 172, 194). This inhibition was competitive with respect to ethanol suggesting competition between the scavengers and alcohol for oxidation. The external addition of iron-EDTA, which is known to increase the production of $^{\bullet}OH$ in biological systems (120-123), also increased the oxidation of ethanol and 1-butanol (124). The inhibition by the $^{\bullet}OH$ scavenging agents was specific since these agents had no effect on catalase-dependent oxidation of ethanol, on microsomal drug metabolism (aminopyrine demethylation or aniline hydroxylation) or on microsomal electron transfer (170, 172, 194). These latter results tend to dissociate the mechanism of MEOS from that of microsomal drug metabolism. It has therefore been concluded that the $^{\bullet}OH$ radical is responsible, at least in part, for the ability of liver microsomes to oxidize ethanol (124, 160, 170-172, 194, Winston, G. and A. I. Cederbaum, manuscript in press).

This conclusion raises the possibility that the increased rate of microsomal ethanol oxidation in response to chronic ethanol consumption may reflect an increase in $^{\bullet}OH$ generation by these microsomes. Therefore, a second major goal of this dissertation research was to test this hypothesis by studying the metabolism of the $^{\bullet}OH$ probe which was developed in the earlier part of this work, namely, the production of formaldehyde from Me_2SO as catalyzed by microsomes from ethanol-fed rats and their pair-fed controls. The results of the studies to further illucidate the mechanism of MEOS and to study factors which are responsible for the increase in MEOS activity after chronic ethanol consumption will also be presented in this dissertation.

1.7. Introduction and Goals of Dissertation Research

The hydroxyl radical appears to be generated by many biological reaction systems (108-113). This radical is a powerful oxidizing agent which reacts readily with many biological compounds (125, 126), and is therefore highly toxic in biological systems (26, 112, 127-138).

In view of these considerations, the ability to detect hydroxyl radicals has become important. There are limitations in the use of many of the probes (110, 111, 120, 151-168) which are currently used to detect hydroxyl radicals.

Dimethylsulfoxide is a potent hydroxyl radical scavenging agent (125, 159). The production of methane gas from dimethylsulfoxide has been used to detect the generation of hydroxyl radicals by several biological systems (111, 113, 160, 161). However, it has been observed that when dimethylsulfoxide, methional and 2-keto-4-thiomethylbutyric acid are used as hydroxyl radical scavenging agent, the production of methane from dimethylsulfoxide is at least an order of magnitude less than the generation of ethylene from either methional or 2-keto-4-thiomethylbutyric acid under the same experimental conditions (111, 160). Therefore, the detection of methane gas in these systems is limited by the

small amounts which are generated.

One of the major goals of this dissertation research was to demonstrate and to characterize the generation of another product from the interaction of dimethylsulfoxide with hydroxyl radicals. This product is formaldehyde. Three model hydroxyl radical generating systems were used to generate $^{\bullet}OH$. They were 1) the oxidation of xanthine by xanthine oxidase, 2) the iron-catalyzed oxidation of ascorbic acid and 3) NADPH-dependent electron transfer by rat liver microsomes. Formaldehyde was produced in a time-dependent manner by all three systems and was inhibited by competing hydroxyl radical scavenging agents. In all cases, formaldehyde, rather than methane, represented a major product of the interaction of dimethylsulfoxide with hydroxyl radicals.

A second major aim of this dissertation research was to apply this tool to the determination of the role of hydroxyl radicals in the molecular mechanism of the microsomal ethanol oxidizing system. This system, which is a normal, although a minor pathway of ethanol metabolism, becomes important with respect to the metabolism of high concentrations of ethanol, after chronic ethanol consumption, and in relation to the interaction of ethanol with microsomal drug metabolism (179, 180,

187-193). It has been suggested that the microsomal ethanol oxidizing system may reflect the ability of ethanol to scavenge hydroxyl radicals which are generated during NADPH-dependent electron transfer (111, 124, 160, 170, 172, 172, 194).

As a result of chronic ethanol consumption, the ability of microsomes to oxidize ethanol is increased. In this investigation, it was proposed that the increased rate of ethanol oxidation found after chronic ethanol consumption may be due to an increased rate of hydroxyl radical production by the microsomes obtained from chronic ethanol-fed rats. It was observed that rat liver microsomes from chronic ethanol-fed rats produced formaldehyde from dimethylsulfoxide,as well as ethylene from 2-keto-4-thiomethylbutyric acid,at rates which were two-to-three-fold greater than microsomes from pair-fed controls. The increased rate of hydroxyl radical production correlated with the increased rate of ethanol metabolism. Cross competition for oxidation among ethanol, dimethylsulfoxide and 2-keto-4-thiomethylbutyric acid was observed. Thus increased production of hydroxyl radicals may contribute to the increase in ethanol oxidation by microsomes from chronic ethanol-fed rats. This increased rate of hydroxyl radical production may play a role in, or contribute to, the cytoxic

damage of the liver cell which is found after chronic ethanol consumption.

The detection of formaldehyde from Me_2SO appears to represent a new, sensitive and convenient technique for the detection of $^{\bullet}OH$ which may be useful in evaluating the role of hydroxyl radicals in biochemical reaction systems.

CHAPTER 2

Materials and Methods

2.1. The Xanthine-Xanthine Oxidase Model Hydroxyl Radical Generation System.

The oxidation of xanthine by xanthine oxidase was used as a model $^{\bullet}OH$-generating system (109, 110). The standard reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.4, 0.4 mM xanthine, 0.1 mM tetrasodium EDTA, 0.018 units of xanthine oxidase (in 20 mM potassium phosphate buffer) and 33 mM Me_2SO (unless otherwise indicated) in a final volume of 3.0 ml. All incubations were carried out in triplicate in a Dubnoff metabolic shaking incubator at 37°C. The reactions were initiated by the addition of xanthine oxidase and were terminated by the addition of 1.0 ml. ice-cold 17.5% (w/v) trichloroacetic acid. The production of formaldehyde was assayed by mixing a 1.5 ml aliquot of the terminated reaction mixture with 1.5 ml of Nash reagent. Nash reagent is a solution which consists of 3M ammonium acetate, 0.05 M acetic acid and 0.02 M acetylacetone. The samples were then incubated in the dark at 60°C for ten (10) minutes. In view of the small amounts of formaldehyde

which were made by the xanthine-xanthine oxidase system (as compared to other systems - see Results) a more sensitive fluorometric modification (195) of the method of Nash (196) was used. A Perkin-Elmer fluorescence spectrophotometer (model 650-10S) was used at an excitation wavelength of 415 nm and at an emission wavelength of 505 nm. It was necessary to construct a standard curve with known amounts of formaldehyde for each experimental condition in order to compensate for the effect of various constituents on the fluorescence emission intensity. For example, hydrogen peroxide quenched the flourescence emission intensity. Hence when experiments with H_2O_2 were conducted, standard curves of formaldehyde in H_2O_2 were constructed. Since the samples are light sensitive, all fluorescent measurements were made under dim illumination to minimize decay of the fluorescent emission intensity. Control experiments verified identical scanning patterns with a maximum excitation wavelength at 415 nm and maximum emission wavelength at 505 nm for both standards and experimental samples. Evidence that an aldehyde was produced from Me_2SO was provided by the observation that the contents of the experimental flasks promoted the oxidation of NADH in the presence of alcohol dehydrogenase. In some experiments iron-EDTA was added as a

1:2 mixture of ferrous ammonium sulfate in aqueous tetrasodium EDTA.

2.2. The Ascorbic Acid-Iron-EDTA Model Hydroxyl Radical Generating System

The iron-catalyzed oxidation of ascorbic acid was used as a second model $^{\bullet}OH$-generating system (108, 109). The standard reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, a mixture of 167 uM iron-333uM EDTA, 0.1 mM, EDTA 2mM ascorbic acid and 33mM Me_2SO in a final volume of 3.0 ml. All incubations were carried out in duplicate in a Dubnoff metabolic shaking incubator at 37°C. The reactions were initiated by the addition of ascorbic acid. At time intervals, 1.0 ml of ice-cold 17.5% (w/v) trichloroacetic acid was added and a 1.5 ml aliquot was assayed for formaldehyde by the spectrophotometric method of Nash (196). The optical density was measured in a Gilford spectrophotometer (model 240) at 415 nm. An extinction coefficient of 8.0 $cm^{-1}mM^{-1}$ was used to calculate the amount of formaldehyde which was produced.

2.3. The Generation of Hydroxyl Radicals by Rat Liver Microsomes During NADPH-Dependent Electron Transfer.

Liver microsomes were prepared from male Sprague-

Dawley rats by standard techniques in our laboratory as has been previously described (197). The rats were stunned by a blow to the head, killed by decapitation and bled from the neck. The livers were excised, weighed and rinsed with an ice-cold solution of 0.25M sucrose containing 0.01 M Tris-HCl, pH 7.4 and 0.001 M EDTA. The livers were minced with a pair of stainless steel scissors and were rinsed off once again with the sucrose solution to remove residual blood from the tissues. The livers were then homogenized in the Sucrose-Tris-EDTA buffer by three passes with a Potter-Elvehjim homogenizer consisting of a teflon pestle and a glass homogenizing vessel. The resulting homogenate was subsequently adjusted to achieve a one to ten dilution (gm wet weight of livers/volume of Sucrose-Tris-EDTA). The microsomes were then obtained by differential centrifugation. The homogenates were first spun at 750xg for ten (10) minutes (2,500 RPM) in a Sorvall RC-2 or RC-5 centrifuge (equipped with a SS-34 rotor) to remove cellular debris and nuclei. The pellets were discarded and the supernatant was spun at 8,700 xg for ten (10) minutes (8,500 RPM) to remove mitochondria. The resulting supernatant was spun once again at 19,000xg for five (5) minutes (12,500 RPM) to remove the lysosomal pellet. The supernatant from this spin was then spun at 100,000xg for one hour (36,000 RPM) in a Beckman Model L ultracen-

trifuge (equipped with either a Type 42.1 or a Titanium 60 rotor) to isolate the microsomal fraction. The microsomes were washed once in 125 mM KCl at 100,000xg in the ultracentrifuge and were finally resuspended in 125 mM potassium chloride with an all glass homogenizer. Protein was determined by the method of Lowry et al (198).

The standard reaction mixture for the assay of the metabolism of Me_2SO consisted of 83 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 10 mM potassium pyrophosphate, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 10mM glucose-6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 3 to 5 mg micosomal protein and 33 mM Me_2SO in a final volume of 3.0 ml. Unless otherwise indicated, azide was present at a final concentration of 1.0 mM. All reactions were carried out in duplicate in a Dubnoff metabolic shaking incubator at 37^oC. The reaction was initiated by the addition of a mixture of glucose-6-phosphate and glucose-6-phosphate dehydrogenase and was terminated, usually after 30 minutes, by the addition of 1.0 ml of ice-cold 17.5% (w/v) trichloroacetic acid. When KTBA was used as the substrate, in place of Me_2SO, 0.3 ml of perchloric acid (70% w/v) was used to stop the reaction. The samples were then spun in a clinical centrifuge to remove microsomal protein. A 1.5 ml aliquot of the supernatant was assayed for formaldehyde according to the spectrophotometric

method of Nash (196), an extinction coefficient of 8.0 cm^{-1} mM^{-1} was used to calculate the amount of formaldehyde which was produced. In some experiments, iron-EDTA was added as a 1:2 mixture of ferrous ammonium sulfate in aqueous tetrasodium EDTA.

The production of formaldehyde from aminopyrine (microsomal aminopyrine demethylase activity) was assayed in the same reaction mixture except that aminopyrine (10mM) was substituted for Me_2SO. All other steps were identical to those which were described above for Me_2SO. Cumene hydroperoxide (final concentration of 0.5mM) and tertiary-butyl hydroperoxide (final concentration of 2.5 mM) were used in some experiments which were concerned with the ability of organic hydroperoxides to support the production of formaldehyde from either Me_2SO or aminopyrine. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were omitted from the reaction mixture for these experiments. The reactions were initiated by the addition of the organic hydroperoxide to flasks which already contained the reaction mix, substrate and microsomes. To study the effect of carbon monoxide on the production of formaldehyde from either Me_2SO or aminopyrine, pure carbon monoxide was bubbled for twenty (20) seconds into flasks which contained reaction mixture, substrate and microsomes. The

flasks were then sealed with serum caps and the reaction was started by the addition of NADPH-generating system through the serum caps. Pure nitrogen was bubbled into other flasks to serve as a control for anaerobiosis which may have been produced by the pure carbon monoxide.

2.4. Method for the Measurement of Hydrocarbon Gases

In some experiments, both formaldehyde and methane were measured in the same flasks in order to compare the yields of each product from Me_2SO. In these experiments, the flasks were sealed with serum caps instead of stoppers. After terminating the reaction with trichloroacetic acid, an aliquot of the head space (0.5 ml) from each flask was removed with a gas-tight, 1-ml plastic and rubber syringe equipped with a side-venting needle. To ensure that the gas phase was thoroughly mixed before each sample was removed, the syringe plunger was drawn back and forth twelve times before sampling. Methane, which was produced from the metabolism of Me_2SO, was measured by injecting a 0.5 ml aliquot of the head space directly into a Hewlett-Packard model 5750 gas (for the early experiments) or a Hewlett-Packard 5840A gas chromatograph (for the recent experiments). The gas chromatograph was equipped with a six-foot column of Poropak N (50-80 mesh) and a flame ionization detector. Operating

conditions were as follows: column temperature 60°C, inlet temperature 190°C, detector temperature 190°C, helium or nitrogen (carrier) flow rate 30 ml/minute, air flow rate 250 ml/minute, and hydrogen flow rate 25 ml/minute. The retention time for methane under these conditions was 0.5 minutes. After the assay for methane the flasks were opened, and aliquots were removed for the measurement of formaldehyde.

In some experiments the production of ethylene from 10mM KTBA, or of ethane from Me_2SO, were also assayed by head space chromatography. The operating conditions of the gas chromatograph were identical to those which were described for methane detection. The retention time for ethylene and ethane under these conditions were 1.7 minutes and 2.3 minutes, respectively.

2.5. Measurement of Carbon Monoxide and Substrate Binding Spectra

The carbon monoxide difference spectra of the liver microsomes was determined by the method of Omura and Sato (199) with a Perkin-Elmer model 554-dual beam spectrophotometer. Microsomes were suspended in 0.1M potassium phosphate buffer, pH 7.4, containing 20% glycerol. An equal amount of the preparation was placed in the reference and sample cuvette of the spectrophotometer. A few

crystals of dithionite were added to both cuvettes. After the baseline was read, pure carbon monoxide was gently bubbled through the sample cuvette for thirty seconds and the carbon monoxide difference spectra was taken over the wavelength range of 500 nm to 350 nm. The substrate binding spectra were determined by the method of Peterson et al (200). Microsomes were suspended in 0.1 M phosphate buffer, pH 7.4, containing 20% glycerol. Both the reference and the sample cuvettes contained the microsomes. Upon the addition of either 18 mM aniline (Type II substrate) or 5 mM hexobarbital (Type I substrate) or 33 mM to 133 mM Me_2SO, the difference spectra was recorded over the wavelength range of 500 nm to 350 nm.

2.6. Generation of Alkoxy Radicals

Alkoxy ($^{\bullet}OR$) radicals were generated from organic hydroperoxides via a Fenton-type reaction between organic hydroperoxides and ferrous iron. The organic hydroperoxides which were used were cumene hydroperoxide (2.5 mM) and tertiary-butyl hydroperoxide (2.5mM). Since ferrous iron has a half-life of only 15 seconds in phosphate buffer, it is necessary to stabilize and to maintain the iron in the ferrous state, i.e., to

prevent its oxidation to the ferric state. This was accomplished by the method of Cohen et al (201). The basic reaction mixture consisted of 50 mM potassium phosphate buffer to which 1.2 mM diethylenetriaminepentaacetic acid (DETAPAC) (an iron-chelating agent) was added. After these were thoroughly mixed, 1.0 mM ferrous ammonium sulfate was added and the solution was vigorously mixed once again. To this mixture either Me_2SO (33 mM) or KTBA (10 mM) were added as substrate. Finally either hydrogen peroxide (3 mM) or cumene hydroperoxide (2.5 mM) or tertiary-butyl hydroperoxide (2.5 mM) were added to start the reaction by generating either $^{\bullet}OH$, the cumene alkoxy radical or the tertiary-butyl alkoxy radical, respectively. Reactions were carried out at room temperature. The generation of methane or ethane from Me_2SO or of ethylene from KTBA were measured by gas chromatography.

2.7. Hydroxyl Radical Production and Ethanol Oxidation by Microsomes from Chronic Ethanol-Fed Rats and Their Pair-Fed Controls.

Chronic ethanol-fed rats and their pair-fed controls were obtained from Dr. Charles S. Lieber at

the Bronx Veterans Administration Hospital. , These animals were housed and fed at that facility. Ethanol was incorporated into a nutritionally adequate liquid diet to the extent of 36% of total calories (202). The control animals were pair-fed the same diet in which ethanol had been isocalorically replaced by dextrose. The ethanol-containing diet consisted of 36% of calories as ethanol, 11% as carbohydrate, 18% as protein and 35% as fat. Each day, the amount of diet which was consumed by the ethanol-fed rats was recorded and an identical amount of control diet was fed to the pair-fed control rats. The rats gain weight under these conditions (202). At the end of four to five weeks, the chronic ethanol-fed rats serve as a good model for the chronic alcoholic state since they exhibit some of the characteristics which are associated with chronic alcoholism in humans, e.g., fatty livers, physical dependence and metabolic tolerance to ethanol.

Liver microsomes were prepared from either the chronic ethanol-fed rats or their pair-fed controls, washed once and were resuspended in 125 mM KCl as was described in a previous section (2.3.). The standard reaction mixture was described in section 2.3. The

substrates which were studied were either 10 mM KTBA or 50 mM ethanol or 33 mM Me_2SO. The microsomal protein concentrations were adjusted before each experiment so that the final protein concentrations of the microsomes from the ethanol-fed rats and the pair-fed controls were identical. Unless otherwise indicated, azide was present at a final concentration of 1.0 mM. Reactions were initiated by the addition of the NADPH-generating system and were terminated by the addition of either 1.0 ml of ice-cold 17.5% (w/v) trichloroacetic acid (Me_2SO or ethanol as substrate) or 0.3 ml of 70% (w/v) perchloric acid (KTBA as substrate). Formaldehyde production was measured by the method of Nash as described in section 2.3. The generation of ethylene from KTBA was assayed by head space chromatography using either a Hewlett-Packard 5750 or 5840A gas chromatograph as described in section 2.4. Ethanol oxidation was determined by trapping the acetaldehyde in center-wells which contained 0.6 ml of 15 mM semicarbizide in 180 mM phosphate buffer, pH 7.4. After an overnight diffusion period, the optical density of the acetaldehyde-semicarbizone complex was determined at 224 nm. The

extinction coefficient for acetaldehyde in this system is 9.4 $cm^{-1}mM^{-1}$.

2.8. Materials

All materials were of the highest available grade from commercial sources. Materials were obtained from the following sources: DETAPAC and sodium KTBA, Sigma Chemical Company (St. Louis, MO); Xanthine, Eastman Kodak Company (Rochester, NY); xanthine oxidase (0.45 units/mg protein) catalase (65,000 units/mg protein), glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$, Boehringer Mannheim (Indianapolis, IN); Superoxide dismutase, Biotics Research Corporation (Houston, TX); Chelex-100 resin (200-400 mesh), Biorad Laboratories (Richmond, CA); Cumene hydroperoxide, ICN Pharmaceuticals (Plainview, NY); tertiary-butyl hydroperoxide and metyrapone, Aldrich Chemical Company (Milwaukee, WI); and SKF-525A, Smith, Kline and French (Philadelphia, PA). All gases were zero grade and were obtained from T.W. Smith (Brooklyn, NY). The air-tight syringes which were used for the gas chromatography and the side-venting needles were obtained from Becton-Dickinson (Rutherford, NJ) and Supelco, Inc. (Bellefonte, PA) respectively. Rats for the microsomal experiments

were obtained from Zivic-Miller (Allison, PA).

Water for the preparation of all solutions was purified by passage through a Millipore system. This water was subsequently distilled in an all glass apparatus. To remove extraneous iron, the water, the potassium phosphate, the pyrophosphate and the potassium chloride solutions were passed through a Kontes column (35 x 3 cm) which was packed with Chelex-100 resin.

2.9. Statistics

All values refer to the mean ± S.E.M. Statistical analyses were performed by Student's t-test. The number of experiments is indicated on the table or in the figure legends.

CHAPTER 3

RESULTS

3.1. The Xanthine-Xanthine Oxidase Model Hydroxyl Radical Generating System

3.1.1. The Generation of Formaldehyde from Dimethylsulfoxide During the Oxidation of Xanthine by Xanthine Oxidase

During the oxidation of xanthine by xanthine oxidase, $O_2^{\cdot-}$, H_2O_2 and $^{\bullet}OH$ are produced (110). The production of $^{\bullet}OH$ probably represents the interaction of $O_2^{\cdot-}$ and H_2O_2 in an iron-catalyzed Haber - Weiss reaction (120).

