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by

Graciela Krikun

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APPROVAL PAGE

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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Graciela Krikun

Adviser: Dr. A.I. Cederbaum

Isolated rat liver microsomes can oxidize a variety of alcohols to their corresponding aldehydes. Recent experiments have suggested that microsomes have the potential to oxidize alcohols by two pathways: one dependent on hydroxyl radicals, the other apparently dependent on cytochrome P450.

Studies in this thesis were carried out to determine the loci of each pathway in intact microsomes. As was the case in reconstituted systems, it was seen that the hydroxyl radical dependent pathway was dependent on NADPH-cytochrome-P450 reductase. It was also shown that the cytochrome P450 pathway could be inhibited by levels of carbon monoxide which did not cause anaerobiosis and hence did not inhibit the formation of oxygen radicals.

The role of each pathway was compared for microsomes isolated from chronic alcohol induced rats versus their pair fed controls. It was seen that the increase in ethanol oxidation after alcohol treatment was due to the increase in both pathways and that the percent contribution of each pathway was the same as it was for control rats.

Stereospecificity studies were carried out with microsomes from normal and differently induced rats. Results show that neither pathway was stereospecific with microsomes from control, phenobarbital in-

duced or dextrose pair fed animals. On the other hand, microsomes isolated from ethanol treated rats displayed stereospecificity via the cytochrome P450 pathway for the (+) isomer of 2-butanol. Initial experiments showed that 2-butanol was a particularly good alcohol substrate for the cytochrome P450 pathway, especially with microsomes from ethanol treated rats. 2-Butanol displayed a binding spectrum with microsomes from alcohol induced rats but not with microsomes from control or phenobarbital treated animals. Similar results were observed with dimethyl sulfoxide.

Injecting rats with pyrazole for 3 days seemed to induce a cytochrome P450 isozyme with properties similar to those described for chronic ethanol consumption.

The use of spectra and stereospecificity in conjunction with increased microsomal oxidation of alcohols can therefore be used to study the induction of the alcohol preferring P450 isozyme(s).

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To my beloved parents, thank you for always being there when I needed you, for your encouragement and your belief in me. I hope this day will bring you all the "naches" that you deserve.

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LIST OF ABBREVIATIONS

CO	carbon monoxide
Co IX	cobalt protoporphyrin IX
DMSO	dimethyl sulfoxide
ESR	electron spin resonance
Fe	iron
H_2O_2	hydrogen peroxide
KTBA	2-keto-4-thiomethyl butyric acid
MEOS	microsomal ethanol oxidizing system
$NADP^{(+)}$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$O_2^{\cdot -}$	superoxide anion radical
·OH	hydroxyl radical (or a species with the oxidizing power of the hydroxyl radical)
SOD	superoxide dismutase

CHAPTER I INTRODUCTION AND BACKGROUND

Microsomes

Electron micrographs of rat hepatocytes reveal the endoplasmic reticulum to be present throughout the cytoplasm as an extensive network of tubules, vesicles, and lamellae (1,2). The functions of this organelle include, among others, the metabolism of xenobiotics, steroids and fatty acids by the cytochrome P450 system. Treatment of animals with phenobarbital, carcinogens and other xenobiotics is well known to cause an increase in the volume and surface area of the endoplasmic reticulum together with a substantial increase in activity of the cytochrome P450 drug metabolizing system (3,4). Chronic alcohol consumption has also been shown to have similar effects (5-7). This adaptability and functional importance of the endoplasmic reticulum have resulted in its being the object of numerous isolations, subfractionations, and other investigations.

The membrane vesicles formed from the endoplasmic reticulum, following disruption of cell structure by homogenization are termed "microsomes". These particles of broken and resealed endoplasmic reticulum remain in the supernatant after centrifuging a liver homogenate at 10,000 xg for up to 20 minutes but which are sedimented at 100,000 xg for 1 hour. The studies to be described in this thesis were carried out using liver microsomes isolated from induced and uninduced rats.

Cytochrome P450

Introduction

The protoporphyrin nature of cytochrome P450 was first reported in

1962 by Omura & Sato (8). The authors proposed the tentative name "P-450", meaning "a pigment with an absorption at 450". This typical absorption spectra was observed upon bubbling carbon monoxide into a dithionite reduced microsomal suspension. They also found that upon treatment of microsomes with detergent the pigment was quantitatively converted to another spectrally distinct solubilized form which still retained the capacity to combine with carbon monoxide in its reduced state. The new peak absorbed at 420 and was therefore named "P-420". Cytochrome P420 turned out to be the inactivated product of cytochrome P450.

Subsequent developments occurred rapidly. Cytochrome P450 was first shown to function in hydroxylation reactions in adrenal cortex microsomes (9) and later was shown to have a similar function in liver microsomal drug oxidation (10). It now appears that cytochrome P450 is the most versatile catalyst known since it is capable of catalyzing the oxidation of fatty acids, alkanes, steroids, polycyclic hydrocarbon carcinogens and a variety of drugs and other foreign compounds.

Although cytochrome P450 is found in extrahepatic tissues such as the lung, the intestine, the kidney, the adrenal cortex, the placenta, the testis and the skin (11), the major site of activity and content is the liver.

The wide distribution of hemoproteins belonging to the class of cytochrome P450 in mammals, birds, fish, insects, bacteria, yeasts and plants emphasizes the important biological role of this oxidase.

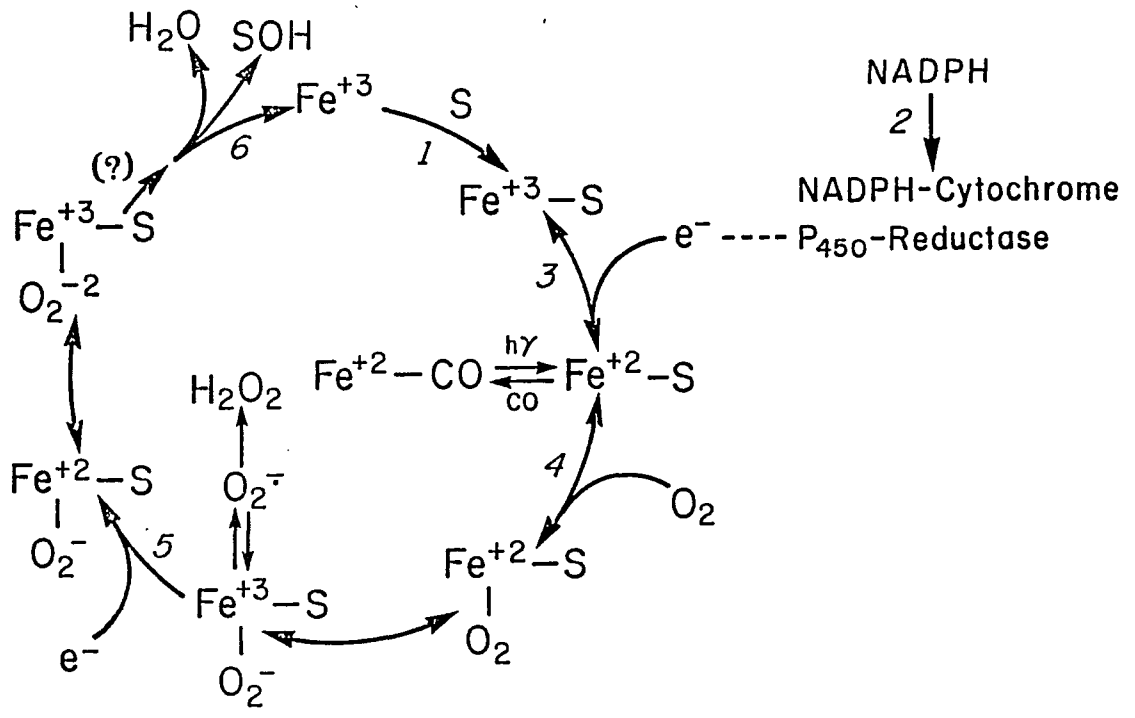
Mechanism of Action

Cytochrome P450 dependent microsomal mixed function oxidation of

most drugs, steroids, fatty acids and hydrocarbons in liver microsomes is generally thought to involve the following steps (Scheme 1):