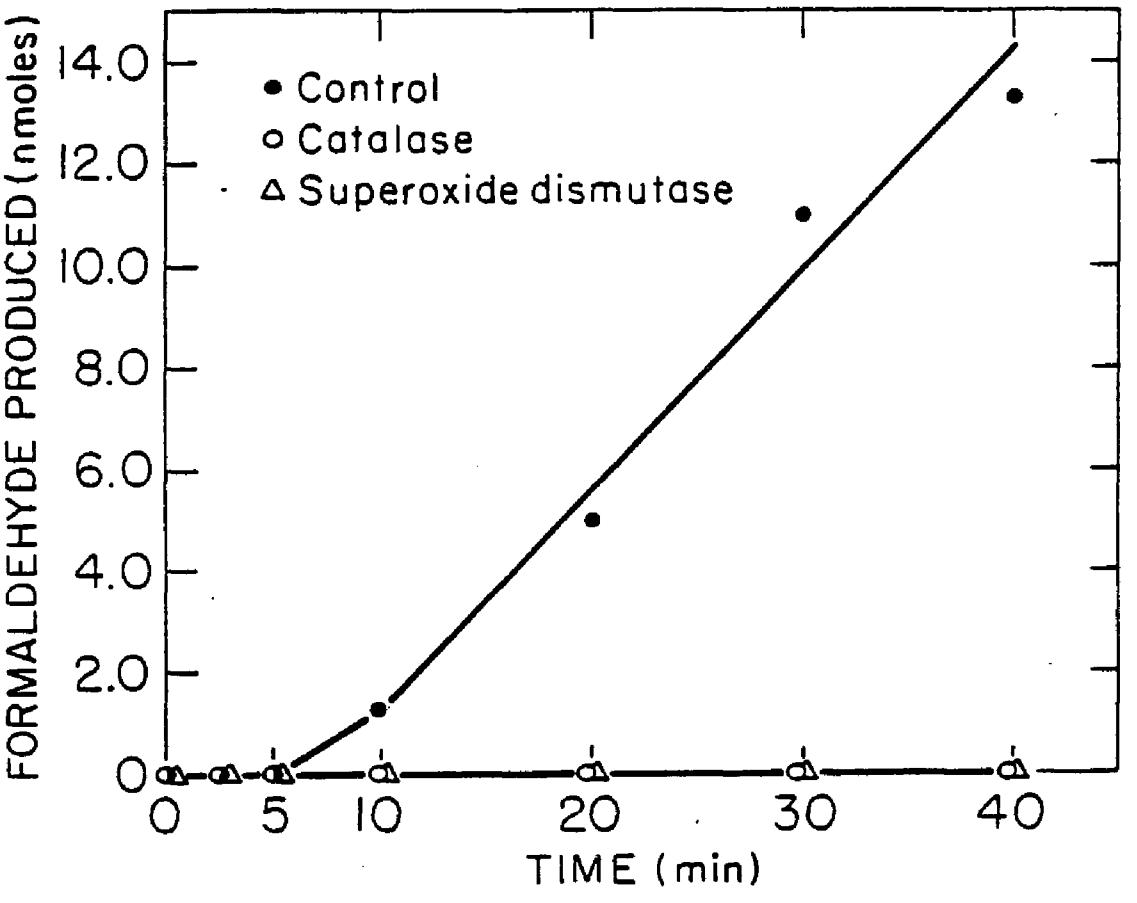
$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe} {}^{\bullet}OH + OH^- + O_2 \qquad (16).$$

Figure 4 illustrates the time course of the production of formaldehyde from Me_2SO during the oxidation of xanthine by xanthine oxidase. After an initial lag period of approximately five (5) minutes, the rate of formaldehyde production was linear for at least an additional thirty-five minutes. The lag period is generally considered to represent the amount of time which is required for the accumulation of sufficient H_2O_2 to serve as a precursor for $^{\bullet}OH$ (120, see below). In control experiments, formal-

Figure 4.

The Production of Formaldehyde from Dimethylsulfoxide During the Oxidation of Xanthine by Xanthine Oxidase and the Effects of Superoxide Dismutase and Catalase.

The standard reaction mixture consisted of 50mM potassium phosphate buffer, pH 7.4, 0.4 mM xanthine, 0.1 mM tetrasodium EDTA, 0.018 units of xanthine oxidase and 33 mM Me_2SO in a final volume of 3.0 ml. Superoxide dismutase and catalase were added at zero time to final concentrations of 58 µg/ml and 67µg/ml. respectively. The results of a typical experiment are presented.


FORMALDEHYDE PRODUCED (nmoles)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
• Control
o Catalase
Δ Superoxide dismutase
0
5
10
20
30
40
TIME (min)

dehyde was not produced when either Me_2SO or xanthine or xanthine oxidase was omitted from the reaction mixture (Table 1). Formaldehyde was not produced when H_2O_2 (0.4 mM) was added to the reaction mixture in the absence of either xanthine or xanthine oxidase (Table 1). Therefore, formaldehyde production does not occur as a result of a direct reaction between Me_2SO and H_2O_2.

Figure 4 also illustrates the effect of the addition of either catalase (which removes H_2O_2) or superoxide dismutase (which removes $O_2^{\cdot-}$) to the reaction mixture at zero time. The production of formaldehyde was completely inhibited by either enzyme. These results demonstrate a requirement for both $O_2^{\cdot-}$ and H_2O_2 in the production of formaldehyde by this system. The addition of either enzyme ten (10) minutes after the initiation of the reaction, instead of at zero time, prevented further accumulation of formaldehyde after that time (data not shown). In contrast, bovine serum albumin, which was used as an inert protein control, had no effect on the generation of formaldehyde (Table 1).

Although formaldehyde production does not occur as a result of a direct interaction between Me_2SO and H_2O_2, the addition of H_2O_2 to the complete reaction mixture stimulated the rate of formaldehyde production (Figure 5). Since H_2O_2 is a precursor of $^{\bullet}OH$, the increased

TABLE 1

Control Experiments for the Production of Formaldehyde From Dimethylsulfoxide During the Oxidation Of Xanthine by Xanthine Oxidase.

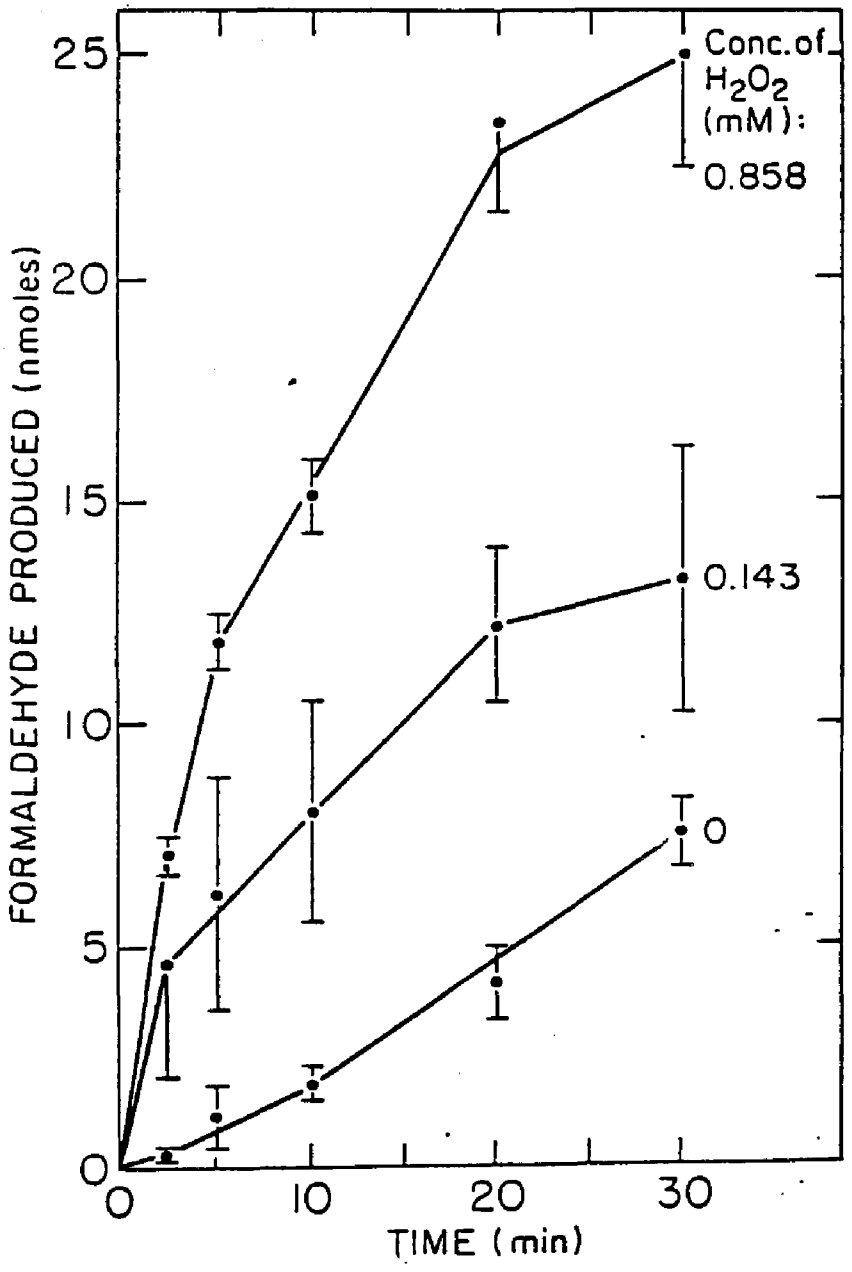
COMPONENTS	Formaldehyde Produced (30 Minutes)
	n Moles
Complete Reaction Mixture	9
Dimethylsulfoxide Omitted	0
Xanthine Omitted	0
Xanthine Oxidase Omitted	0
Xanthine Omitted, 0.4mM H_2O_2 Added	0
Xanthine Oxidase Omitted, 0.4mM H_2O_2 Added	0
Superoxide Dismutase Added	0
Catalase Added	0
Bovine Serum Albumin Added	7.2

[a]The standard reaction mixture is described in the legend to Figure 4. Results are from a typical experiment.

Figure 5

The Effect of the Addition of Hydrogen Peroxide on the Generation of Formaldehyde from Dimethylsulfoxide during the Oxidation of Xanthine by Xanthine Oxidase.

The standard reaction mixture is described in the legend to Figure 4. H_2O_2 was added to achieve the final concentrations which are indicated in the figure. The results represent the mean$\pm$ S.E.M. from three experiments.


25
20
15
10
5
0
0
10
20
30
TIME (min)
FORMALDEHYDE PRODUCED (nmoles)
Conc.of
H_2O_2
(mM):
0.858
0.143
0

rate of production of formaldehyde from Me_2SO may reflect increased production of $^{\bullet}OH$ in the presence of H_2O_2. External addition of H_2O_2 overcame the lag period for the production of formaldehyde, further suggesting that the generation of H_2O_2 is rate limiting for the production of $^{\bullet}OH$.

The concentrations of xanthine, xanthine oxidase and Me_2SO were varied in order to determine optimal conditions for the production of formaldehyde. A reaction period of thirty minutes was used in these experiments. The production of formaldehyde reached a maximum rate at approximately 0.4 mM xanthine and above 1.25 mM Me_2SO and was dependent upon the concentration of xanthine oxidase (Figure 6).

3.1.2. The Effect of Hydroxyl Radical Scavenging Agents

The results which were shown in Figure 4, indicated that the production of formaldehyde from Me_2SO was completely inhibited by either catalase or by superoxide dismutase. These results demonstrate that neither H_2O_2 nor $O_2^{\bullet-}$ radicals alone have the ability to produce formaldehyde from Me_2SO. The effect of $^{\bullet}OH$ scavenging agents on the production of formaldehyde was evaluated. The $^{\bullet}OH$ scavenging agents and their rate constants (10^9 $M^{-1}sec^{-1}$) for interaction with $^{\bullet}OH$ are: Me_2SO, K=7; ethanol K=1.8;

Figure 6.

Titrations of Formaldehyde Production versus the Concentration of Xanthine, Xanthine Oxidase and of Dimethylsulfoxide.

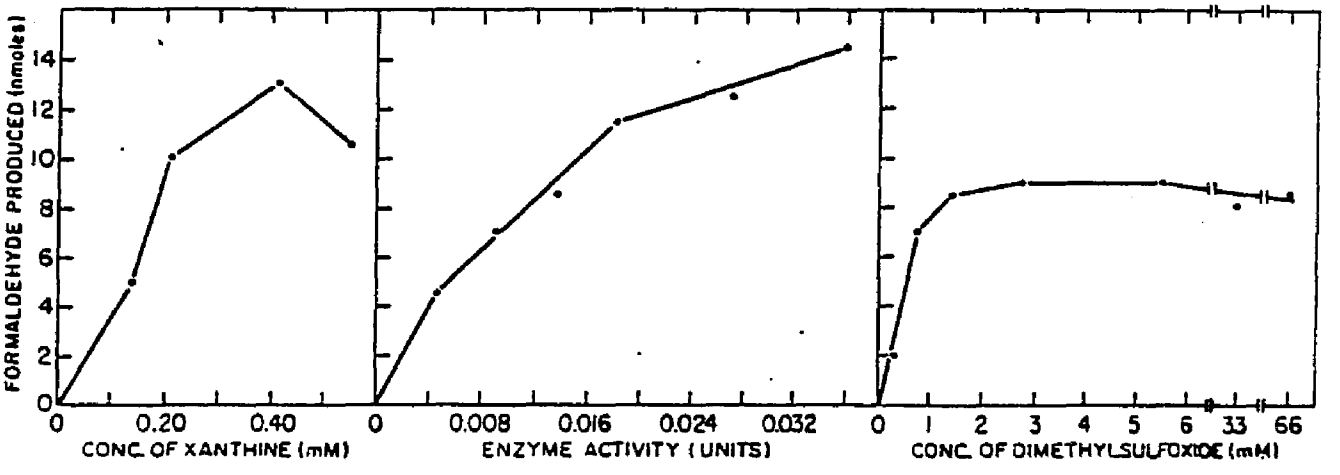
The standard reaction mixture is described in the legend to Figure 4. The modifications of the standard reaction mix are as follows:

6A. The concentration of xanthine was varied from zero to 0.55 mM, while the concentrations of xanthine oxidase and Me_2SO were held constant at 0.018 units and 33mM respectively.

6B. The concentration of xanthine oxidase was varied from zero to 0.036 units while the concentrations of xanthine and Me_2SO were held constant at 0.4 mM and 33 mM respectively.

6C. The concentration of Me_2SO was varied from zero to 66 mM while the concentrations of xanthine oxidase and xanthine were held constant at 0.018 units and 0.4 mM, respectively.

Results are from a typical titration experiment.


FORMALDEHYDE PRODUCED (nmoles)
0
2
4
6
8
10
12
14
0
0.20
0.40
CONC. OF XANTHINE (mM)
0
0.008
0.016
0.024
0.032
ENZYME ACTIVITY (UNITS)
0
1
2
3
4
5
6
33
66
CONC. OF DIMETHYLSULFOXIDE (mM)

butanol, K=3.7; mannitol, K≅1 (125). Two concentrations of Me_2SO, 33 and 3.3 mM, were used. All three $^{\bullet}OH$ scavenging agents suppressed the production of formaldehyde in a dose-dependent manner (Table 2). The extent of inhibition was greater with 3.3 mM Me_2SO than with 33 mM Me_2SO as the substrate (Table 2). The fact that the competing scavengers are more inhibitory against a lower concentration of Me_2SO than against a higher concentration suggests that the scavengers are competing with Me_2SO for the generated $^{\bullet}OH$. Urea, a relatively weak scavenger of $^{\bullet}OH$ ($k= 7 \times 10^5 M^{-1} sec^{-1}$) (159) was used as a negative control in these experiments. Urea did not inhibit formaldehyde production (Table 2).

3.1.3. The Effect of Iron-EDTA

Iron-EDTA is known to stimulate the generation of $^{\bullet}OH$ during the oxidation of xanthine by xanthine oxidase (120-122). This stimulation probably reflects the ability of iron to catalyze the Haber-Weiss reaction. Increased generation of $^{\bullet}OH$ should result in an increase in formaldehyde production. The addition of iron-EDTA stimulated the production of formaldehyde from Me_2SO (Fig. 7). In control experiments, formaldehyde was not produced in the presence of iron-EDTA when either xanthine or xanthine oxidase was omitted from the reaction mixture (Table 3).

TABLE 2

The Effect of Hydroxyl Radical Scavenging Agents on the Production of Formaldehyde from 3.3 mM and 33 mM Dimethylsulfoxide.[a]

Addition	Concentration of Scavenger	Rate of Formaldehyde Production		Effect of Scavenger	
		Concentration of Dimethylsulfoxide			
	(mM)	3.3 mM (nmol/30 min)	33 mM	3.3 mM (%)	33 mM
A. Butanol	0	9.12±.62	9.31±0.61	-	-
	10	4.31±0.38	7.50±0.54	-53[b]	-20[a]
(n=4)	25	2.94±0.22	6.38±0.65	-68[b]	-32
	50	2.31±0.19	5.19±0.72	-75[b]	-44[a]
	100	3.06±0.42	4.06±0.16	-66[b]	-56[b]
B. Ethanol	0	10.78±2.11	7.07±0.54	-	-
(n=7)	10	5.75±0.98	7.14±1.07	-47[b]	+1
	25	4.61±0.86	6.14±0.53	-57[b]	-13
	50	3.43±0.60	5.75±0.99	-68[c]	-19
	100	3.00±0.49	4.86±1.60	-72[c]	-31

C. Mannitol	0	10.88±2.12	12.12±1.82	-	-
(n=3 or 4)	10	8.12±1.52	11.35±1.98	-25	-6
	25	5.98±1.17	11.03±1.77	-45[a]	-7
	50	4.50±1.37	10.12±1.38	-59[c]	-17
	100	2.45±1.30	8.38±1.59	-79[c]	-31
D. Urea	0	12.0±0.5	10.64±1.05	-	-
(n=3)	50	13.3±1.2	11.06±1.61	+10	+4
	100	15.0±2.3	10.50±0.70	+25	+1

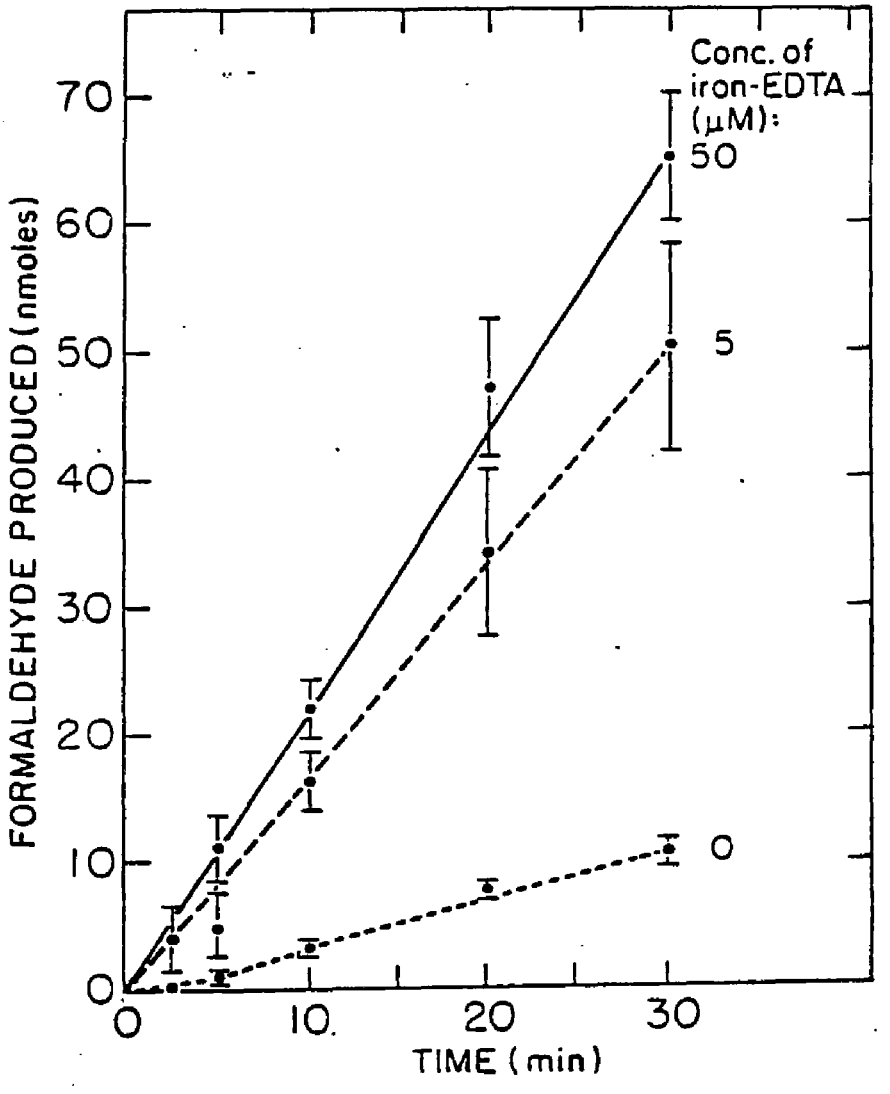
[a] $p < 0.05$ [b] $p < 0.01$ [c] $p < 0.005$

[a]The complete reaction system is described in the legend to Figure 4. The various hydroxyl radical scavenging agents were added to achieve the final concentrations which are indicated on the Table. The number of experiments is indicated in parenthesis in the Table.

Figure 7.

The Effect of Iron-EDTA on the Production of Formaldehyde from Dimethylsulfoxide During the Oxidation of Xanthine by Xanthine Oxidase.

The standard reaction mixture is described in the legend to Figure 4. Iron-EDTA was added as a 1:2 mixture to achieve the final concentrations of iron which are indicated on the figure. The results represent the mean± S.E.M. of 6 or 7 experiments.