1) Association of substrate to oxidized cytochrome P450.
2) Reduction of NADPH cytochrome P450 reductase by NADPH.
3) Reduction of cytochrome P450 substrate complex by reduced NADPH-cytochrome P450 reductase.
4) Addition of oxygen to reduced cytochrome P450 substrate complex to form reduced OXY-P-450-substrate complex.
5) Reduction of oxycytochrome P450 substrate complex by another electron, probably from reduced NADPH cytochrome P450 reductase.
6) Decomposition of peroxycytochrome P450-substrate complex to yield hydroxylated substrate, oxidized cytochrome P450 and water.

A key intermediate between steps 5 and 6 is postulated to be the iron-oxene species $(SH\text{-}FeO)^{3+}$; however this intermediate has never been observed directly.



Scheme 1:

MECHANISM OF
MICROSOMAL CYTOCHROME P_{450}-DEPENDENT HYDROXYLATIONS

Although the oxidation of exogenous compounds frequently prevents lethal doses of ingested compounds from being accumulated within an organism, examples are known where cytochrome P450 linked oxidations lead to the production of toxic and carcinogenic metabolites.

Substrate Interaction with Cytochrome P450

Formation of an enzyme-substrate complex must precede metabolism, so further evidence that the metabolism of a drug involves P450 is that specific binding of the drug to P450 occurs. The kind of spectral changes produced by the binding of a substrate to P450 were categorized and described by Schenkman et al. (12). In rat liver microsomes they were classified as type I, a change in difference spectrum that is characterized by the appearance of an absorption peak at 385-390 nm and a trough at 420 nm and type II, a spectral change characterized by an absorption minimum at 390-405 nm and an absorption peak at 425-435 nm. Spectral changes yielding an absorption maximum at 420 nm and a trough at 388-390 nm were designated as reverse type I. Alcohols typically display a reverse type I spectrum. At least forty drugs and environmental chemicals show binding spectra and might therefore be expected to undergo P450 mediated metabolism. However, the degree of specificity of the P450 binding that is manifested in these spectra, and the extent to which these reflect true enzyme-substrate complexes, are matters of debate. The fact that a drug binds in any of these modes to P450 is no absolute guarantee that it will be metabolized: TCDD (tetrachlorodibenzo-p-dioxin) gives a clear type I spectrum but is minimally metabolized by P450. Octylamine binds in a type II manner yet it is an inhibitor rather than a substrate of P450.

Species variation is a complication: aminopyrine produces a type I spectra with rat liver P450 but reverse type I spectra with human liver P450. The mode of binding may also be altered by induction of P450; nortryptiline produces a type I spectra with normal rat liver P450 but a type II spectra with phenobarbitone induced rat liver P450 (13).

Isolation and Existence of Multiple Forms

Preliminary attempts to solubilize cytochrome P450 were unsuccessful due to the instability of the enzyme in the presence of a variety of solubilizing agents such as detergents, lipases, proteases, organic solvents, and high salt concentrations, which convert P450 to the inactive form P420. Ichikawa and Yamano (14) first reported that polyols stabilize detergent treated cytochrome P450. Shortly thereafter, Lu and Coon (15) successfully solubilized rabbit liver microsomes with deoxycholate in the presence of glycerol and obtained cytochrome P450, NADPH cytochrome P450 reductase, and phosphatidylcholine fractions using DEAE cellulose chromatography. Subsequently, the mixed function oxidase activity of such reconstituted systems was demonstrated using steroids, drugs and alkanes as substrates (16-18).