Conc. of
iron-EDTA
(μM):
50
5
0
FORMALDEHYDE PRODUCED (nmoles)
TIME (min)
0
10
20
30
40
50
60
70

The addition of 50 μM ammonium sulfate in 100 μM EDTA, instead of 50 μM ferrous ammonium sulfate, had no effect on the production of formaldehyde (Table 3). Therefore, the stimulation which was brought about by the ferrous ammonium sulfate-EDTA complex is due to the iron moiety of the complex. The further addition of 10,30, 50 or 100 μM EDTA alone, beyond the normal 100 μM which was usually present in the standard reaction mixture, had no effect on the oxidation of Me_2SO to formaldehyde (data not shown). The addition of either catalase or superoxide dismutase inhibited the production of formaldehyde in the presence of 50 μM iron-EDTA (Table 3). Therefore, both H_2O_2 and $O_2^{\cdot-}$ radicals are involved in the increase of $^{\bullet}OH$ generation which occurs as a result of the addition of iron-EDTA. Further evidence which supports a role for $^{\bullet}OH$ in this system is that benzoate, a competitive $^{\bullet}OH$ scavenger, inhibited the production of formaldehyde in the iron-catalyzed system (Table 3).

3.1.4. The Effect of DETAPAC

The above results suggest that the production of formaldehyde from Me_2SO in the xanthine oxidase system appears to occur as a result of an iron-catalyzed Haber-Weiss reaction (120-122). DETAPAC (diethylenetriamine-

TABLE 3

Control Experiments for the Effect of Iron-EDTA on the Production of Formaldehyde from Dimethylsulfoxide[a]

Concentration of Me_2SO (mM)	Reaction Condition	Rate of Formaldehyde Production (nmol/30min)
33	1) Complete System (5)	53.6±6.8
	2) Iron-EDTA Omitted (3)	10.3±2.0
	3) Xanthine Omitted (3)	0.7±1.2
	4) Xanthine Oxidase Omitted (3)	0
	5) Catalase Added (67 µg/ml) (3)	2.0±2.0
	6) Superoxide Dismutase Added (58µg/ml) (3)	0.8±1.0
	7) 100mM Benzoate Added (5)	35.4±3.0[a]
	8) 50µM Ammonium Sulfate in 100µM EDTA Iron-EDTA Omitted (2)	10.0±3.0
3.3	9) Complete System (3)	51.0±1.6
	10) Superoxide Dismutase Added (58µg/ml) (1)	0
	11) 100mM Benzoate Added (3)	12.3±6.3[a]

[a] $p < 0.05$

[a]The complete system is the same as that described in the legend to Figure 4 except that 50 µM ferrous ammonium sulfate in 100 µm EDTA was added. The number of experiments is indicated in the Table. Ammonium sulfate had no effect on the rate of formaldehyde production in the absence of iron-EDTA (Compare lines 2 and 8).

pentaacetic acid) is an iron-chelating agent which has been shown to prevent the iron-catalyzed Haber-Weiss reaction (121, 122, 151, 158). DETAPAC inhibited formaldehyde generation from Me_2SO during the oxidation of xanthine by xanthine oxidase (Table 4). EDTA was present at a final concentration of 100 μM. In the absence of EDTA, formaldehyde was not detectable regardless of whether or not DETAPAC was added (data not shown).

3.1.5 The Effect of Treating all Solutions with Chelex-100 Resin

The inhibition which was produced by DETAPAC indicates a role for iron in the production of formaldehyde from Me_2SO. What is the source of this iron which catalyzes the Haber-Weiss reaction? Iron may be present as a contaminant of the water or of the phosphate buffer in the reaction mixture. It has been calculated that 50 mM phosphate buffer contains about 10 μM iron present as a contaminant (133). Chelex-100 resin was used to remove extraneous iron. A single passage of the solutions through a column of Chelex-100 resulted in a 50% decrease in the rate of formaldehyde production at all time points tested (Figure 8). These results indicate that there is iron

TABLE 4

The Effect of DETAPAC on the Production of Formaldehyde from Dimethylsulfoxide During the Oxidation of Xanthine by Xanthine Oxidase[a]

Concentration of DETAPAC (mM)	Rate of Formaldehyde Production (nmol/30 min)	Effect of DETAPAC (%)
0	9.00±0.50	-
0.007	10.50±0.57	+17
0.067	7.77±0.44	-14
0.33	4.17±0.73	-54[a]
0.67	3.42±0.12	-62[b]
1.0	3.83±0.33	-57[b]
2.0	2.33±0.15	-74[a]

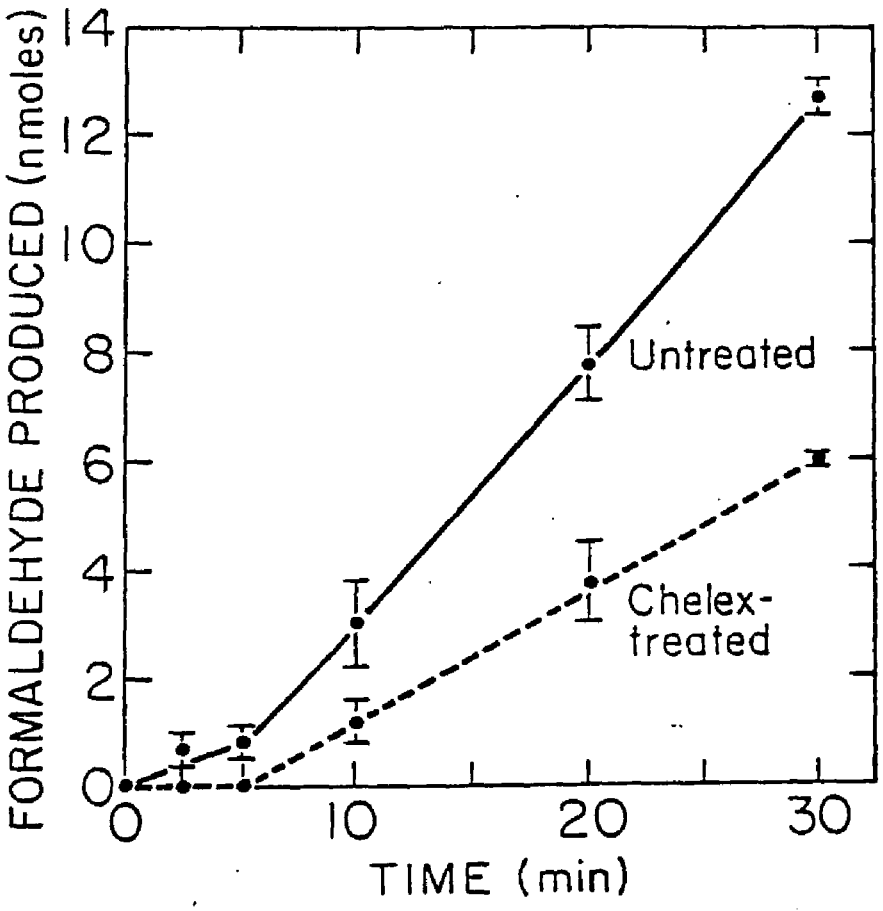
[a] $p<0.005$ [b] $p<0.001$

[a]Formaldehyde production from 33 mM dimethylsulfoxide by the xanthine oxidase system was carried out as described in the legend to Figure 4. Results are from four experiments.

Figure 8.

Effect of Treatment with Chelex-100 Resin on the Production of Formaldehyde from Me_2SO during the Oxidation of Xanthine by Xanthine Oxidase.

Chelex treatment was carried out as described in Materials and Methods. Results are from three experiments.


FORMALDEHYDE PRODUCED (nmoles)
0
2
4
6
8
10
12
14
0
10
20
30
TIME (min)
Untreated
Chelex-
treated

in the solutions which catalyzes the production of $^{\bullet}OH$ and therefore of formaldehyde.

3.2. The Generation of Formaldehyde from Dimethylsulfoxide During the Iron-EDTA-Catalyzed Oxidation of Ascorbic Acid

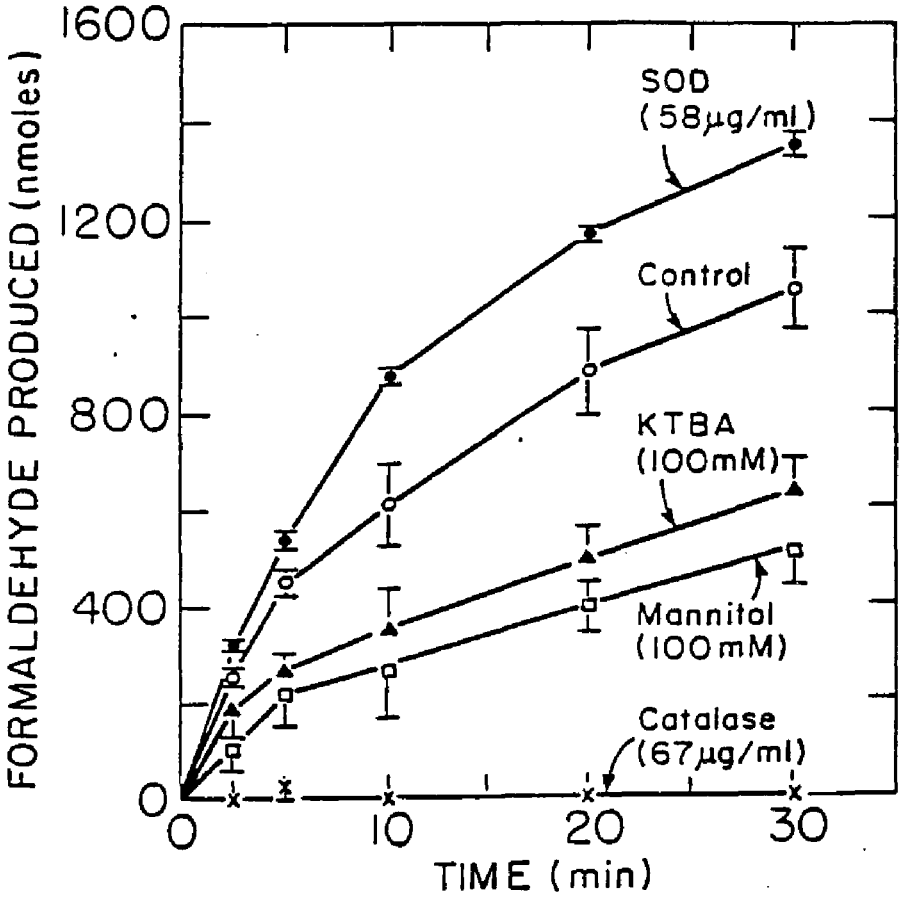
The iron-EDTA catalyzed oxidation of ascorbic acid was used as a second model $^{\bullet}OH$-generating system (108, 109). Figure 9 illustrates the time course for the production of formaldehyde from Me_2SO by this system. The addition of either KTBA or mannitol, two $^{\bullet}OH$ scavenging agents, inhibited the production of formaldehyde (Figure 9). This inhibition suggests that $^{\bullet}OH$ is responsible for the production of formaldehyde by this system. The complete inhibition produced by catalase (Figure 9) indicates that H_2O_2 is a precursor of $^{\bullet}OH$ in this system as it was in the xanthine oxidase system. In contrast, superoxide dismutase which inhibited the production of formaldehyde by the xanthine oxidase system, did not inhibit the production of formaldehyde by the ascorbate system (Figure 9). In fact, the addition of superoxide dismutase resulted in a slight stimulation of formaldehyde production. A working scheme to explain these data will be presented in the Discussion.

The iron-chelator, DETAPAC was a much less effective inhibitor of formaldehyde production from Me_2SO

Figure 9.

The Generation of Formaldehyde from Dimethylsulfoxide During the Iron-Catalyzed Oxidation of Ascorbic Acid.

The standard reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 0.167mM iron-EDTA, 2 mM ascorbic acid and 33 mM Me_2SO in a final volume of 3.0 ml. The final concentrations of the various additions to the basic reaction mixture are indicated in the figure. The results represent the mean± S.E.M. of three experiments.


1600
1200
800
400
0
0
10
20
30
FORMALDEHYDE PRODUCED (nmoles)
TIME (min)
SOD
(58μg/ml)
Control
KTBA
(100mM)
Mannitol
(100mM)
Catalase
(67μg/ml)

during the iron-catalyzed oxidation of ascorbic acid than during the xanthine oxidase reaction (Table 5, compare with Table 4). This relative lack of inhibition may be related to the lack of inhibition by superoxide dismutase (see Discussion).

3.3. Comparison Between the Production of Formaldehyde and the Production of Methane from Dimethylsulfoxide

The production of methane gas from Me_2SO has been used to detect the generation of $^{\bullet}OH$ by several biological systems (111, 113, 160, 161). Since the results which are shown above indicate that formaldehyde is also produced when Me_2SO reacts with $^{\bullet}OH$, experiments were carried out to compare the amounts of formaldehyde and of methane produced under identical reaction conditions. Either the xanthine oxidase system or the ascorbate system was used to generate $^{\bullet}OH$. The rate of formaldehyde production was considerably greater than the rate of methane production from Me_2SO by both model $^{\bullet}OH$ generating systems (Table 6). In fact, the measurements of methane production were carried out near the limits of sensitivity of the gas chromatograph and were barely detectable above the background levels of methane in room air. These data indicate that formaldehyde, rather than methane, represents the predominant product of the

TABLE 5

The Effect of DETAPAC on the Production of Formaldehyde from Dimethylsulfoxide During the Iron-EDTA Catalyzed Oxidation of Ascorbate[a]

Concentration of DETAPAC (mM)	Rate of Formaldehyde Production (nmol/10 min)	Effect of DETAPAC (%)
0	782±56	-
1.0	658±23	-16
2.0	587±32	-25[a]

[a] $p < 0.05$

[a]Formaldehyde production from 33 mM dimethylsulfoxide by the ascorbate system was carried out as described in the legend to Figure 9. Results are from three experiments.

TABLE 6

A Comparison Between the Production of Formaldehyde and of Methane from Dimethylsulfoxide[a].

Reaction System	Reaction Time (min)	Production of Formaldehyde (nMol)	Production of Methane (nMol)
Ascorbate-Iron-EDTA	2.5	292±33	1.60±0.8
	5	380±67	3.01±1.2
	20	849±117	3.87±0.6
	30	1037±140	3.93±0.3
	40	1119±119	3.50±0.8
Xanthine Oxidase	20	5.2±0.5	0.33±0.3
	30	8.2±0.5	1.09±0.6
	40	10.2±0.5	1.2±0.7

[a]The oxidation of 33mM dimethylsulfoxide to either formaldehyde or methane by the xanthine oxidase system or by the ascorbate system was carried out as described in Materials and Methods. After the determination of methane by head space chromatography, formaldehyde was assayed in the same flasks. Results are from three experiments.

interaction of Me_2SO with $^{\bullet}OH$ generated by these two model systems.

3.4. NADPH - Dependent Rat Liver Microsomal Electron Transfer

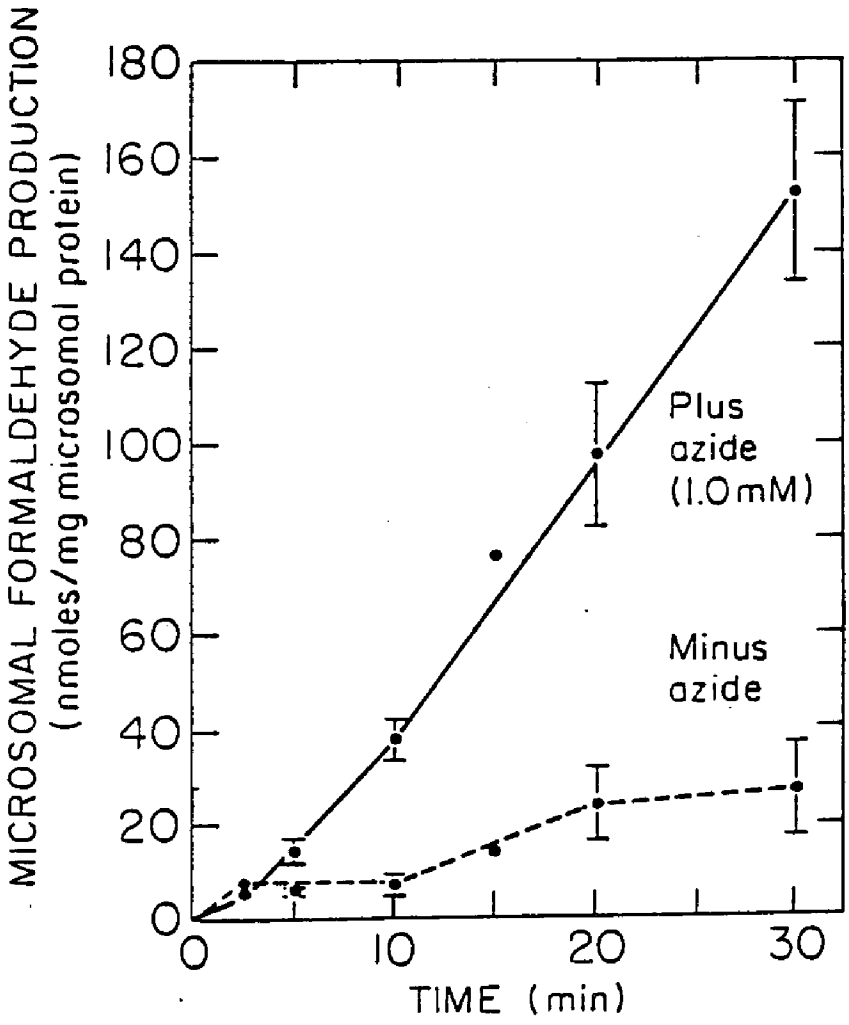
3.4.1. The Generation of Formaldehyde from Dimethylsulfoxide During NADPH-Dependent Electron Transfer by Rat Liver Microsomes.

NADPH-dependent electron transfer by rat liver microsomes was used as a third $^{\bullet}OH$-generating system (111, 160). Rat liver microsomes are invariably contaminated with catalase during the preparation of the microsomal fraction. Therefore, formaldehyde production was measured both in the absence and presence of azide, an inhibitor of catalase activity. The microsomes produced formaldehyde from Me_2SO in a time-dependent manner (Figure 10). Azide, which prevents the decomposition of H_2O_2 by catalase, stimulated the production of formaldehyde. This stimulation by azide is consistent with the presumption that H_2O_2 serves as a precursor of $^{\bullet}OH$ in this system in much the same manner as it did in the other $^{\bullet}OH$-generating systems. Previously, the microsomal oxidation of other $^{\bullet}OH$ scavenging agents such as methional, KTBA and 1-butanol were also shown to be enhanced in the presence of azide (160, 172).

Figure 10.

The Generation of Formaldehyde from Dimethylsulfoxide During NADPH-Dependent Electron Transfer by Rat Liver Microsomes.

The standard reaction mixture consisted of 83 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 10 mM potassium pyrophosphate, 10mM $MgCl_2$, 0.3 MM NADP+, 10 mM glucose-6-phosphate, 7 units of glucose-6-phosphate dehydrogenase, 3 to 5 mg of microsomal protein and 33 mM Me_2SO in a final volume of 3.0 ml. Experiments were carried out in the absence or presence of 1mM sodium azide. Results are from four experiments except for the 2.5 and 15 min. time points where the results are from two experiments.


MICROSOMAL FORMALDEHYDE PRODUCTION
(nmoles/mg microsomal protein)
180
160
140
120
100
80
60
40
20
0
0
10
20
30
TIME (min)
Plus
azide
(1.0mM)
Minus
azide

Formaldehyde was not produced in the absence of either microsomes, the NADPH-generating system or Me_2SO (Table 7). Heat denatured microsomal protein was inactive with respect to formaldehyde production (Table 7). Hydrogen peroxide could not substitute for the NADPH-generating system (Table 7). This suggests that NADPH-dependent electron transfer was required for the production of $^{\bullet}OH$ and therefore for the production of formaldehyde from Me_2SO by rat liver microsomes.

The rate of formaldehyde production from Me_2SO (about 4 nmol/min/mg microsomal protein (Figure 10, Table 7) was of the same order of magnitude as the rate of ethylene generation from KTBA (about 1.5 to 2 nmol/min/mg microsomal protein) (160)). These rates should be contrasted with the rate of microsomal methane production from Me_2SO (about 0.16 nmol/min/mg microsomal protein) (160). These results indicate that formaldehyde, rather than methane, represents a main product of the metabolism of Me_2SO by the microsomal system, similar to that found with the other model $^{\bullet}OH$-generating systems.

The concentration of Me_2SO was varied in order to determine the optimal conditions for the production of formaldehyde. A reaction period of thirty minutes was used in these experiments. The production of formaldehyde was linear with respect to the concentration of

TABLE 7

Control Experiments for the Production of Formaldehyde from Dimethylsulfoxide During NADPH-Dependent Electron Transfer by Rat Liver Microsomes.

Condition	Formaldehyde Production (nMol/30min/mg microsomal protein)
1. Complete System	116
2. Complete System-Microsomes Omitted	0
3. Complete System-Glucose-6-Phosphate Omitted	9
4. Complete System-Boiled Microsomes Substituted for Microsomes	1
5. Complete System-NADPH Generating System Omitted.200 nMoles Hydrogen Peroxide Added	1

The complete reaction system is described in the legend to Figure 10. Results are from a typical experiment.

Me_2SO up to approximately 2.0 mM (Figure 11). Formaldehyde production reached a maximum rate at 33 mM Me_2SO (Figure 11). The apparent Km for Me_2SO was approximately 9.1 mM while Vmax was 3.1 nmol/min/mg microsomal protein. The rate of formaldehyde production also demonstrated a linear dependence upon the amount of microsomal protein up to 7 mg per flask at 10, 20 and 30 minutes (Figure 12).