The very broad substrate specificity of the cytochrome P450 dependent oxygenase activity of liver microsomes appeared to be rather unusual for a reaction catalyzed by a single enzyme species, and there was actually no strong evidence which could discount the presence of more than one molecular species of cytochrome P450 in liver microsomes, each of which might catalyze more or less specific oxygenation reactions. Studies involving drug induced increases of microsomal drug oxidizing activities showed that the rates of oxidation of a variety of

foreign compounds did not always increase in parallel with one another when animals were treated with different inducers. For example, phenobarbital and 3-methylcholanthrene, when used as inducers, showed very different selective effects on the oxidation of various drugs by the liver microsomes of treated animals (3). Such observations were provided with a material basis in 1966 when Sladek and Mannering (19) reported evidence for the presence of a different species of cytochrome P450 in liver microsomes from 3-methylcholanthrene treated rats. Since then, various forms of cytochrome P450 have been isolated from liver microsomes, purified to apparent homogeneity, and characterized on the basis of biochemical, immunochemical, and spectral properties and differences in electrophoretic mobilities. Chronic alcoholism was first shown by Ohnishi & Lieber (6) to induce a distinct form of cytochrome P450 in rat liver microsomes. Similarly, Coon and coworkers (7) have recently shown the induction by alcohol of a distinct form of cytochrome P450 in rabbit liver.

Experiments to be reported in this thesis appear to demonstrate that pyrazole induces the same or similar isozyme(s) of P450 as that induced by ethanol. It is possible that perhaps many other agents may serve to induce the same isozyme(s).

NADPH Cytochrome P450 Reductase

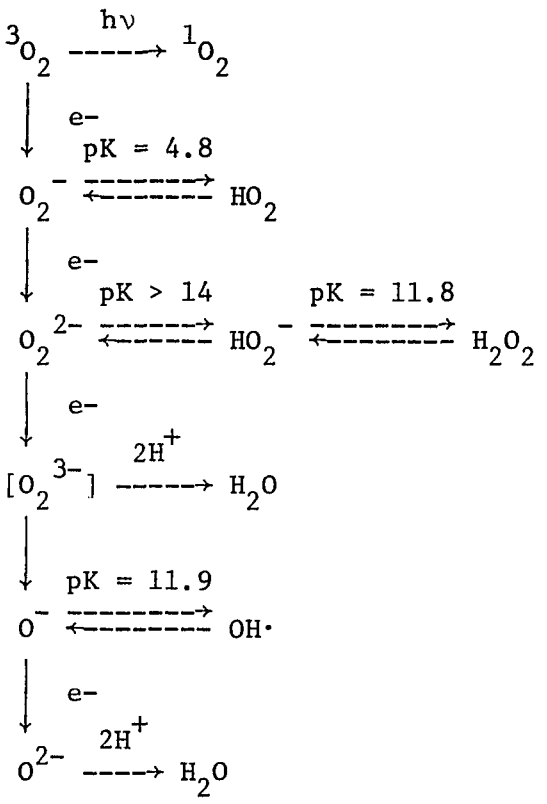
NADPH cytochrome P450 reductase is a membrane bound flavoprotein which contains one molecule each of FAD and FMN (20,21) and is required for electron transfer to cytochrome P450 in microsomes from various tissues. The reductase was first isolated as a protease treated form (22, 23). These procedures yielded a flavoprotein preparation capable of re-

ducing various artificial electron acceptors but unable to support cytochrome P450 dependent substrate hydroxylation. Because in this form the enzyme had high activity in the reduction of cytochrome c as an artificial electron acceptor, it was originally called cytochrome-c reductase. Later, the reductase was purified in native form from a variety of sources following detergent solubilization from microsomal membranes (21,24-26). The native, detergent solubilized reductase functions as a catalyst for the reduction of cytochrome P450, but is somewhat less active than the protease treated form in the reduction of cytochrome c.