3.4.2. The Effect of Hydroxyl Radical Scavenging Agents

To evaluate a role for $^{\bullet}OH$ in the microsomal oxidation of Me_2SO, the effect of $^{\bullet}OH$ scavenging agents on the production of formaldehyde was evaluated. KTBA, mannitol, benzoate, ethanol and 1-butanol all suppressed the production of formaldehyde in a dose-dependent manner (Table 8). KTBA and benzoate, which are more potent $^{\bullet}OH$ scavenging agents than are mannitol and ethanol, were the more effective inhibitors (Table 8). Microsomal drug metabolizing activities such as aniline hydroxylase and aminopyrine demethylase, and microsomal electron transfer, were previously found to be unaffected by $^{\bullet}OH$ scavengers such as mannitol, benzoate or KTBA (160, 172).

3.4.3. The Effects of Iron-EDTA

Previous work has demonstrated that externally added iron-EDTA stimulates the oxidation of ethanol or isopropanol during NADPH-dependent microsomal electron

Figure 11.

Titration of Formaldehyde Production versus the Concentration of Dimethylsulfoxide.

The standard reaction mixture is described in the legend to Figure 10 except that the concentration of Me_2SO was varied. Azide was present at a final concentration of 1.0mM. The results represent the mean$\pm$ S.E.M. of three experiments.

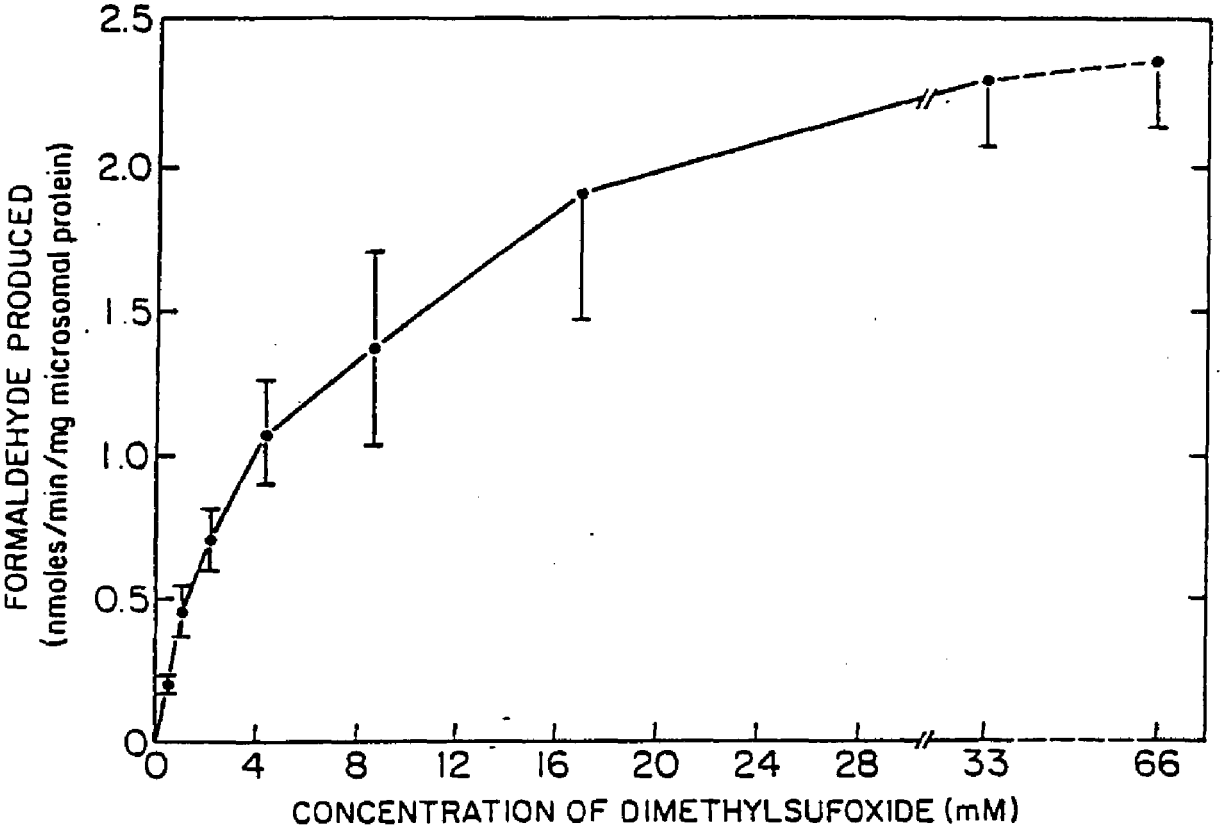

FORMALDEHYDE PRODUCED
(nmoles/min/mg microsomal protein)
2.5
2.0
1.5
1.0
0.5
0
0
4
8
12
16
20
24
28
33
66
CONCENTRATION OF DIMETHYLSUFOXIDE (mM)

Figure 12.

Titration of Formaldehyde Production versus the Concentration of Microsomal Protein.

The complete reaction mixture is described in the legend to Figure 10 except that the concentration of microsomal protein was varied. Results are from a typical experiment at 10, 20 and 30 minutes.

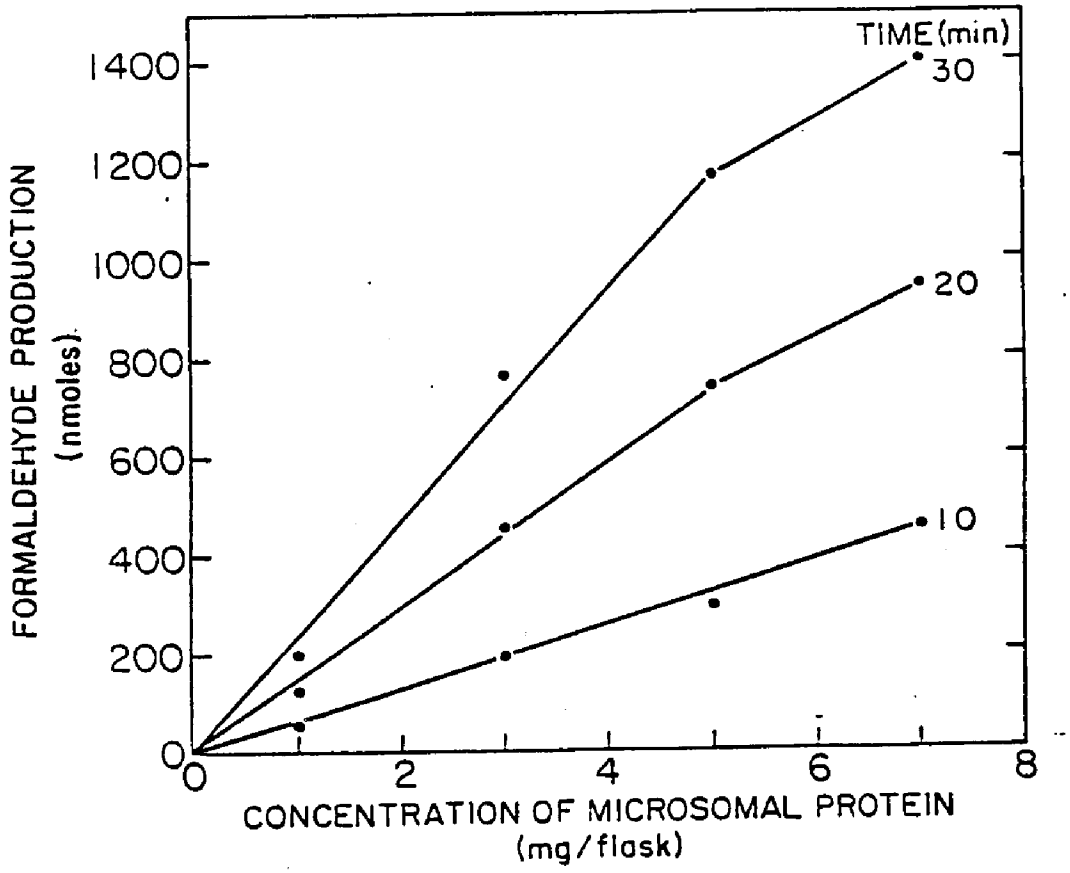

TIME(min)
30
20
10
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
FORMALDEHYDE PRODUCTION
(nmoles)
CONCENTRATION OF MICROSOMAL PROTEIN
(mg/flask)

TABLE 8

The Effect of Hydroxyl Radical Scavenging Agents on the Production of Formaldehyde from 33 mM Dimethylsulfoxide[a].

Scavenger	Concentration of Scavenger (mM)	Rate of Formaldehyde Production (nmol/30min/mg microsomal Protein)	Effect of Scavenger (%)
Control	0	82.60±8.82	-
A. KTBA	1.0	63.90±5.92	-23
	3.3	39.60±3.15	-52[b]
	6.7	26.52±2.11	-68[c]
	10.0	17.76±0.82	-78[c]
B. Mannitol	10.0	80.96±14.80	-2
	33.0	67.59±6.21	-18
	67.0	59.00±5.94	-29
	100.0	53.75±5.08	-35[a]
C. Benzoate	10.0	62.07±10.04	-25
	33.0	48.50±7.44	-41[a]

	67.0	34.67±4.51	-58[b]
	100.0	28.00±2.72	-66[c]
D. Ethanol	10.0	72.20±5.14	-13
	33.0	66.27±3.43	-20
	67.0	60.17±2.51	-27
	100.0	49.93±1.47	-40[a]
E. Butanol	10.0	71.30±4.67	-14
	33.0	64.77±8.51	-22
	67.0	51.33±4.73	-38[a]
	100.0	44.97+5.02	-46[a]

[a] $p < 0.05$ [b] $p \leq 0.01$ [c] $p < 0.005$

[a]The complete reaction system is described in the legend to Figure 10. The various hydroxyl radical scavenging agents were added at the indicated final concentrations. The results represent the mean± S.E.M. of three experiments.

transfer probably by increasing the generation of $^{\bullet}OH$ (124, 171). The stimulation of microsomal $^{\bullet}OH$ production by iron-EDTA is probably due either to the ability of ferrous ion to catalyze the decomposition of H_2O_2 (the Fenton reaction, equation 14) or to the ability of iron to catalyze the Haber-Weiss reaction (equations 13 and 14). The effect of added iron on Me_2SO oxidation was therefore determined. Iron-EDTA stimulated the production of formaldehyde from Me_2SO by rat liver microsomes (Figure 13). The stimulation was significant at all concentrations of iron-EDTA and at all time points which were tested (Figure 13). Iron-EDTA had no effect on microsomal drug metabolizing activities such as aniline hydroxylase and aminopyrine demethylase (124). Therefore, the effect of iron-EDTA appeared to be relatively specific with regard to stimulation of $^{\bullet}OH$ production. To confirm that this increase in formaldehyde production by iron-EDTA involved increased production of $^{\bullet}OH$, the effect of competitive $^{\bullet}OH$ savenging agents was evaluated. Experiments were carried out in the presence of 50 μM iron-EDTA. The $^{\bullet}OH$ scavenging agents KTBA, benzoate and 1-butanol, inhibited the iron-EDTA catalyzed rate of formaldehyde production in a dose dependent manner (Table 9). These results are consistent with a role for $^{\bullet}OH$ in the stimulatory action of iron-EDTA on formaldehyde production

Figure 13.

The Effect of Iron-EDTA on the Production of Formaldehyde from Dimethylsulfoxide by Rat Liver Microsomes in the Presence of Azide.

The standard reaction mixture is described in the legend to Figure 10. The rate of formaldehyde production was determined in the presence and absence of the indicated concentration of iron-EDTA. The results represent the mean± S.E.M. of three experiments.

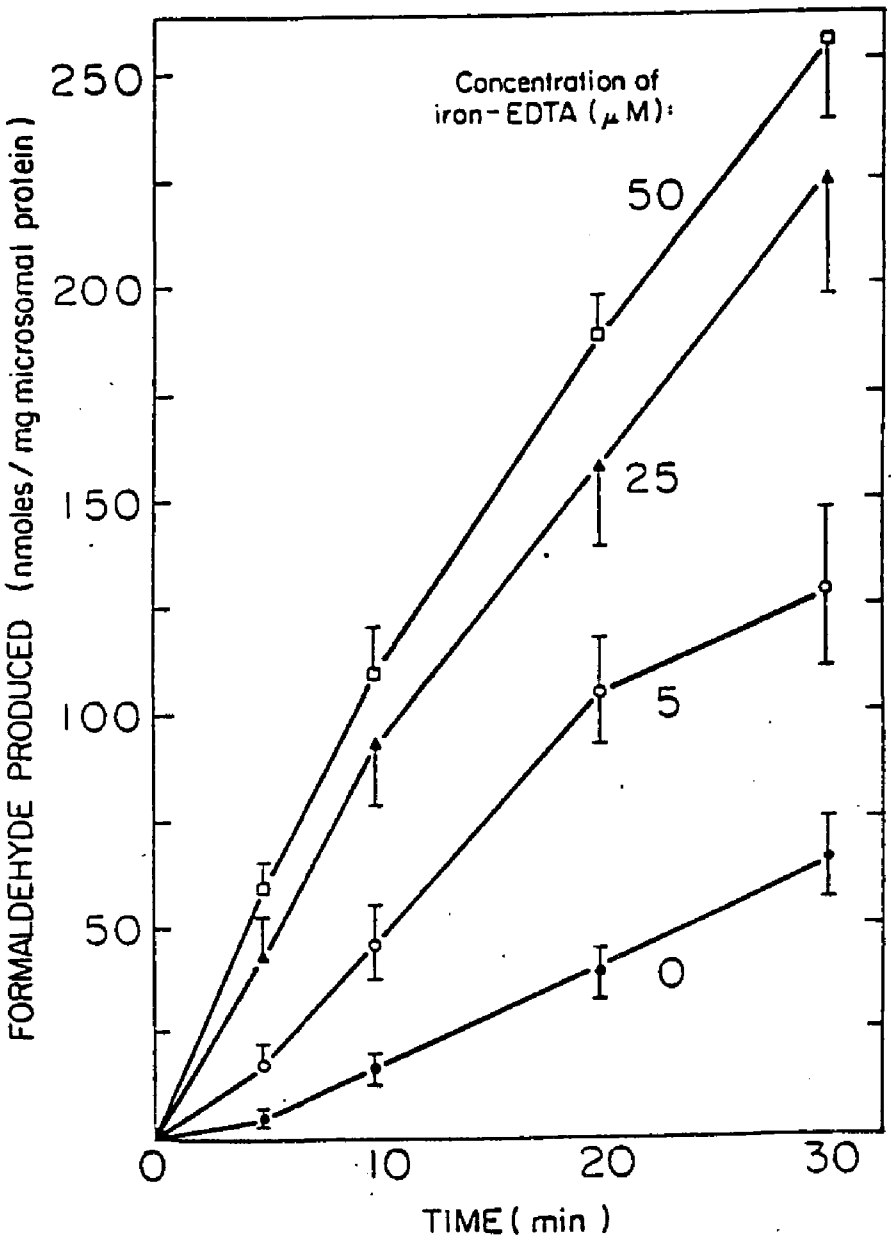

Concentration of
iron-EDTA (μM):
50
25
5
0
250
200
150
100
50
0
10
20
30
FORMALDEHYDE PRODUCED (nmoles / mg microsomal protein)
TIME (min)

TABLE 9

The Effect of Hydroxyl Radical Scavenging Agents on the Stimulation by Iron-EDTA of Formaldehyde Production from Dimethylsulfoxide in the Presence of Azide[a].

Scavenger	Concentration of Scavenger (mM)	Rate of Formaldehyde Production (nmol/30min/mg microsomal Protein)	Effect of Scavenger (%)
Control (4)	-	332.10±15.98	-
A. KTBA (4)	10	214.18±13.61	-36[c]
	33	110.35±7.24	-67[d]
B. Benzoate (4)	33	210.68±23.67	-37[b]
	67	176.90±29.11	-47[c]
	100	131.45±18.17	-60[d]
Control (3)	-	248.00±27.70	-
C. Butanol (3)	33	192.10±17.20	-22
	67	168.50±17.46	-32[a]
	100	139.70±14.70	-44[b]

[a] $0.1 > p > 0.05$ [b] $p < 0.05$ [c] $p < 0.01$ [d] $p < 0.001$

[a] The complete reaction system is described in the legend to Figure 10. Iron-EDTA was added at a final concentration of 50uM.

from Me_2SO by the microsomal system.

The low rate of formaldehyde production in the absence of azide (Figure 10) is due to decomposition of H_2O_2 (the precursor of $^{\bullet}OH$) by catalase which is present as a contaminant in the microsomes. In the absence of azide, iron-EDTA stimulated the production of formaldehyde from Me_2SO by rat liver microsomes (Figure 14). Since added iron increases $^{\bullet}OH$-generation, could iron compete with catalase for the metabolically generated H_2O_2? The stimulation increased as the concentration of iron-EDTA increased (Figure 14). This increased rate of formaldehyde production was inhibited by competing $^{\bullet}OH$ scavenging agents (Table 10). These results indicate a role for $^{\bullet}OH$ in the stimulatory effect of iron-EDTA on formaldehyde production even in the absence of azide. A working scheme to explain these results is presented in the Discussion.

3.4.4. The Effect of Treating all Solutions with Chelex-100 Resin

Since formaldehyde is produced by rat liver microsomes in the absence of exogenously added iron-EDTA, there may be an endogenous source of iron which catalyzes the production of $^{\bullet}OH$ by rat liver microsomes. It is possible that iron contaminants in the water or especially

Figure 14.

The Effect of Iron-EDTA on the Production of Formaldehyde from Dimethylsulfoxide by Rat Liver Microsomes in the Absence of Azide.

The standard reaction mixture is described in the legend to Figure 10, except that azide was omitted. The rate of formaldehyde production was determined in the presence and absence of the indicated concentrations of iron-EDTA. The results represent the mean $\pm$ S.E.M. of three experiments.

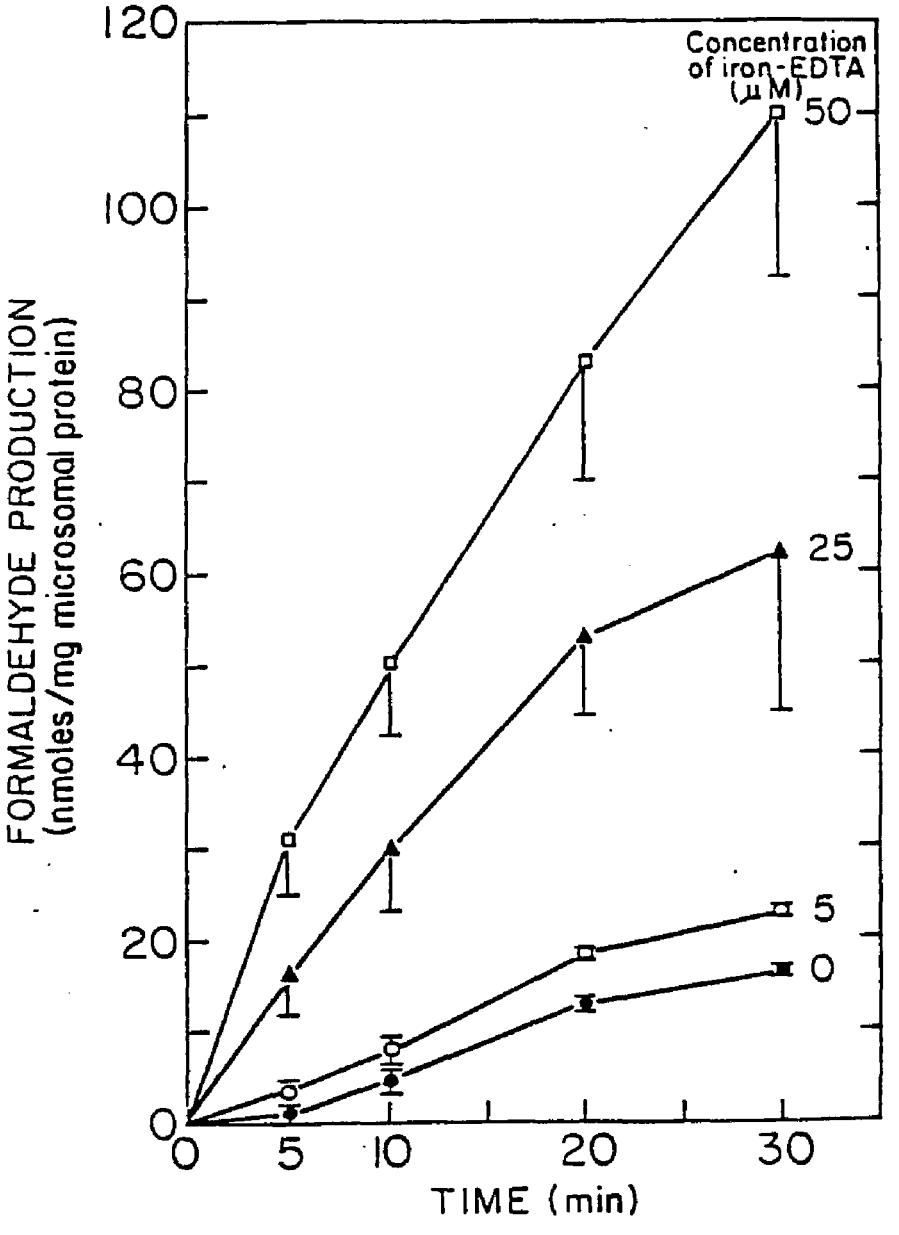

120
100
80
60
40
20
0
0
5
10
20
30
TIME (min)
FORMALDEHYDE PRODUCTION
(nmoles/mg microsomal protein)
Concentration
of iron-EDTA
(μM)
50
25
5
0

TABLE 10

The Effect of Hydroxyl Radical Scavenging Agents on the Stimulation by Iron-EDTA of Formaldehyde Production from Dimethylsulfoxide in the Absence of Azide[a].

Scavenger	Concentration of Scavenger (mM)	Rate of Formaldehyde Production (nmol/30min/mg microsomal Protein)	Effect of Scavenger (%)
Control (4)	-	181.75±15.00	-
A. KTBA (4)	10	153.30±17.16	-16
	33	116.68±23.12	-36
B. Benzoate (4)	33	151.08±28.72	-17
	67	114.40±24.47	-37
	100	84.23±16.49	-54
Control (3)	-	107.90±15.20	-
C. Butanol (3)	33	80.50±11.50	-25
	67	65.60±12.30	-39
	100	64.80±11.50	-40

[a]The complete reaction mixture is described in the legend to Figure 10 except that azide was omitted. Iron-EDTA was added at a final concentration of 50μM.

in the phosphate-pyrophosphate buffer system catalyze $^{\bullet}OH$ generation as described for the xanthine oxidase system. Another possible source may be iron in the microsomes or catalysis by microsomal heme proteins such as cytochrome P-450. To test the first possibility, Chelex-100 resin was used to remove extraneous iron from the water in which all solutions were made and from the phosphate buffer and the pyrophosphate solutions. A comparison of the microsomal oxidation of Me_2SO to formaldehyde in chelex-treated and untreated solutions was made. The rate of formaldehyde production was not significantly different whether the solutions were chelex-treated or not (Figure 15). It is therefore unlikely that iron contaminants in the buffer solutions play a role in these experiments, in contrast to results with the xanthine oxidase system (see Discussion). As a precautionary measure, all experiments were routinely carried out in chelex-treated solutions.

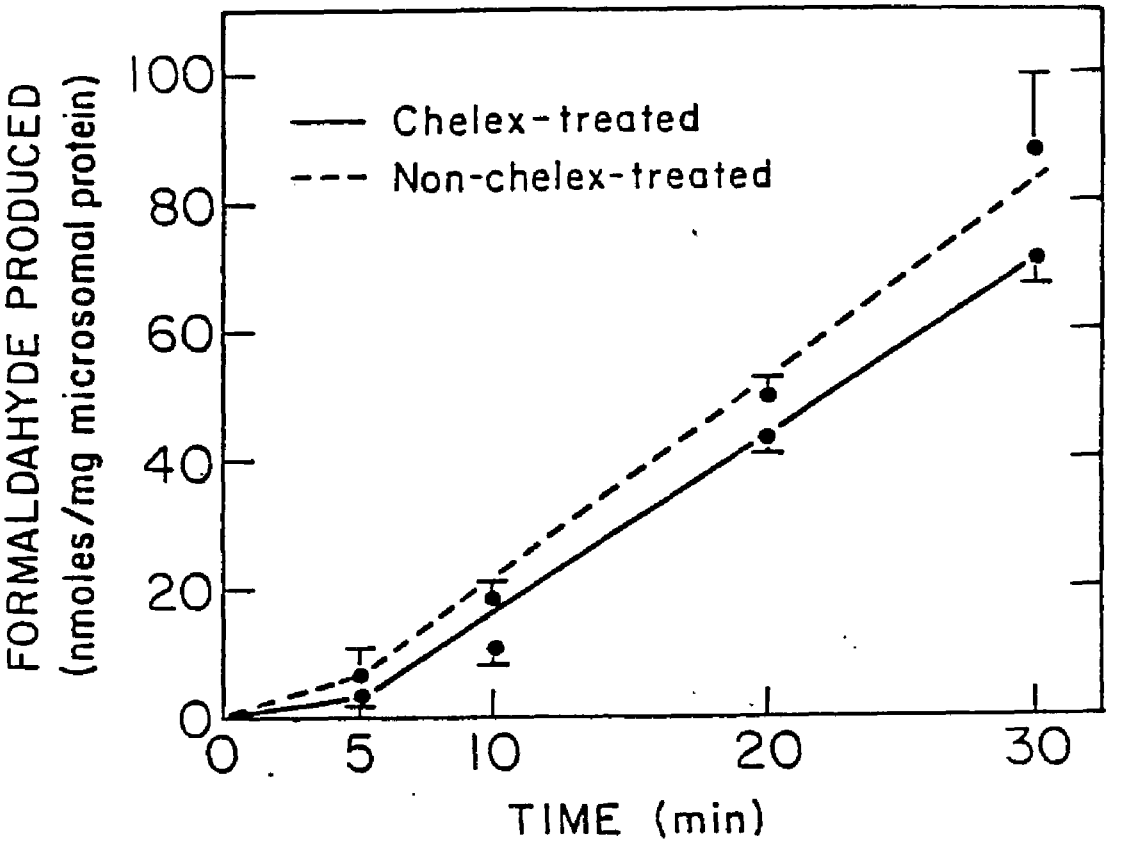
3.4.5. The Effect of DETAPAC

The above results suggest that the production of $^{\bullet}OH$ by rat liver microsomes may be due to an iron-catalyzed Haber-Weiss reaction. In view of the lack of effect of chelex treatment, the microsomes themselves are probably the source of the iron which catalyzes the production of

Figure 15.

The Effect of Treating all Solutions with Chelex-100 Resin on the Microsomal Production of Formaldehyde from Dimethylsulfoxide.

The standard reaction mixture is described in the legend to Figure 10. Solutions were treated with Chelex-100 resin as described in Materials and Methods. The results represent the mean± S.E.M. of two experiments.


FORMALDAHYDE PRODUCED
(nmoles/mg microsomal protein)
100
80
60
40
20
0
Chelex-treated
Non-chelex-treated
0
5
10
20
30
TIME (min)

$^{\bullet}OH$. DETAPAC prevents the iron-catalyzed Haber-Weiss reaction (121, 122, 150, 158), probably by preventing reduction of ferric iron by $O_2^{\cdot-}$ (201). Therefore, DETAPAC can be used to determine if $^{\bullet}OH$ production by a reaction system is mediated by the iron-catalyzed Haber-Weiss reaction. DETAPAC inhibited the microsomal production of formaldehyde in a dose-dependent manner (Table 11). This inhibition suggests that microsomal iron catalyzes the production of $^{\bullet}OH$ from its precursor, H_2O_2. DETAPAC had no effect on microsomal drug metabolism. EDTA (0.1 mM) was normally present as a component of the reaction mixture. In the absence of EDTA, the rate of formaldehyde production (5.4 $\pm$ 1.0 nmol/30 min/mg microsomal protein)was considerably lower than in the presence of EDTA, (77.1$\pm$ 2.5 nmol/30 min/mg microsomal protein). It appears, therefore, that in order to effectively participate in the production of $^{\bullet}OH$, the endogenous microsomal iron must be in a chelated form, (i.e., iron-EDTA).

3.4.6. The Effect of Mixed Function Oxidase Inhibitors.

Since there does not appear to be sufficent iron in the solutions to catalyze the modified Haber-Weiss reaction, another possible source of microsomal iron may be microsomal heme proteins such as cytochrome P-450.

TABLE 11

The Effect of DETAPAC on the Production of Formaldehyde from Dimethylsulfoxide[a].

Concentration of DETAPAC (mM)	Rate of Formaldehyde Production (nmol/30min/mg microsomal Protein)	Effect of DETAPAC (%)
0	77.1±2.5	-
0.007	68.0±1.9	-12[a]
0.067	34.2±5.0	-56[b]
0.33	21.4±5.3	-72[c]
0.67	18.0±4.0	-77[c]
1.00	14.9+2.1	-81[c]

[a] $p < 0.05$ [b] $p < 0.01$ [c] $p < 0.001$

[a]The complete reaction system is described in the legend to Figure 10. DETAPAC was added at the indicated concentrations. The results represent the mean± S.E.M. of three experiments.

This protein is an important component of the microsomal mixed function oxidase system for the detoxification of drugs and for the metabolism of endogenous compounds such as steroids. The effect of two mixed function oxidase inhibitors, metyrapone and SKF-525A, on the metabolism of Me_2SO was compared to their effect on the metabolism of aminopyrine, a typical substrate of the mixed function oxidase system. Formaldehyde was produced from both substrates by the microsomes (Table 12). Whereas aminopyrine n-demethylase activity was inhibited by the mixed function oxidase inhibitors, metyrapone and SKF-525A had no significant effect on the microsomal oxidation of Me_2SO (Table 12). Neither SKF-525A nor metyrapone inhibited the production of H_2O_2 in the presence of either aminopyrine or Me_2SO (data not shown). Therefore, even in the presence of these mixed function oxidase inhibitors, H_2O_2 is produced which can serve as a precursor of $^{\bullet}OH$. These results, plus experiments indicating that typical $^{\bullet}OH$ scavengers do not affect microsomal drug metabolism (124, 172) tend to differentiate the overall mechanisms of the microsomal cytochrome P-450 dependent mixed function oxidase system from the mechanism which is responsible for the microsomal production of $^{\bullet}OH$.

TABLE 12

The Effect of Metyrapone and SKF-525A on the Production of Formaldehyde from 33 mM Dimethylsulfoxide or from 10 mM Aminopyrine[a].

Inhibitor	Concentration of Inhibitor (mM)	Rate of Formaldehyde Production From: (nmol/30min/mg microsomal protein)		Effect (%)	
		Me_2SO	Aminopyrine	Me_2SO	Aminopyrine
Control	0	78.9±3.7	164.0±23.5	-	-
A. Metyrapone	0.1	86.1±8.1	115.0±7.0	+9	-30
	0.3	86.7±3.6	99.9±4.3	+10	-39[a]
	1.0	79.4±1.9	79.4±9.3	+1	-52[b]
B. SKF-525A	0.1	77.9±3.7	107.1±12.1	-1	-35[a]
	0.3	71.3±0.7	90.6±9.1	-10	-45[b]
	1.0	57.9+12.3	82.5+9.9	-27	-50[b]

[a] $0.1 > p > 0.05$ [b] $p < 0.05$

[a]The complete reaction system is described in the legend to Figure 10. Metyrapone and SKF-525A were added at the indicated final concentrations. Metyrapone and SKF-525A had no statistically significant effect on the oxidation of Me_2SO. The results represent the mean ± S.E.M. of three experiments.

3.4.7. Organic Hydroperoxide Supported Mixed Function Oxidation of Dimethylsulfoxide and Aminopyrine

Organic hydroperoxides can be used in place of the NADPH-generating system to support microsomal mixed function oxidase activities (204, 205). Organic hydroperoxides react directly with cytochrome P-450 to form an oxy-cytochrome P-450 complex (181) which catalyzes the metabolism of drugs. Cumene hydroperoxide (0.5mM) and tertiary-butyl hydroperoxide (2.5mM) effectively supported aminopyrine demethylase activity (Figure 16). However, when Me_2SO was the substrate in these assays, no formaldehyde was produced when the organic hydroperoxides replaced NADPH (Figure 16). These results, together with the results with metyrapone and SKF-525A, point to a different mechanism for the microsomal mixed function oxidase system and for the microsomal production of $^{\bullet}OH$.

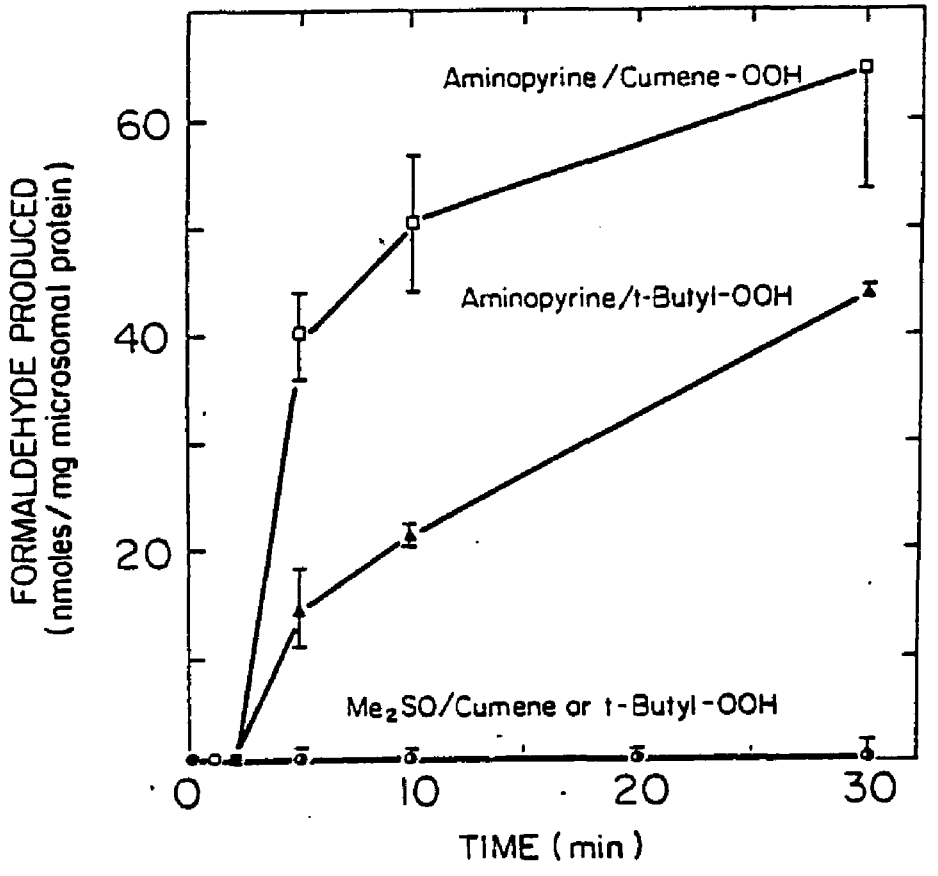
3.4.8. Cytochrome P-450 and Substrate Binding Spectra

Typical substrates of the cytochrome P-450-dependent mixed function oxidase system, such as aniline and hexabarbital, produce a characteristic spectra in the presence of microsomes or of cytochrome P-450 when these substrates bind to the cytochrome P-450 (200).

Figure 16.

Organic Hydroperoxide Supported Oxidation of Aminopyrine and Dimethylsulfoxide by Rat Liver Microsomes.

The standard reaction mixture is described in the legend to Figure 10. Cumeme-hydroperoxide, tertiary-butyl-hydroperoxide, Me_2SO and aminopyrine were present at final concentrations of 0.5 mM, 2.5 mM, 33 mM and 10 mM, respectively. The results represent the mean± S.E.M. of three experiments.


Aminopyrine / Cumene - OOH
Aminopyrine / t-Butyl-OOH
Me2SO/Cumene or t-Butyl-OOH
FORMALDEHYDE PRODUCED
(nmoles / mg microsomal protein)
60
40
20
0
10
20
30
TIME (min)

If hexabarbital is the substrate, then the difference spectrum typically shows a peak at 385 nm and a trough at 420 nm (a Type I difference spectrum). The addition of aniline produces a spectrum with a peak at 430 nM and a trough at 390 nM (a Type II difference spectrum). These "substrate-induced" difference spectra should be contrasted with the carbon-monoxide difference spectra which is used to quantify the amount of cytochrome P-450 and to determine the purity of the cytochrome P-450 preparation. This spectrum exhibits a peak at 450 nm and is due to the formation of a ferrocytochrome P-450-carbon monoxide complex. Figure 17a shows a carbon-monoxide reduced spectrum of rat liver microsomes prepared as described in Materials and Methods. The typical peak at 450 nm is apparent. Figure 17b shows the typical Type I and Type II binding spectra of hexabarbital and aniline, respectively. However, the addition of dimethylsulfoxide produced neither a Type I nor a Type II difference spectrum (Figure 17c). These results indicate that Me_2SO does not bind to cytochrome P-450 as do other typical substrates of the microsomal mixed-function oxidase system. Moreover, Me_2SO had no effect on the carbon monoxide binding spectrum (Figure 17c) or the aniline binding spectrum (data not shown). Therefore,

Figure 17.

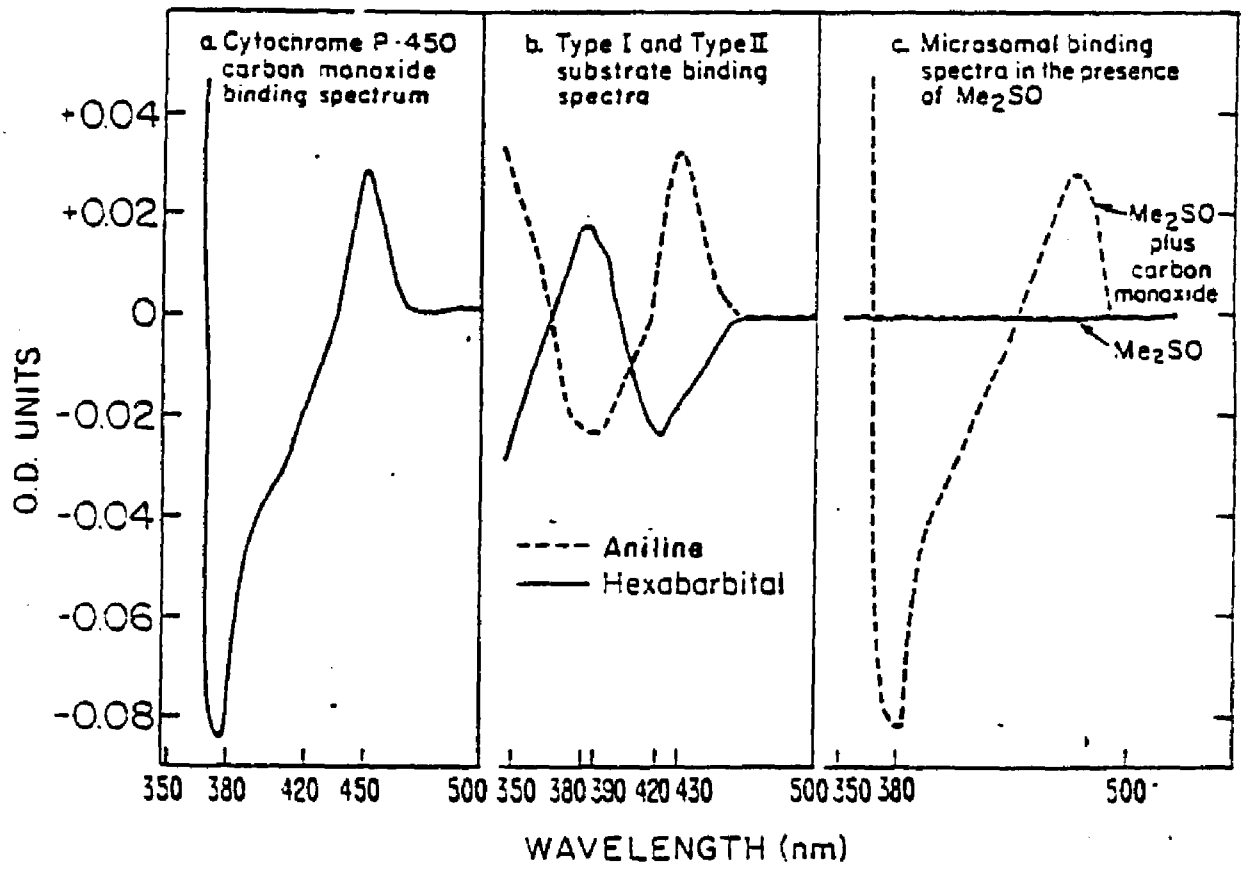
Cytochrome P-450 and Substrate Binding Spectra.

Experiments were carried out as described in Materials and Methods.

a) Carbon monoxide difference spectra,

b) Aniline and hexabarbital binding spectra,

c) Addition of either Me_2SO alone or Me_2SO plus carbon monoxide. Neither a Type I nor a Type II binding spectra was observed upon addition of Me_2SO. Results represent a typical experiment.


a. Cytochrome P-450 carbon monoxide binding spectrum
b. Type I and Type II substrate binding spectra
c. Microsomal binding spectra in the presence of Me2SO
+0.04
+0.02
0
-0.02
-0.04
-0.06
-0.08
O.D. UNITS
350 380 420 450 500
350 380 390 420 430 500
350 380 500
Aniline
Hexabarbital
Me2SO plus carbon monoxide
Me2SO
WAVELENGTH (nm)

in the presence of Me_2SO at these concentrations (33 to 133 mM) there were no obvious solvent effects of Me_2SO interferring with microsomal membrane integrity.

3.4.9. The Effect of Carbon Monoxide on the Production of Formaldehyde from Dimethylsulfoxide and Aminopyrine

Carbon monoxide inhibits cytochrome P-450 mixed function oxidase activity by competing with oxygen for binding to the oxygen-binding site on cytochrome P-450. Table 13 demonstrates the carbon monoxide dependent inhibition of microsomal aminopyrine demethylase activity. Carbon monoxide also inhibited the production of formaldehyde from Me_2SO. At first glance, this suggests that cytochrome P-450 is involved in the microsomal production of $^{\bullet}OH$. However, bubbling with carbon monoxide resulted in the production of anaerobiosis since under identical conditions, Me_2SO oxidation by the ascorbate-iron-EDTA system was inhibited by carbon monoxide (data not shown). Anaerobiosis produced by bubbling with nitrogen also inhibited the oxidation of Me_2SO and aminopyrine (data not shown). Therefore, the inhibition of Me_2SO oxidation by carbon monoxide cannot be taken as evidence for or against a role for cytochrome P-450 in generating $^{\bullet}OH$.