Studies have shown that cytochrome P450 reductase is capable of generating a powerful oxidant with the properties of the hydroxyl radical (·OH) (27-29). More recently it's been shown that the reductase can directly reduce ferric EDTA by a superoxide dismutase insensitive reaction (30). Autoxidation of the reductase or the ferrous EDTA can thus produce precursors for the formation of ·OH (see below).

Oxygen Radicals

Oxygen is essential for the life of aerobes, but it has long been known to be toxic to them when supplied at concentrations only slightly greater than those in normal air (31). What is unclear, however, is the actual mechanism of oxygen toxicity. It is well recognized that superoxide ($O_2^{-\cdot}$) and H_2O_2 are produced in a wide range of biological systems involved in the metabolism of O_2 (32,33). In addition, $O_2^{-\cdot}$ and H_2O_2 are precursors of the very powerful oxidant, the hydroxyl radical. The relationship between the reduction products is shown in the scheme below:



In order to permit survival in an aerobic environment, a variety of mechanisms have evolved to scavenge and destroy these toxic intermediates. Following is a summary describing the formation of oxygen radicals together with the specific cellular defensive mechanisms which prevent their toxicity.

The Superoxide Anion Radical ($O_2^{-\cdot}$):

A number of biological reactions have been shown to produce $O_2^{-\cdot}$. These include the autoxidation of hydroquinones, leukoflavins, catecholamines, thiols and tetrahydropterins (34-39). Hemoglobin and myoglobin slowly liberate $O_2^{-\cdot}$ as they are converted to methemoglobin and metmyoglobin (40,41). A number of enzymes, including xanthine oxidase and aldehyde oxidase produce $O_2^{-\cdot}$ as do several flavin-containing dehydrogenases (32,42). Subcellular organelles such as mitochondria and micro-

somes produce $O_2^{\cdot-}$ (43). Phagocytic cells have been shown to liberate large amounts of $O_2^{\cdot-}$ during the respiratory burst that accompanies active phagocytosis (44). It has been suggested that the toxicity of many drugs such as alloxan (45) or paraquat (46) is due to a large production of $O_2^{\cdot-}$. 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 powerful oxidizing agent such as the hydroxyl radical. Since the pioneering work of McCord and Fridovich (47), it has been clearly established that O_2 utilizing organisms contain an enzyme known as superoxide dismutase (SOD) which catalyzes the reaction described by Equation 1 and brings about a rapid removal of $O_2^{\cdot-}$ at physiological pH values.

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

Three types of SOD's, falling into two evolutionary families, have thus far been described. These are the Cu/Zn-, and the Mn- and Fe-containing SOD's. The Mn-SOD's and the Fe SOD's are closely related, as shown by amino acid sequence homologies, whereas the CuZn-SOD's represent an independent line of descent (48). The CuZn-SOD's are characteristically found in the cytosol of the eukaryotes. The Mn-SOD's have most often been found in prokaryotes and also occur within mitochondria (48). Fe-SOD's are found in prokaryotes and in certain plants (49).

Exposure of many animal, plant and bacterial systems to increased O_2 concentrations causes them to increase their SOD activity, which increases their ability to tolerate the higher O_2 concentrations (50, 51). Organisms such as Bacillus subtilis, which cannot respond in this

way cannot adapt to elevated O_2 concentrations (52). The presence of SOD in some anaerobes may well relate to the fact that many so called "strict anaerobes" can survive brief exposure to O_2, albeit with growth inhibition (50). Paraquat greatly increases the production of $O_2^{-\cdot}$ under aerobic conditions and hence has been used as a defoliant agent (53). Experiments have shown that paraquat tolerant lines of Lolium perenne and Conyza possess higher activities of SOD (54,55). Evidence of this type indicates that SOD serves as an essential defense mechanism against oxygen and superoxide mediated toxicity.