TABLE 13

The Effect of Carbon Monoxide on the Microsomal Production of Formaldehyde from Aminopyrine and From Dimethylsulfoxide[a].

	Rate of Formaldehyde Production (nmol/20min/mg microsomal Protein)		Effect of Carbon Monoxide (%)	
	Aminopyrine	Me_2SO	Aminopyrine	Me_2SO
Control	108.7	57.4	-	-
Carbon Monoxide	24.3	10.4	77.6	81.9

[a]Experiments were carried out as described in Materials and Methods. The results represent the mean of two experiments.

3.5. The Interaction of Dimethylsulfoxide with Alkoxy Radicals.

Can Me_2SO interact with radicals other than $^{\bullet}OH$? Alkoxy radicals ($^{\bullet}OR$) are also strong oxidizing agents. Experiments were conducted to determine whether or not Me_2SO can react with $^{\bullet}OR$. Cumene alkoxy and tertiary-butyl alkoxy radicals were generated by a Fenton-type reaction between ferrous-DETAPAC and either cumene or tertiary-butyl hydroperoxide. As a control, $^{\bullet}OH$ was generated in a similar manner via a Fenton reaction between H_2O_2 and ferrous-DETAPAC. A large burst of $^{\bullet}OH$ or $^{\bullet}OR$ is generated instantaneously in these systems upon the addition of the appropriate peroxide. Formaldehyde was not detectable with any of these systems under these conditions (see Discussion for explanation). Rather, methane and ethane are the predominant products of the interaction of $^{\bullet}OH$ generated by a classical Fenton reaction (Table 14, 160). Methane and ethane were also produced when cumene - or tertiary-butyl hydroperoxide replaced H_2O_2 (Table 14). However, the yields of methane and ethane by these two alkoxy radical generating systems were considerably less than the yields found when Me_2SO reacted with $^{\bullet}OH$ (Table 14). Cumene and tertiary-butyl hydroperoxide but not H_2O_2, produced large amounts of methane and

TABLE 14

Comparison of the Yields of Methane and Ethane from the Interaction of Dimethylsulfoxide with Either $^{\bullet}OH$ or Alkoxy Radicals ($^{\bullet}OR$)[a].

Reaction System	Product Formation (nmol)	
	Methane	Ethane
$H_2O_2 \longrightarrow {}^{\bullet}OH$	2864±181	3871±479
Cumene OOH $\longrightarrow$ Cumene Alkoxy Radical	422±328	326±394
t-Butyl OOH $\longrightarrow$ t-Butyl Alkoxy Radical	680±275	833±75

[a] $^{\bullet}OH$ and $^{\bullet}OR$ were generated from the interaction of H_2O_2 or cumene hydroperoxide (cumene alkoxy radical) or tertiary-butyl hydroperoxide (tertiary-butyl alkoxy radical) with ferrous-DETAPAC as described in Materials and Methods. Methane and ethane were measured by head-space gas chromatography. Formaldehyde was not detectable in any of these systems under these conditions. Results represent the mean± S.E.M. of three experiments.

ethane even in the absence of Me_2SO. Apparently, the hydroperoxides interact directly with ferrous-DETAPAC. Therefore, all values in Table 14 represent the net increase in the yield of methane and ethane when Me_2SO was added to the reaction system. For the organic hydroperoxide systems, these blanks, (in the absence of Me_2SO) were very high compared to the comparable experimental samples (in the presence of Me_2SO). The results in Table 14 suggest that Me_2SO may be considerably less reactive with alkoxy radicals compared to $^{\bullet}OH$.

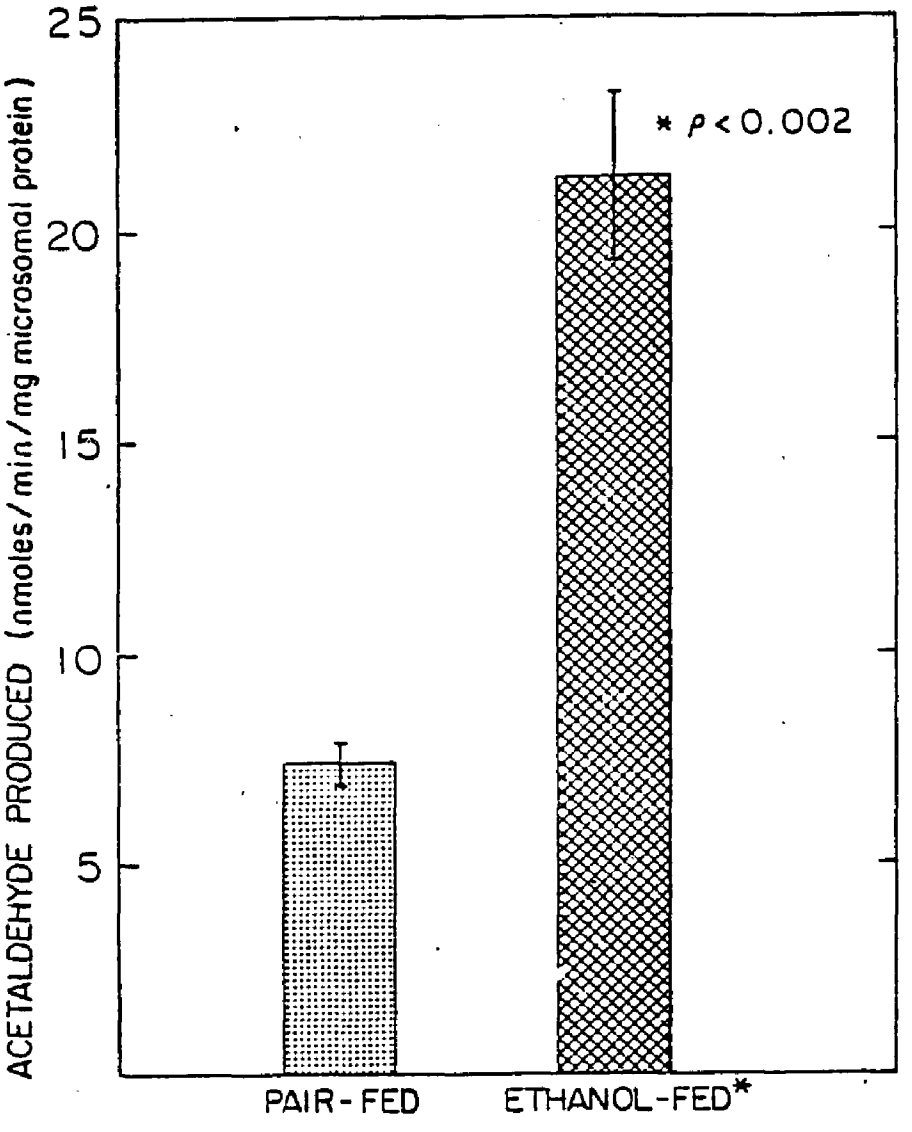
3.6. Application of Technique - The Production of Hydroxyl Radicals by Rat Liver Microsomes from Chronic Ethanol-Fed Rats and their Pair-Fed Controls.

The production of formaldehyde from the interaction of Me_2SO with $^{\bullet}OH$ generated by microsomal electron transfer was used as a probe to determine the role of $^{\bullet}OH$ in the increased rate of ethanol oxidation by microsomes obtained from chronic ethanol-fed rats. Initial experiments confirmed the increased rate of ethanol oxidation by microsomes from chronic ethanol-fed rats (Figure 18). Ethanol oxidation by microsomes was increased approximately three-fold after chronic

Figure 18.

The Production of Acetaldehyde from Ethanol by Rat Liver Microsomes Prepared from Ethanol-Fed Rats and their Pair-Fed Controls.

The standard reaction mixture is described in the legend to Figure 10 except that 50 mM ethanol was substituted for Me_2SO. The statistical significance of the effect of ethanol feeding is indicated on the figure. The results represent the mean $\pm$ S.E.M. of five experiments.


25
20
15
10
5
ACETALDEHYDE PRODUCED (nmoles/min/mg microsomal protein)
* p < 0.002
PAIR-FED
ETHANOL-FED*

ethanol consumption (Figure 18). If ethanol oxidation is due in part to interaction with $^{\bullet}OH$, then the increase in ethanol oxidation may be associated with an increase in $^{\bullet}OH$ generation. The production of formaldehyde from Me_2SO and of ethylene from KTBA were used to detect the generation of $^{\bullet}OH$ by the microsomes obtained from the chronic ethanol-fed rats and their pair-fed controls (Figures 19 and 20). The oxidation of Me_2SO (Figure 19) and KTBA (Figure 20) by microsomes was increased by chronic ethanol consumption. The increase in the oxidation of Me_2SO (approximately 3-fold) and KTBA (about 2-fold) was within the same range as the increase in ethanol oxidation (Figure 18). There was no production of formaldehyde or ethylene in zero-time controls (acid added before microsomes) or in the absence of either microsomes or the NADPH-generating system with both microsomal preparations (data not shown).

Previous results demonstrated that azide, which prevents the decomposition of H_2O_2 by catalase, stimulated the production of formaldehyde from Me_2SO by rat liver microsomes from chow fed rats (Figure 10). Similar results were obtained with microsomes from

Figure 19.

The Production of Formaldehyde from Me_2SO by Rat Liver Microsomes Prepared from Ethanol-Fed Rats and their Pair-Fed Controls.

The standard reaction mixture is described in the legend to Figure 10. Azide was present at a final concentration of 1.0 mM. The statistical significance of the effect of ethanol-feeding is indicated on the figure. The results represent the mean $\pm$ S.E.M. of seven experiments.

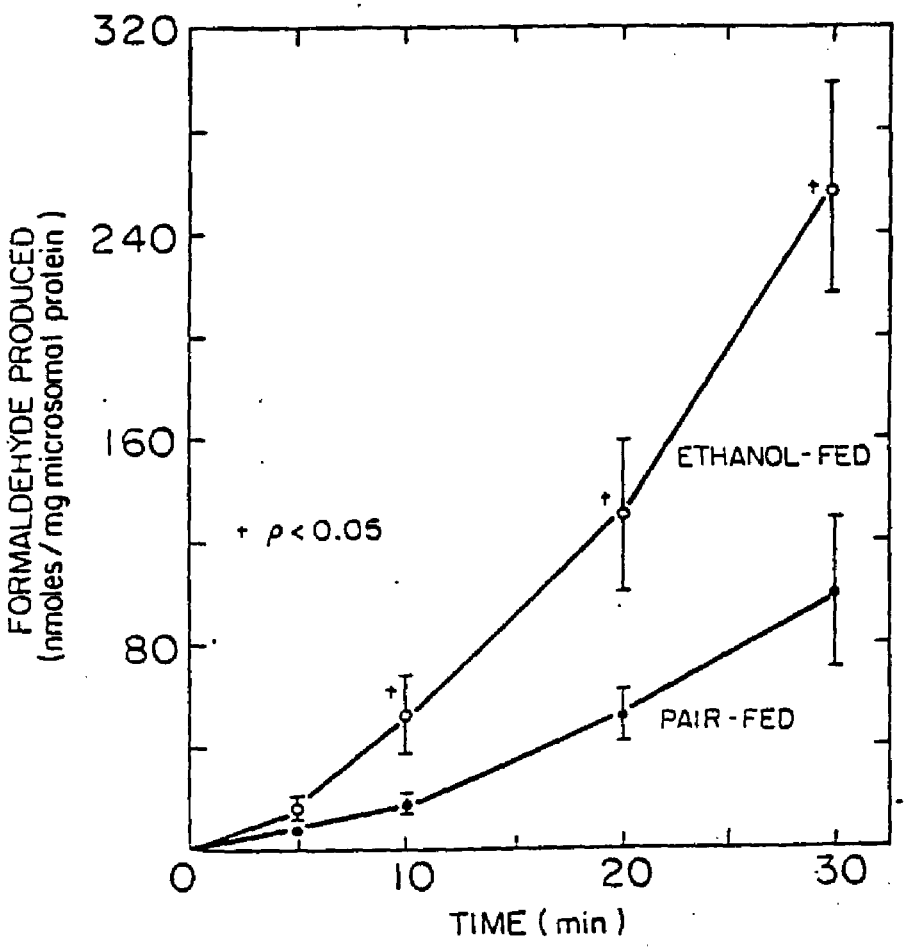
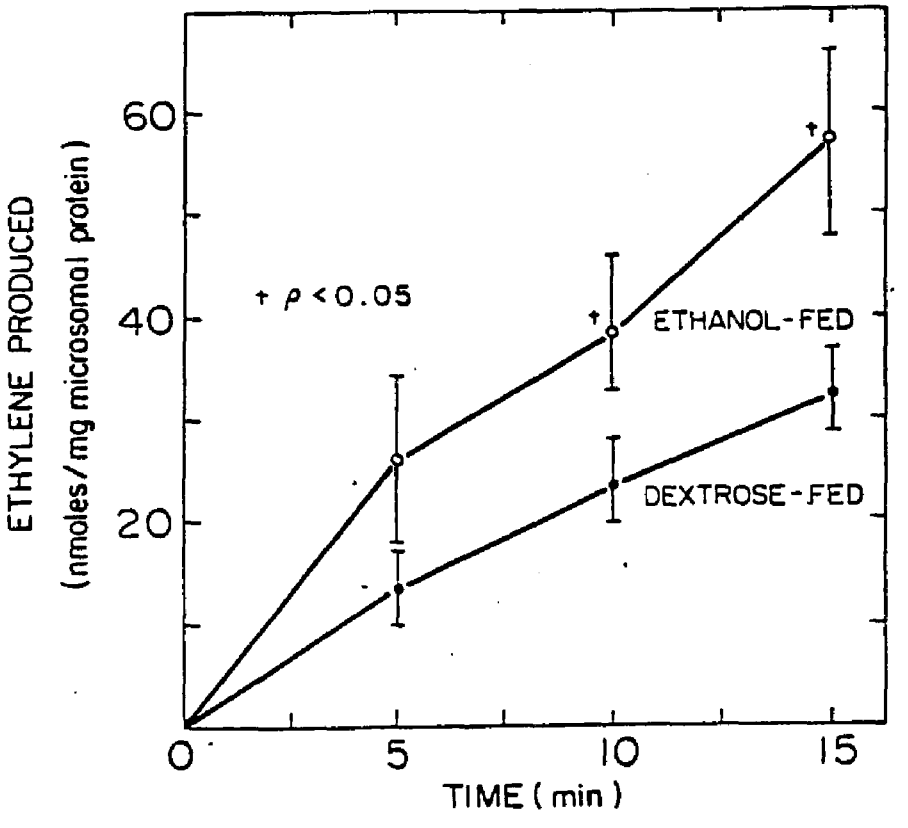

320
240
160
80
0
10
20
30
TIME (min)
FORMALDEHYDE PRODUCED
(nmoles / mg microsomal protein)
+ p < 0.05
ETHANOL-FED
PAIR-FED

Figure 20.

The Production of Ethylene from KTBA by Rat Liver Microsomes Prepared from Ethanol-Fed Rats and their Pair-Fed Controls.

The standard reaction mixture is described in the legend to Figure 10 except that 10 mM KTBA was substituted for Me_2SO. The statistical significance of the effect of ethanol feeding is indicated on the figure. The results represent the mean$\pm$ S.E.M. of seven experiments.


ETHYLENE PRODUCED
(nmoles/mg microsomal protein)
60
40
20
0
5
10
15
TIME (min)
† p < 0.05
ETHANOL-FED
DEXTROSE-FED

chronic ethanol-fed rats and their pair-fed controls (Table 15). Azide stimulated the oxidation of both Me_2SO and KTBA by the microsomes from the ethanol-fed rats and their pair-fed controls, consistent with a role for H_2O_2 as a precursor of $^{\bullet}OH$ in both microsomal preparation. The oxidation of Me_2SO and KTBA was greater by the microsomes from the ethanol-fed rats, both in the absence as well as in the presence of azide (Table 15).

To correlate the oxidation of Me_2SO, KTBA and ethanol with a common oxidizing intermediate, cross-competition experiments among these substrates was carried out. If the oxidation of Me_2SO, KTBA and ethanol by microsomes from ethanol-fed rats and pair-fed controls reflected, at least in part, interaction with $^{\bullet}OH$, then it can be anticipated that the three substrates should compete with each other for metabolically generated $^{\bullet}OH$. The results of the cross competition experiments are presented in Figures 21 through 23. Me_2SO and KTBA decreased the rate of ethanol oxidation by both the ethanol-fed and the pair-fed microsomal preparations (Figure 21). Similarly, KTBA and ethanol decreased the rate of formaldehyde production from Me_2SO in both microsomal preparations (Figure 22). Finally, Me_2SO and ethanol decreased the

TABLE 15

The Oxidation of Me_2SO and KTBA by Rat Liver Microsomes in the Presence and Absence of Azide[a].

Substrate	Rate of Product Formation (nmol/min/mg microsomal protein)			
	-Azide		+Azide	
	Dextrose Fed	Ethanol Fed	Dextrose Fed	Ethanol Fed
Me_2SO (5)	0.75±0.15	1.58±0.28	3.02±0.57[a]	8.07±1.46[a]
KTBA (7)	0.90±0.18	1.78±0.37	2.19±0.27[b]	3.82±0.62[b]

[a] $p < 0.05$ [b] $p < 0.001$

[a]The standard reaction mixture is described in the legend to Figure 10 except that 10 mM KTBA was used as substrate in some experiments. When present, azide was added at a final concentration of 1.0 mM. The statistics refer to the effect of the addition of azide. The number of experiments is indicated in the Table. The results represent the mean± S.E.M.

Figure 21.

The Effect of KTBA and Me_2SO on the Production of Acetaldehyde from Ethanol.

The standard reaction mixture is described in the legend to Figure 10 except that 50 mM ethanol was substituted for Me_2SO. Either KTBA (10 mM) or Me_2SO (33 mM) was added as competing scavenger. The effect of these additions is indicated on the figure as percent decrease. The results represent the mean$\pm$ S.E.M. of five experiments.

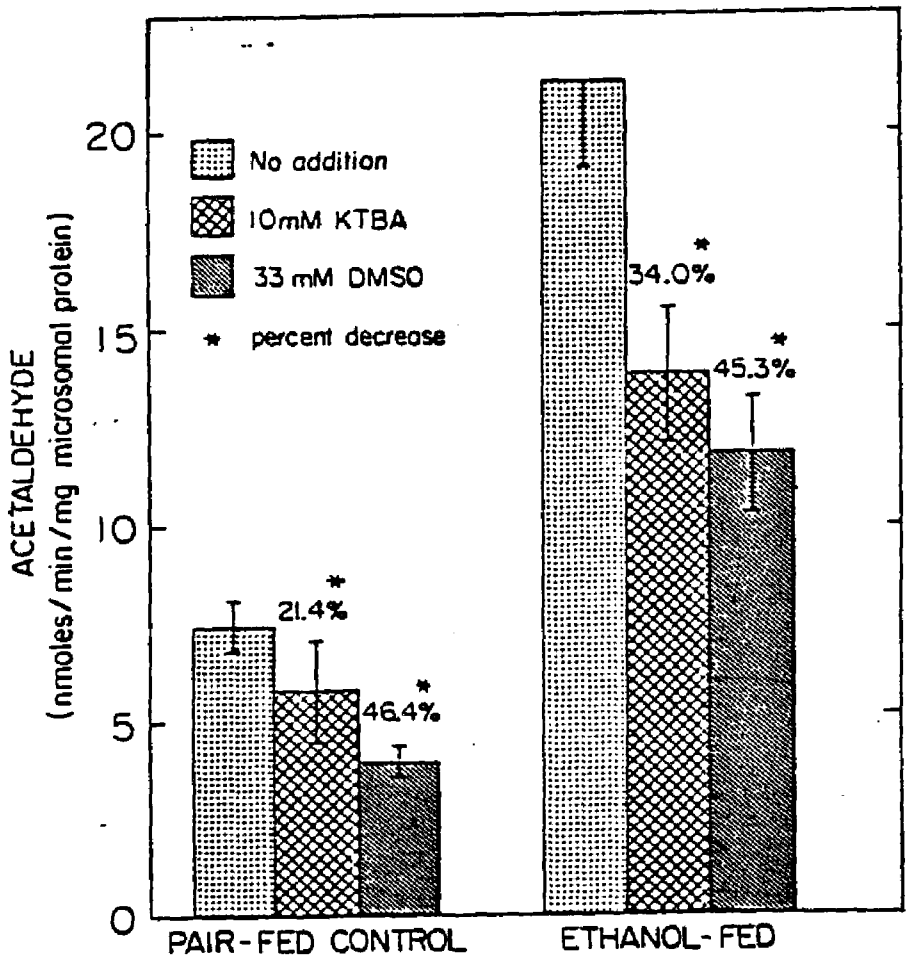

ACETALDEHYDE
(nmoles/min/mg microsomal protein)
20
15
10
5
0
No addition
10mM KTBA
33 mM DMSO
* percent decrease
21.4%
46.4%
34.0%
45.3%
PAIR-FED CONTROL
ETHANOL-FED

Figure 22.

The Effect of KTBA and Ethanol on the Production of Formaldehyde from Me_2SO.

The standard reaction mixture is described in the legend to Figure 10. Either KTBA (10 mM) or ethanol (100 mM) was added as competing scavenger. The effect of these additions is indicated on the figure as percent decrease. The results represent the mean$\pm$ S.E.M. of five experiments.