Hydrogen Peroxide

Several cellular enzymes are oxidases which transfer two electrons onto each O_2 molecule used, to make hydrogen peroxide:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \qquad (2)$$

Examples are urate oxidase and L-amino acid oxidase. In addition, all reactions that produce $O_2^{-\cdot}$ are potential producers of H_2O_2 via dismutation of the anion, i.e., hydrogen peroxide is produced by the autoxidation of some of the components of the electron transport chain (56). Isolated microsomes incubated with NADPH generate large quantities of H_2O_2 (57). H_2O_2 at high concentrations will kill most cells even though H_2O_2 per se is not very reactive. In the presence of transition metal ions, however, H_2O_2 can serve as the precursor to the more reactive hydroxyl radical.

Two types of enzymes exist to remove H_2O_2 within cells. They are catalase, which catalyzes the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (3)$$

and the peroxidases, which bring about the general reaction:

$$SH_2 + H_2O_2 \rightarrow S + 2H_2O \qquad (4)$$

in which SH_2 is a substrate that becomes oxidized, e.g., ethanol, formate.

Animal tissues contain the enzyme glutathione peroxidase which is specific for glutathione. The enzyme is located mainly in the cytosol, although some activity is present in the mitochondria (58). The second enzyme, catalase, is located in the peroxisomes. Catalase has an enormous V_{max} and breaks down high concentrations of H_2O_2 very rapidly, but is not very effective at decomposing low concentrations because of its low affinity for this substrate. Because it is compartmentalized in peroxisomes, catalase is probably responsible for decomposing H_2O_2 generated in this organelle, while glutathione peroxidase may be more important in decomposing cytosolic or mitochondrial H_2O_2.

The Hydroxyl Radical

The hydroxyl radical (or a species with the oxidizing power of the hydroxyl radical) is generated indirectly by many biological reaction systems. For example, ·OH is generated during the iron catalyzed oxidation of ascorbic acid (59); the oxidation of xanthine by xanthine oxidase (60); NADPH dependent microsomal electron transfer (61); and by phagocytosing polymorphonuclear leukocytes (62). Two H_2O_2 dependent pathways are often invoked as sources of ·OH in biological systems as outlined in the following equations:

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot \qquad (1)$$

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

$$Fe^{+2}\text{-chelate} + H_2O_2 \rightarrow Fe^{+3}\text{-chelate} + OH^- + \cdot OH \qquad (3)$$

Reaction (1) is known as the Haber-Weiss reaction (63) and reaction (3)

is known as the Fenton reaction (64). It is now generally accepted that the rate constant for the uncatalyzed Haber-Weiss reaction is too slow to account for the formation of ·OH in biological systems (65). However, the Haber-Weiss reaction can be catalyzed by transition metals, especially iron. Taken together, equations (2) and (3) represent the iron catalyzed Haber-Weiss reaction. The feasibility of reactions (2) and (3) was demonstrated by the ability of iron EDTA to stimulate the generation of ·OH during the oxidation of xanthine by xanthine oxidase (66) and during NADPH dependent microsomal electron transfer (67). In addition to its feasibility, the iron catalyzed Haber-Weiss reaction can account for the inhibition of ·OH formation either by SOD, catalase or by certain iron chelating agents in many of the reaction systems that were mentioned above.

Unlike the case for $O_2^{\cdot -}$ or H_2O_2, the cell does not contain a specific enzymatic scavenging system for ·OH. By the combined actions of SOD, catalase and peroxidase the levels of $O_2^{\cdot -}$ and H_2O_2 are kept very low, thus, minimizing ·OH production. Fortunately, most of the iron in biological systems is stored in forms that do not appear to catalyze the Fenton or 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.

The Microsomal Alcohol Oxidizing System

The liver represents the main site for the oxidation of ethanol. The main pathway of ethanol metabolism in the liver is via the cytosolic enzyme, alcohol dehydrogenase, which utilizes nicotinamide ade-

nine dinucleotide as its co-factor. A minor microsomal pathway, the importance of which is greatly increased after chronic alcohol consumption, was also found to be present in the liver.