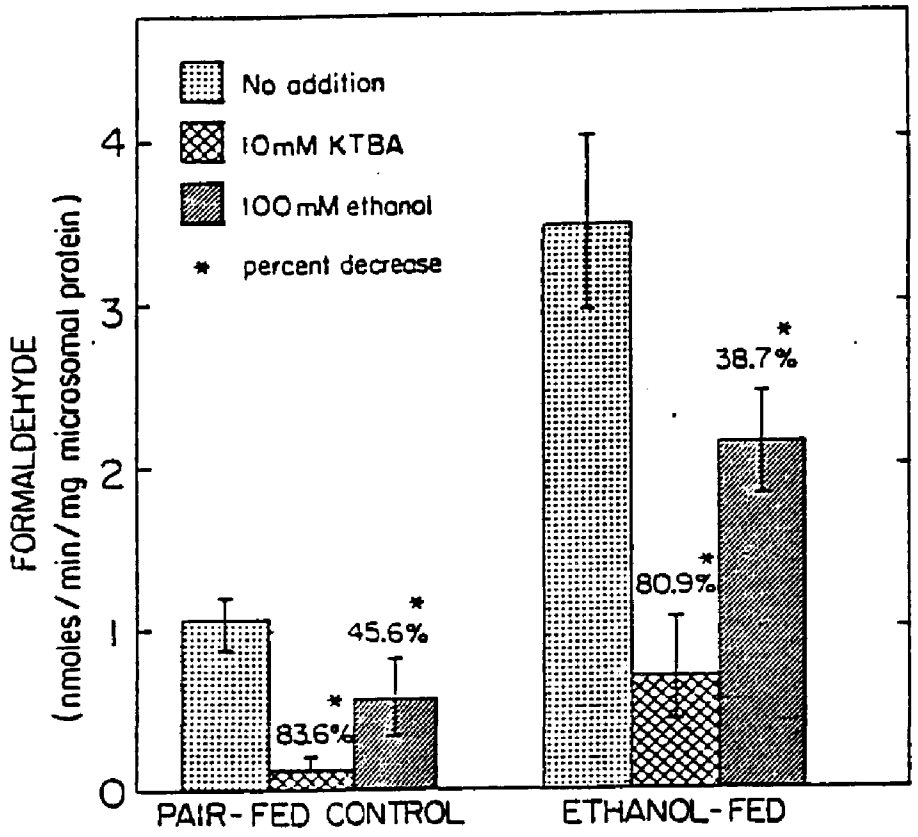
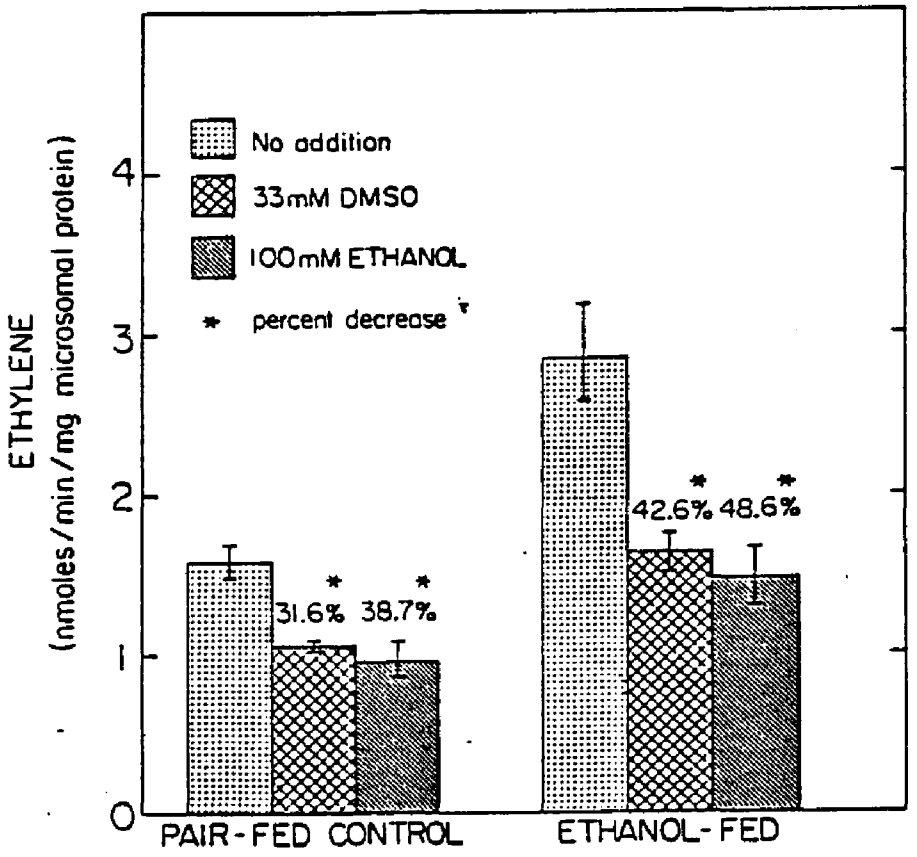

No addition
10mM KTBA
100mM ethanol
* percent decrease
FORMALDEHYDE
(nmoles/min/mg microsomal protein)
0
1
2
3
4
83.6%
45.6%
80.9%
38.7%
PAIR-FED CONTROL
ETHANOL-FED

Figure 23.

The Effect of Me_2SO and Ethanol on the Generation of Ethylene from KTBA.

The standard reaction mixture is described in the legend to Figure 10 except that 10 mM KTBA was substituted for Me_2SO. Either Me_2SO (33 mM) or ethanol (100 mM) was added as competing scavenger. The effect of these additions is indicated on the figure as percent decrease. The results represent the mean$\pm$ S.E.M. of five experiments.


ETHYLENE
(nmoles/min/mg microsomal protein)
No addition
33mM DMSO
100mM ETHANOL
* percent decrease
0
1
2
3
4
31.6%
38.7%
42.6%
48.6%
PAIR-FED CONTROL
ETHANOL-FED

rate of KTBA oxidation in both microsomal preparations (Figure 23). Thus cross-competition among the three substrates for $^{\bullet}OH$ was observed. Moreover, comparable extents of inhibition were found with both microsomal preparations (Figures 21-23). Competition among the substrates for interaction with $^{\bullet}OH$ would be expected to result in comparable extents of inhibition of the oxidation of the same substrate by both microsomal preparations.

Additional experiments were carried out with two other compounds that can interact with $^{\bullet}OH$. These were benzoate and 5, 5-dimethyl-1-pyroline-n-oxide (DMPO). DMPO is a free radical spin-trapping agent which has been used to detect the production of $^{\bullet}OH$ by rat liver microsomes and other $^{\bullet}OH$-generating systems (151-155). DMPO inhibited the oxidation of ethanol and KTBA by microsomes from chow-fed rats (194). Benzoate was a good inhibitor of Me_2SO oxidation by microsomes from chow-fed animals (Table 8). The effect of DMPO and benzoate on the oxidation of Me_2SO, KTBA and ethanol by liver microsomes obtained from the ethanol-fed rats and their pair-fed controls is described in Table 16. Both DMPO and benzoate inhibited the oxidation of Me_2SO, KTBA and ethanol in a dose-dependent manner. Comparable extents of inhibition were observed with both

TABLE 16

The Effect of DMPO and Benzoate on the Oxidation of Me_2SO, KTBA and Ethanol by Liver Microsomes from Ethanol-Fed Rats and Their Pair-Fed Controls[a].

Substrate	Addition	(mM)	Inhibition of Product Formation Pair-Fed (%)	Ethanol-Fed (%)
Me_2SO (33mM)	DMPO	3.3	12	3
		10	28	27
		33	56	59
	Benzoate	67	63	64
		100	71	70
KTBA (10mM)	DMPO	3.3	14	21
		10	44	47
		33	71	69
	Benzoate	67	75	83
		100	82	84
Ethanol (50mM)	DMPO	3.3	34	34
		10	51	52
		33	54	69

[a]The standard reaction mixture is described in the legend to Figure 10. Substrates were either 33mM Me_2SO, 10mM KTBA or 50mM Ethanol. The results are presented as % inhibition of product formation by either DMPO or benzoate and they represent the mean effect of three experiments.

microsomal preparations (Table 16) as was the case with the cross-competition experiments. Taken as a whole, these results suggest that ethanol, Me_2SO and KTBA compete for a common intermediate, e.g. $^{\bullet}OH$ for their oxidation by microsomes from chow-fed animals, ethanol-fed rats and their pair-fed controls.

CHAPTER 4

DISCUSSION

The ability of Me_2SO to be oxidized to formaldehyde was characterized in three different systems which are known to produce oxygen radicals. These systems are 1) the coupled oxidation of xanthine by xanthine oxidase; 2) the iron-catalyzed oxidation of ascorbate and 3) NADPH-dependent microsomal electron transfer. During the oxidation of xanthine by xanthine oxidase, formaldehyde production from Me_2SO is linear with time after an initial lag period (Figure 4). The lag period is overcome by the addition of H_2O_2, the precursor of $^{\bullet}OH$. The production of formaldehyde is inhibited by either superoxide dismutase or by catalase or by $^{\bullet}OH$ scavenging agents (Figure 4). These characteristics of formaldehyde production from Me_2SO are similar to those described for the interaction of other scavengers with the $^{\bullet}OH$ which is generated during the xanthine oxidase reaction e.g., for the production of acetaldehyde from ethanol (109) or the production of ethylene from methional (110). The addition of H_2O_2 in the absence of either xanthine or xanthine oxidase is ineffective in promoting the generation of formaldehyde (Table I). However, the addition of H_2O_2 to the complete system in the presence of xanthine and xanthine oxidase stimulates the production of formaldehyde (Figure 5). The stimulation by H_2O_2 and

the inhibition by catalase suggest that H_2O_2 serves as a precursor of $^{\bullet}OH$ during the oxidation of xanthine by xanthine oxidase. The addition of $^{\bullet}OH$ scavenging agents inhibits the production of formaldehyde in a dose-dependent manner and in a manner which is consistent with a competition between the scavengers and Me_2SO for the generated $^{\bullet}OH$ (Table 2). In view of the data with chelex-100 treatment (Figure 8), production of $^{\bullet}OH$ by the xanthine oxidase system (and therefore the production of formaldehyde from Me_2SO by this system) appears to be mediated by iron that is present in the solutions, most likely the phosphate buffer. This conclusion is supported by the observation that DETAPAC, which inhibits $^{\bullet}OH$ production via the iron catalyzed Haber-Weiss reaction (121, 122, 151, 158) also inhibits the production of formaldehyde from Me_2SO (Table 4). Iron-EDTA, which is known to stimulate the generation of $^{\bullet}OH$ during the oxidation of xanthine by xanthine oxidase (120-122, 124) also stimulates the production of formaldehyde from Me_2SO (Figure 7). This stimulation by added iron-EDTA is sensitive to the addition of superoxide dismutase, catalase and benzoate thereby implicating a role for increased production of $^{\bullet}OH$ in the mechanism of action of iron-EDTA in this system (Table 3). Taken as a whole, these observation indicate that a) the production of formaldehyde from Me_2SO during the oxidation of xanthine by

xanthine oxidase is due to the interaction of Me_2SO with $^{\bullet}OH$ which is generated by this reaction system, and that b) the generation of $^{\bullet}OH$ by this system occurs as a result of an iron-catalyzed Haber-Weiss type reaction between superoxide anion and hydrogen peroxide (equations 13 and 14 and see below).

Formaldehyde is also produced from dimethylsulfoxide during the iron-EDTA-catalyzed oxidation of ascorbic acid (Figure 9). Formaldehyde production by this system is sensitive to the addition of $^{\bullet}OH$ scavenging agents, analogous to results found with the xanthine oxidase system. Another similarity between the xanthine oxidase system and the ascorbate system is that the production of $^{\bullet}OH$ by both systems is catalyzed by iron. Essentially no formaldehyde is produced from Me_2SO when iron-EDTA is omitted from the ascorbate reaction mixture (data not shown). Both systems also share similar sensitivities to catalase i.e. H_2O_2 is the precursor of $^{\bullet}OH$ in both systems. However, the xanthine oxidase system and the ascorbate system differ in their sensitivities to superoxide dismutase and DETAPAC. These two differences suggest that the reducing agent which is utilized by each system to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is different. Iron is rapidly oxidized by oxygen to the ferric state (half-life less than 15 sec)

in the presence of EDTA and phosphate buffer (205). Therefore, reduction of Fe^{3+} back to Fe^{2+} is required when iron acts to catalyze the production of $^{\bullet}OH$ from H_2O_2. In the xanthine oxidase system, $O_2^{\cdot-}$ reduces the ferric iron to the ferrous state.

$$Fe^{3+}\text{-EDTA} + O_2^{\cdot-} \longrightarrow Fe^{2+}\text{-EDTA} + O_2 \qquad (13)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + {}^{\bullet}OH + OH^- \qquad (14)$$

$$\text{Net:}\quad O_2^{\cdot-} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2 \qquad (12)$$

This requirement for $O_2^{\cdot-}$ explains why superoxide dismutase and DETAPAC (which prevents the reduction of Fe^{3+} by $O_2^{\cdot-}$) are strongly inhibitory in the xanthine oxidase system. However, in the ascorbate system, ferric iron is reduced by ascorbate to the ferrous state (206, 207, equation 17). Therefore ascorbate serves as the functional analog of $O_2^{\cdot-}$. Autoxidation of Fe^{2+} produces H_2O_2 (equation 18). A Fenton reaction between Fe^{2+} and H_2O_2 (equation 19) results in the production of $^{\bullet}OH$. The net reaction for the iron-catalyzed oxidation of ascorbate is shown in equation 20.

$$3Fe^{3+} + 3 \text{ Ascorbate} \longrightarrow 3 \text{ Dehydroascorbate} + 3Fe^{2+} \quad (17)$$

$$2Fe^{2+} + O_2 \xrightarrow{2H^+} 2Fe^{3+} + H_2O_2 \quad (18)$$

$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (19)$$

Net: $3 \text{ Ascorbate} + O_2 \longrightarrow {}^{\bullet}OH + OH^- + 3 \text{ Dehydroascorbate}$ (20).

The general lack of sensitivity of the ascorbate system to either superoxide dismutase or DETAPAC is therefore due to the fact that ascorbate, rathar than $O_2^{\cdot-}$ reduces the iron in this system. The different sensitivities of the xanthine oxidase and the ascorbate systems to inhibition by DETAPAC may reflect the greater reactivity of the iron-DETAPAC complex with ascorbate then with $O_2^{\cdot-}$ (Tables 4 and 5). It has been suggested that DETAPAC inhibits the iron-catalyzed Haber-Weiss reaction as a result of it ability to interfere with superoxide-dependent reduction of iron (equation 13) rather than the Fenton Type reaction (equation 14) (201). Therefore, DETAPAC inhibits the production of ${}^{\bullet}OH$ and of formaldehyde from Me_2SO) in the xanthine oxidase system where $O_2^{\cdot-}$ is the reducing agent but has a relatively weak effect in the ascorbate system where ascorbic acid, rather than $O_2^{\cdot-}$, is the reducing agent. It is also possible that the different rates of formaldehyde production by the ascorbate system as compared to the xanthine oxidase system may con-

tribute to the different sensitivities of the two systems to superoxide dismutase and DETAPAC.

In summary, $^{\bullet}OH$ is generated by both the xanthine oxidase system and by the ascorbate system via a Fenton reaction in which the reduction of iron is brought about either by superoxide anion radicals or by ascorbate, respectively. Therefore, the inability of H_2O_2 alone, in the absence of either xanthine or xanthine oxidase or ascorbate, to promote formaldehyde production, is probably due to the lack of an agent, namely $O_2^{\cdot-}$ or ascorbate, to reduce Fe^{3+} to Fe^{2+}. If superoxide dismutase was added to the xanthine oxidase system after ten minutes of incubation, then further production of formaldehyde from Me_2SO was prevented. It appears that continuous generation of $O_2^{\cdot-}$ is required for the continuous generation of H_2O_2 and for the continuous reduction of ferric to ferrous iron in the xanthine oxidase system.

The ability of rat liver microsomes to produce formaldehyde from Me_2SO during NADPH-dependent electron transfer has also been characterized (Figure 10). This system of oxidizing Me_2SO to formaldehyde is a) inhibited by competing $^{\bullet}OH$ scavengers (Table 8), b) stimulated by azide (Figure 10) and c) stimulated by iron-EDTA (Figures 13 and 14) in a reaction which is blocked by competing $^{\bullet}OH$ scavenging agents (Table 9 and 10). Similar properties have been ob-

served for the microsomal metabolism of other $^{\bullet}OH$ scavenging agents (111, 124, 160, 170-173). Therefore, it appears that the production of formaldehyde during the metabolism of Me_2SO by rat liver microsomes is due to the interaction of Me_2SO with $^{\bullet}OH$, or a species with the oxidizing power of $^{\bullet}OH$, which is generated during microsomal electron transfer.

The oxidation of NADPH by microsomes generates H_2O_2 (181, 208). In the presence of azide to prevent the decomposition of H_2O_2 by contaminating catalase, the production of formaldehyde is stimulated approximately four-fold (Figure 10, 175). This extent of stimulation by azide is similar to that observed with other $^{\bullet}OH$-scavenging agents. The addition of H_2O_2 in the absence of the NADPH-generating system, however, did not result in the production of formaldehyde from Me_2SO (Table 7). Therefore, it appears that H_2O_2 plays an indirect, although a major pivotol role, in the generation of $^{\bullet}OH$, and subsequently in formaldehyde production from Me_2SO. As described earlier, H_2O_2 may serve as a precursor of $^{\bullet}OH$ via a Fenton reaction or a modified, i.e. iron-catalyzed, Haber-Weiss reaction with iron-chelates (equations 13 and 14). External addition of iron-EDTA stimulates the microsomal oxidation of Me_2SO in a

reaction which is sensitive to inhibition by competing $^{\bullet}OH$ scavengers. This observation suggests that in the microsomal system, conditions may be appropriate for a Fenton or modified Haber-Weiss reaction to occur, in analogy to the conditions which were described above for the xanthine oxidase system or the ascorbate system. Of considerable interest is the observation that even in the absence of azide, iron-EDTA stimulates the production of formaldehyde, and competing $^{\bullet}OH$ scavengers block this stimulation (Figure 14 and Table 10). These results suggest that the added iron-chelate, compete with catalase for metabolically generated H_2O_2 (equation 21).

$$^{\bullet}OH \xleftarrow[b]{Fe^{2+}\text{-EDTA}} H_2O_2 \xrightarrow[a]{\text{catalase}} \text{Compound 1} \qquad (21)$$

Since, in the absence of azide, catalase will decompose H_2O_2, there may be little utilization of H_2O_2 to produce $^{\bullet}OH$. Hence formaldehyde production is low (equation 21a). However, added iron-EDTA may compete effectively with catalase for H_2O_2 and promote the generation of $^{\bullet}OH$ (equation 21b) by a Fenton-like mechanism. This results in an increase in the production of formaldehyde in the absence of azide.

These results suggest that formaldehyde is produced during NADPH-dependent microsomal electron transfer

by interaction of Me_2SO with $^{\bullet}OH$ which is generated via a reaction between metabolically generated H_2O_2 and iron-chelates. In the absence of exogenously added iron-EDTA, there is probably some endogenous source(s) of iron which catalyzes the production of $^{\bullet}OH$ by rat liver microsomes. Further support for a role for endogenous iron in the catalysis of the microsomal production of $^{\bullet}OH$ is the observation that DETAPAC inhibits the production of formaldehyde from Me_2SO (Table 11). The inhibition which is produced by DETAPAC strongly suggests that $^{\bullet}OH$ is generated by the microsomal system via an iron-catalyzed Haber-Weiss reaction, in analogy to the xanthine oxidase system. Prevention (by DETAPAC) of the $O_2^{\cdot-}$ dependent reduction of ferric iron to ferrous iron interferes with iron cycling between the reduced and oxidized forms. Thus, DETAPAC diminishes microsomal $^{\bullet}OH$ generation and therefore formaldehyde production is decreased.

In contrast to the xanthine oxidase system, chelex-100 treatment has no significant effect on formaldehyde production from Me_2SO by the microsomal system (Figure 15). Therefore, there must be a sufficient source of iron which is endogenous to the microsomes that contributes to the production of $^{\bullet}OH$ in this reaction system. Possible sources of these endogenous iron-chelates are

microsomal hemoproteins, e.g. cytochrome P-450, heme itself and/or adventitious iron-chelates which are present in the microsomes. At the present time, the specific nature of the endogenous microsomal iron is not known. It is believed that cytochrome b_5 and NADH-cytochrone b_5 reductase do not play a major role in microsomal $^{\bullet}OH$ generation since NADH cannot substitute for NADPH in these experiments and NADH does not enhance the rate of $^{\bullet}OH$ generation which is found in the presence of NADPH (A.I. Cederbaum, unpublished observations). Several experiments tend to dissociate the microsomal production of $^{\bullet}OH$ from the classical cytochrome P-450 mixed function oxidase system. DETAPAC was a much less effective inhibitor of aminopyrine demethylase (A.I. Cederbaum, unpublished observations) than it was against the microsomal production of formaldehyde from Me_2SO. If cytochrome P-450 catalyzes both $^{\bullet}OH$ production and aminopyrine demethylation by a similar mechanism, then one would expect iron-chelators, such as DETAPAC, to have the same effect on both microsomal activities. Similar results were found with another iron-chelator, namely desferrioxamine (E. Dicker and A.I. Cederbaum, unpublished observations). Moreover, mixed function oxidase inhibitors such as metyrapone and SKF-525A inhibit the cytochrome P-450 dependent production of formaldehyde from aminopyrine but have no effect on the

$^{\bullet}OH$ dependent production of formaldehyde from Me_2SO (Table 12). In addition organic hydroperoxides, such as cumene-hydroperoxide and tertiary-butyl hydroperoxide, cannot support the production of formaldehyde from Me_2SO under reaction conditions which support cytochrome P-450 dependent mixed function oxidase activity (Figure 16 and 181, 203, 204). Me_2SO did not appear to react with cytochrome P-450 since no substrate binding spectrum was observed when Me_2SO was added to the microsomes (Figure 17). Taken together, these data indicate that a different mechanism exists for the microsomal generation of $^{\bullet}OH$ and for the metabolism of substrates of the cytochrome P-450 dependent mixed function oxidase system. This conclusion is further supported by the fact that externally-added iron-EDTA and competing $^{\bullet}OH$ scavenging agents affect the microsomal metabolism of other $^{\bullet}OH$-scavenging agents but have no effect on cytochrome P-450 dependent drug metabolizing reactions of microsomes (124, 172).