In 1965, Orme-Johnson and Ziegler (68) described a microsomal system that was capable of oxidizing methanol and ethanol. Iseri et al. (69) showed in rats that chronic administration of ethanol, as an isocaloric replacement for carbohydrate (36 percent of total calories) led to an increase in hepatic endoplasmic reticulum. It seemed likely that chronic ethanol administration to man would lead to similar results since examination of liver biopsy specimens from patients with alcoholic liver disease revealed an increase in endoplasmic reticulum (70).

Since factors other than ethanol consumption play a role in the toxicity observed with alcoholics (i.e., nutritional status, concomitant ingestion of drugs, amount or duration of ethanol consumption and intercurrent disease), studies were conducted in which the environment and diet of several volunteers could be controlled. Hence, volunteers were fed a nutritionally optimal diet and also had six drinks a day (a total of 10 ounces of 86 proof alcohol). Under these conditions, not only was there proliferation of the endoplasmic reticulum by 9 to 16 days, but in addition, routine thin needle biopsy revealed an eightfold increase in fat accumulation over the 18-day course of the experiment. Here was the characteristic "fatty liver", the first (and usually reversible) stage of liver disease (71,72). These liver changes were brought about by ingesting quantities of alcohol which did not result in any clinical signs of intoxication. Hence, drunkenness is not a prerequisite for liver damage.

Because human studies are difficult to conduct, much of the data involving the microsomal alcohol oxidizing system has been obtained

from animal models. Subsequently studies by Lieber and co-workers, using rats as their experimental animal, led to the full characterization of the microsomal alcohol oxidizing system (MEOS) (73,74). Chronic ethanol feeding increased the microsomal content of cytochrome P450, phospholipid and the activity of the NADPH cytochrome P450 reductase in microsomes (75,76). These effects of ethanol are similar to those of drugs that are metabolized by the microsomal cytochrome P450 dependent mixed function oxidase system. The dependence of MEOS on NADPH, the partial inhibition by CO and the ability of ethanol to competitively inhibit the metabolism of typical substrates (drugs) for the mixed function oxidase led to the hypothesis that MEOS involved components of the mixed function oxidase system (73). Furthermore, two hemoproteins, resolved by SDS-polyacrylamide gel electrophoresis, increased after chronic ethanol treatment. One of them was distinct from the hemoproteins induced by phenobarbital or 3-methylcholanthrene (6). Recently, Coon and coworkers have purified and characterized a unique ethanol induced cytochrome P450 isozyme (7,77) from rabbit liver. In the past, MEOS had been ascribed by many as due to contamination of isolated microsomes by alcohol dehydrogenase or catalase (78,79). Differentiation of MEOS from alcohol dehydrogenase was based on differences in the Km values for ethanol, cofactor requirements, pH optimum and effects of pyrazole, an inhibitor of alcohol dehydrogenase (73). However, differentiation of MEOS from catalase has been a subject of great controversy. Microsomes can generate H_2O_2 from added NADPH, and ethanol is a known peroxidatic substrate for catalase. Oxidation of ethanol by reconstituted systems containing purified cytochrome P450, physical separation of MEOS from catalase and alcohol dehydrogenase by column chromatography procedures (80,81), the use of azide (a potent

catalase inhibitor) in microsomal systems, the metabolism of 1-butanol (not a substrate for catalase) by microsomes (6) and alcohol metabolism catalyzed by microsomes from acatalesimic mice (82) are all clear indications that MEOS is a separate and unique system.

Although MEOS constitutes a minor pathway for alcohol metabolism under normal conditions, chronic ethanol consumption results in an induction of the microsomal pathway. This induction appears to play a role in the metabolic adaptation to ethanol displayed by alcoholics. When rats were fed a chronic alcohol diet for six weeks, the rate of ethanol oxidation was significantly increased as compared to the dextrose fed controls (73).