Recent experiments were carried out concerning the metabolism of ethanol and $^{\bullet}OH$ scavengers by reconstituted systems containing cytochrome P-450 and NADPH-cytochrome P-450 reductase which were purified from microsomes from phenobarbital-treated rats (G. Winston, unpublished observations). In preliminary experiments, it has been observed that the production of formaldehyde from Me_2SO

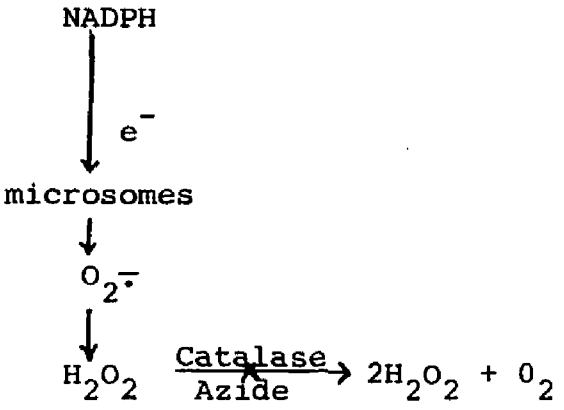
is independent of the concentration cytochrome P-450 but is directly related to the concentration of NADPH-cytochrome P-450 reductase. Thus it would appear that the reductase plays the major role in generating $O_2^{\cdot-}$, and subsequently H_2O_2, when NADPH is oxidized by this enzyme. The reductase contains FMN and FAD as cofactors. Autoxidation of reduced flavins can produce $O_2^{\cdot-}$ in biological systems (9). It has been directly demonstrated, using spin-trapping agents that NADPH cytochrome P-450 reductase can generate $^{\bullet}OH$ (152). It was suggested that the reductase produced $^{\bullet}OH$ directly from $O_2^{\cdot-}$ (152) which is known to be generated by this enzyme (32, 45).

Based on these results, a conceptual scheme to describe the mechanism of $^{\bullet}OH$ generation during microsomal electron transfer is described in Figure 24. NADPH transfers electrons to the microsomal NADPH cytochrome P-450 reductase. During this process, $O_2^{\cdot-}$ and H_2O_2 are produced. In the presence of azide to inhibit contaminating microsomal catalase, H_2O_2 accumulates so that it may serve as a precursor of $^{\bullet}OH$. Microsomal ferric iron-chelates (of unknown origin) then catalyze a Haber-WEiss reaction in which the iron-chelates are alternately

Figure 24.

The Generation of $^{\bullet}OH$ During NADPH-Dependent Microsomal Electron Transfer.

During microsomal NADPH-dependent electron transfer, $O_2^{\cdot-}$ and H_2O_2 are produced. In the presenct of azide, H_2O_2 interacts with microsomal iron-chelates to produce $^{\bullet}OH$.



$$\text{microsomal } Fe^{3+}\text{-chelate} + O_2^{\cdot-} \longrightarrow \text{microsomal } Fe^{2+}\text{-chelate} + O_2$$

$$\underline{\text{microsomal } Fe^{2+}\text{-chelate} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + \text{microsomal } Fe^{3+}\text{-chelate}}$$

reduced and oxidized. It is assumed that the microsomal ferric-chelates are reduced to ferrous-chelates either by interaction with $O_2^{\cdot-}$ or by reaction with reduced components of the microsomal electron transfer chain e.g. reduced flavin. Since superoxide dismutase does not readily penetrate the microsomal membrane, it is not possible to test the role of $O_2^{\cdot-}$ directly. However, DETAPAC, which prevents the reduction of ferric iron-chelates by $O_2^{\cdot-}$ (201, and results in this report), inhibits the $^{\bullet}OH$-dependent production of formaldehyde from Me_2SO. Therefore, it appears likely that $O_2^{\cdot-}$ is in fact responsible for the reduction of microsomal ferric iron-chelates. EDTA stimulates the oxidation of Me_2SO (see Results). This stimulation suggests that iron must be in a chelated form (e.g. iron-EDTA) to catalyze $^{\bullet}OH$ production. In fact, when EDTA was not added, very low concentrations of DETAPAC could potentiate the microsomal production of formaldehyde (data not shown) probably by chelating the endogenous microsomal iron. Thus $^{\bullet}OH$ is produced during NADPH-dependent microsomal electron transfer via an iron-catalyzed Haber-Weiss reaction with endogenous microsomal iron-chelates. When Me_2SO is introduced into this system, formaldehyde is produced due to interaction with $^{\bullet}OH$.

In view of the suggested role for $^{\bullet}OH$ in the pathway of ethanol oxidation by microsomes from chow-fed rats (160, 170, 172, 194), experiments were carried out to determine the role of these radicals in ethanol oxidation by microsomes from ethanol-fed rats. Specifically, these experiments were carried out to evaluate whether the increase in ethanol oxidation by these induced microsomes correlates with an increase in the generation of $^{\bullet}OH$. To determine the rate of production of $^{\bullet}OH$ by rat liver microsomes from ethanol-fed rats and their pair-fed controls, the rate of production of formaldehyde from Me_2SO was determined as was the rate of production of ethylene gas from KTBA. Microsomes from chronic ethanol-fed rats catalyze the oxidation of both Me_2SO and KTBA at rates which are two to three-fold faster than the rates which are observed with the microsomes from control rats (Figures 19 and 20). The increase in the oxidation of these $^{\bullet}OH$ scavenging agents correlates with the increase in the oxidation of ethanol (three-fold) (Figure 18).

To correlate the oxidation of Me_2SO, KTBA and ethanol with a common intermediate, cross competition experiments were carried out. The results of these experiments suggest that in the microsomes from both the ethanol-fed

rats as well as from the pair-fed controls, the oxidation of these three substrates, i.e. Me_2SO, KTBA and ethanol, is in fact brought about by a common intermediate, namely, the hydroxyl radical (Figures 21-23). Both microsomal preparations exhibit comparable extents of inhibition by a competitor against any one of the substrates. These results indicate that the mechanism by which inhibition is brought about by the competitor is similar in both preparations, namely, competition for $^{\bullet}OH$. It would be expected that the extent of competition, and hence the extent of inhibition, would be the same even though the rates of $^{\bullet}OH$ production in the two microsomal preparations may be different. These conclusions are further supported by the observation that both DMPO and benzoate, two other potent $^{\bullet}OH$ scavenging agents, inhibit Me_2SO, KTBA and ethanol oxidation in a dose-dependent manner and to similar extents in both microsomal preparations (Table 16).

These results suggest that the increase in the rate of ethanol oxidation found with microsomes from chronic ethanol-fed rats may be due, at least in part, to an increase in the rate of production of $^{\bullet}OH$ by these induced microsomes. The mechanism(s) which are responsible for this increase in $^{\bullet}OH$ production and of ethanol oxidation remain to be elucidated. It has been

demonstrated that reconstituted systems which contain purified cytochrome P-450 oxidize ethanol (186, 187). The cytochrome P-450 which was purified from chronic ethanol-fed rats was more active in these reconstituted systems in catalyzing ethanol oxidations than was cytochrome P-450 from pair-fed controls (187). Hence, the increase in ethanol oxidation and of the oxidation of $^{\bullet}OH$ scavengers such as Me_2SO and KTBA may reflect induction by chronic ethanol of a more active cytochrome P-450. However, as was described above, recent experiments indicate that cytochrome P-450 may not play a significant role in the production of $^{\bullet}OH$, and the mechanism of microsomal $^{\bullet}OH$ production and of the microsomal mixed function oxidase system have been differentiated. Alternatively, after chronic ethanol consumption, the rate of H_2O_2 production by microsomes is increased due to an increase in the activity of microsomal NADPH oxidase (183, 210). Increased generation of H_2O_2 results in increased $^{\bullet}OH$ production by the microsomes from the ethanol-fed rats. Azide was shown to increase the oxidation of Me_2SO and KTBA in both microsomal preparations (Table 15). This stimulation by azide confirms the pivotal role of H_2O_2 as a precursor of $^{\bullet}OH$. Other possibilities, such as increased iron content in these

microsomes from the chronic ethanol-fed rats may also contribute to the increase in $^{\bullet}OH$ production which is found with these microsomes. Indeed, chronic ethanol consumption is associated with iron-overload in the liver (211). Regardless of the mechanism, the increase in ethanol oxidation by microsomes from chronic ethanol-fed rats is associated with an increase in $^{\bullet}OH$ production.

Does this increase in $^{\bullet}OH$ production play a role in the increased rate of ethanol clearance found *in vivo* after chronic ethanol consumption? The possibility that increased production of $^{\bullet}OH$ may contribute to the cytotoxic damage of the liver cell found after chronic ethanol consumption remains an interest speculation. Normally, the various cellular defensive mechanisms and antioxidants prevent or limit $^{\bullet}OH$ generation *in vivo*. However, as described earlier (see Literature Review), cellular defensive mechanism can be overwhelmed under certain conditions. In this context it is interesting that glutathione levels are decreased after ethanol consumption (212-214). Glutathione is a major component of the cellular defense mechanism against oxygen radical damage. Increased production of oxygen radicals, coupled to a decrease in the level of protective agents such as glutathione, may result in oxygen radical induced damage to the liver cell and

thereby contribute to the cytotoxicity found after chronic ethanol consumption.

Previously, the oxidation of Me_2SO to methane gas was utilized to detect $^{\bullet}OH$ which was generated by several biological reaction systems (111, 113, 160, 161). In all three reaction systems which were tested in the current report, the oxidation of Me_2SO is found to yield formaldehyde, rather than methane, as a predominant product (Table 6 and microsomal results). Moreover, in the microsomal system, the rate of formaldehyde production from Me_2SO was of the same order of magnitude as the rate of ethylene generation from KTBA or methanol (160). In contrast, methane production from Me_2SO by this system was an order of magnitude less than the generation of ethylene from KTBA or methional. Therefore, in all three systems which were tested, formaldehyde, rather than methane represents a main product of the metabolism of Me_2SO by $^{\bullet}OH$. The usefulness of this tool for the detection of $^{\bullet}OH$ is enhanced by the fact that the detection of formaldehyde by the Nash method is simple and inexpensive to execute. In addition, the assay for formaldehyde is extremely sensitive since it is possible to measure formaldehyde spectrofluorometrically. The spectrofluorometric measurement of formaldehyde is at least three orders of magnitude more sensitive than the spectrophotometric

assay.

The mechanism by which formaldehyde is produced from Me_2SO may involve the following. Dimethylsulfoxide interacts with $^{\bullet}OH$ radicals to give rise to methyl radicals ($^{\bullet}CH_3$, see Figure 2) (167, 168). The $^{\bullet}CH_3$ radical may abstract a hydrogen atom from available substrates to form methane or it may dimerize to form ethane (160). Under conditions in which a large pulse of $^{\bullet}OH$ is generated instantaneously, e.g. during the pure Fenton reaction between H_2O_2 and Fe^{2+}, large amounts of methane and ethane are produced from Me_2SO. These are conditions which produce large steady state levels of $^{\bullet}CH_3$ and hence hydrogen abstraction to form methane, or dimerization to produce ethane, is favored. When $^{\bullet}OH$ is slowly generated, e.g. by the systems which are described in this dissertation, $^{\bullet}CH_3$ is produced at a slower rate and formaldehyde, rather than methane or ethane, is the predominant product. Under these conditions, $^{\bullet}CH_3$ reacts with molecular oxygen to produce the methyl peroxy radical (CH_3-O-O$^{\bullet}$) (215). Organic peroxy radicals can undergo a chain termination reaction in which two organic peroxy radicals interact to produce the corresponding alcohol and aldehyde with the evolution of molecular oxygen (the Russell mechanism, (216)). The decomposition of the methyl peroxy radical via a Russell type mechanism can

result in the production of formaldehyde and methanol (equation 22).

$$2\ CH_3\text{-}O\text{-}O^{\bullet} \longrightarrow CH_2O + CH_3OH + O_2 \qquad (22)$$

To evaluate whether the Russell mechanism is responsible for the production of formaldehyde, assays for both formaldehyde and methanol as the end-products of the interaction of Me_2SO with $^{\bullet}OH$ were carried out. Methanol was assayed by treating aliquots of the reaction system (ascorbate-iron-EDTA system) with NAD^+ plus alcohol dehydrogenase and monitoring the absorbance change at 340nm. It was anticipated that equimolar amounts of formaldehyde and methanol would be produced. However, no methanol could be detected in these experiments under conditions in which large amounts of formaldehyde are produced. Therefore, although the Russell mechanism remains attractive, the exact mechanism by which formaldehyde is produced from Me_2SO, and from $^{\bullet}CH_3$, remains to be elucidated.

It has recently been reported that alkoxy radicals ($^{\bullet}OR$) and peroxy radicals ($^{\bullet}OOR$) give rise to ethylene gas upon interaction with methional (161, 162). Therefore the production of ethylene gas from methional is not a specific indicator of the presence of $^{\bullet}OH$. Similar results have recently been obtained for the production of ethylene from KTBA, or acetaldehyde from ethanol, or acetone from isopropanol by cumene alkoxy radicals and

tertiary-butyl alkoxy radical (A.I. Cederbaum, unpublished observations). In these experiments, the rate of product generation from KTBA, ethanol or isopropanol was measured in the presence of $^{\bullet}OH$, cumene alkoxy radicals and tertiary-butyl alkoxy radicals. These radicals were generated via a Fenton reaction between iron-diperidyl and either H_2O_2, or cumene hydroperoxide or tertiary-butyl hydroperoxide, respectively. The rates of ethylene, acetaldehyde or acetone production by the cumene and the tertiary-butyl alkoxy radicals were similar to rates that were observed with $^{\bullet}OH$ (H_2O_2 system). Experiments were carried out to determine whether the oxidation of Me_2SO is specific for interaction with $^{\bullet}OH$ or whether Me_2SO also interacts with organic peroxy radicals. Formaldehyde was not produced when $^{\bullet}OH$ was generated via the Fenton reaction between H_2O_2 and ferrous-DETAPAC. Instead, large amounts of methane and ethane were produced (Table 14). As described above, a large pulse of $^{\bullet}OH$ is generated instantaneously during the Fenton reaction. This pulse of $^{\bullet}OH$ results in the production of large amounts of $^{\bullet}CH_3$ from Me_2SO. It appears that this instaneous production of $^{\bullet}CH_3$ favors hydrogen abstraction and dimerization to produce methane and ethane, respectively, instead of the production of formaldehdye. Therefore,

the end - products of the oxidation of Me_2SO by $^{\bullet}OH$ that is generated via the Fenton reaction are mainly methane and ethane. When cumene hydroperoxide or tertiary-butyl hydroperoxide replaced H_2O_2, some methane and ethane was produced. Formaldehyde was not produced from Me_2SO by these alkoxy radical generating systems. However, the production of methane and ethane in both the cumene hydroperoxide and tertiary-butyl hydroperoxide experiments (i.e., the alkoxy radical systems) were very much less than that which is produced in the H_2O_2 experiments (i.e., by $^{\bullet}OH$). This lower yield of products suggests that Me_2SO is considerably less reactive with cumene and tertiary-butyl alkoxy radicals than with $^{\bullet}OH$. To further investigate this possibility, the ability of 33mM Me_2SO to inhibit the production of ethylene from 10mM KTBA by the $^{\bullet}OH$-generating system and the alkoxy radical-generating system was compared. Me_2SO inhibited ethylene production from KTBA by 49.8% when ethylene production was catalyzed by the H_2O_2-ferrous-diperidyl system ($^{\bullet}OH$-dependent). The production of ethylene by either the cumene hydroperoxide or the tertiary-butyl hydroperoxide-ferrous-diperidyl systems ($^{\bullet}OR$-dependent) was inhibited 22.0% and 13.4% respectively by the same concentration of Me_2SO (A.I. Cederbaum, unpublished observations). Therefore, it

appears that Me_2SO reacts as readily with $^{\bullet}OH$ as does KTBA, but is considerably less reactive with alkoxy radicals than KTBA. These results indicate that Me_2SO is more specific than KTBA as a scavenger for $^{\bullet}OH$.

The production of formaldehyde from Me_2SO appears to be a useful technique by which to detect and to evaluate the role of $^{\bullet}OH$ in some biological reaction systems. Although it is clear that $^{\bullet}OH$ is produced by many biological reaction systems <u>in vitro</u>, the production of $^{\bullet}OH$ <u>in vivo</u> is much more difficult to demonstrate directly. In spite of this lack of direct evidence, some theories of aging (65), hyperbaric oxygen toxicity (55), radiation damage (129, 130) and carcinogenesis (63, 138) invoke $^{\bullet}OH$ and other free radicals as having an important etiological role. Therefore, the ability to measure the production of $^{\bullet}OH$ <u>in vivo</u> is an important one. As described earlier, many of the current techniques for the detection of $^{\bullet}OH$ (even <u>in vitro</u>) suffer from lack of specificity or from difficulty in either the execution of the method or interpretation of the results.

Recently the measurement of exhaled ethane and to a lesser extent, pentane was shown to be a useful tool by which to measure lipid peroxidation <u>in vivo</u> (217-223). However, this technique is a very indirect measure of

oxygen radical and $^{\bullet}OH$ radical production since there are many intervening steps between the initiation of lipid peroxidation by oxygen radicals and the evolution of these hydrocarbon gases (223). It would be useful, therefore, to have a more direct measurement of $^{\bullet}OH$ production *in vivo*. The production of radioactive formaldehyde from the interaction of radioactive (^{14}C-labeled) Me_2SO with $^{\bullet}OH$ may help to provide such a measurement. Formaldehyde is oxidized to formate via formaldehyde (aldehyde) dehydrogenase. The formate is subsequently oxidized to radiolabeled carbon dioxide via 10-formyltetrahydrofolate synthetase and 10-formyltetrahydrofolate dehydrogenase in the cytosol (224). The carbon dioxide may then be collected after exhallation, and measured by scintillation counting. Such measurements of radioactive carbon dioxide have been used to estimate the kinetics of hepatic ^{14}C-labeled aminopyrine demethylation *in vivo* (225). In analogy, it is possible that the rate of exhalation of ^{14}C-labeled carbon dioxide from ^{14}C-labeled Me_2SO may be useful to measure the rate of $^{\bullet}OH$ generation *in vivo*.

This proposed methodology may help to answer many inportant questions in free radical biology. For example, is the hydroxyl radical actually produced *in vivo* in the presence of superoxide dismutase, catalase and other

cellular antioxidants? If so, is it produced via an iron-catalyzed Haber-Weiss reaction? What are the sources, if any, of endogenous iron-chelates that may participate in the production of $^{\bullet}OH$? Does the hydroxyl radical actually play a role in the cytotocity of antitumor antibiotics and hyperbaric oxygen and even in chronic ethanol consumption in vivo? Is the cytotoxic action of $^{\bullet}OH$ exacerbated by certain nutritional conditions such as vitamin E or selenium deficiency? Most importantly, can the postulated cytotoxic action of $^{\bullet}OH$ in vivo be ameliorated or prevented by the dietary or local administration of $^{\bullet}OH$ scavenging agents? The answers to these questions would not only lay to rest many controversies in the field of free radical biology but would also open new vistas of research in this exciting area of biochemistry.

CHAPTER 5

CONCLUSIONS

The following conclusions may be drawn based on the foregoing thesis dissertation:

1) Formaldehyde is produced from the interaction of dimethylsulfoxide with hydroxyl radicals which are generated by three model hydroxyl radical-generating systems.

2) Formaldehyde, rather than methane, represents a major product of the interaction of dimethylsulfoxide with hydroxyl radicals which are generated either during the oxidation of xanthine by xanthine oxidase, or the iron-catalyzed oxidation of ascorbic acid, or during NADPH-dependent electron transfer by rat liver microsomes.

3) Hydrogen peroxide is a precursor of hydroxyl radicals in all three systems.

4) Hydroxyl radicals are generated in the xanthine oxidase and the microsomal hydroxyl radical-generating systems via an iron-catalyzed Haber-Weiss reaction. Hydroxyl radicals are produced by the ascorbate system via an ascorbate driven Fenton-type reaction.

5) Rat liver microsomes from chronic ethanol-fed rats generate hydroxyl radicals at an increased rate.

6) The increased rate of hydroxyl radical generation by microsomes from ethanol-fed rats may be responsible, at least in in part, for the increased rate of ethanol oxidation by these preparations.

7) The production of formaldehyde from Me_2SO may represent a convenient technique to detect and to evaluate the role of hydroxyl radicals in some biological systems.

8) In view of the production of formaldehyde, dimethyl-sulfoxide should not be considered to be an inert solvent in biological systems.

* * *

CHAPTER 6

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