Part of the increase reflected an increased activity of MEOS (5). At low concentrations of ethanol, the contribution of MEOS to ethanol oxidation is approximately 10%. However, as the concentration of ethanol is elevated, the rate of ethanol oxidation is increased and the contribution of MEOS towards ethanol oxidation increases to up to 50% (83). 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 (73,84). Alcohol dehydrogenase has a Km for ethanol of about 1-2 mM whereas the Km for ethanol for MEOS is approximately tenfold higher. In view of the low Km of alcohol dehydrogenase for ethanol, the increase in the rate of ethanol metabolism at elevated concentrations of ethanol appears to reflect the activity of MEOS and not alcohol dehydrogenase. Recently, a report by Petersen et al. (85) has indicated that in mice an acute dose of alcohol stimulates the microsomal alcohol oxidizing system as well.

MEOS may be responsible at least in part for the effects seen with

drug-alcohol interactions. Ethanol has been shown to inhibit the hepatic metabolism of a variety of drugs both in vitro and in vivo (86, 87). In view of the above observations, it is important to understand the biochemical mechanism of the microsomal alcohol oxidizing system.

Role of Oxygen Radicals in MEOS

As previously mentioned, microsomes generate $O_2^{\cdot-}$ and H_2O_2 during NADPH oxidation. In the presence of catalytic amounts of iron, these organelles have the potential to produce ·OH. Recent studies have reported that microsomes are capable of generating a potent oxidant with properties similar to the ·OH radical during NADPH dependent electron transfer. Typical scavengers (probes) of ·OH such as methional, KTBA, DMSO, t-butanol or benzoate were oxidized by liver microsomes to products known to result from the interaction of these scavengers with ·OH (88-90). This provided chemical evidence for the production of ·OH by microsomes. Recent studies which used spin trapping agents to detect the presence of ·OH, provide physical evidence for the production of ·OH during microsomal electron transfer (91). Interestingly, one commonly used spin trapping agent, DMPO, inhibits the activity of MEOS by competing with ethanol for the generated ·OH (92).

The oxidation of ethanol by ·OH yields acetaldehyde. This suggested the possibility that the molecular mechanism for the oxidation of alcohols by microsomes may be due, at least in part, to the interaction of the alcohols with ·OH generated during microsomal electron transport. Microsomal oxidation of alcohols was competitively inhibited by a variety of ·OH scavengers (88,93). External addition of iron EDTA, which is known to increase production of ·OH in biological systems (66), increased the oxidation of alcohols and probes (67).

Addition of the iron chelating agent, desferrioxamine, known to block the generation of ·OH from various systems, was found to inhibit the following microsomal reactions: production of formaldehyde from either DMSO or t-butanol; generation of ethylene from KTBA; release of $^{14}CO_2$ from 7-^{14}C-benzoate; production of acetaldehyde from ethanol and production of butyraldehyde from 1-butanol (94).

Recent results from our laboratory have suggested that two pathways may be responsible for the microsomal oxidation of primary alcohols. Desferrioxamine had no effect on a typical mixed function oxidase activity, the N-demethylation of aminopyrine. Whereas desferrioxamine inhibited the oxidation of scavengers (i.e., DMSO, KTBA, t-butanol and benzoate) by more than 90%, the oxidation of the primary alcohols ethanol and 1-butanol could not be decreased by more than 45 to 60% (94).

The desferrioxamine insensitive rate of ethanol oxidation was not inhibited by competing hydroxyl radical scavengers. In addition it was shown that organic hydroperoxides, which can oxidize drugs in the absence of NADPH, NADPH cytochrome P450 reductase and molecular oxygen, also supported the oxidation of primary alcohols such as ethanol and 1-butanol (95). In contrast, the oxidation of hydroxyl radical scavengers was not supported by organic hydroperoxides (96) nor did the addition of hydroxyl radical scavengers inhibit the hydroperoxide supported oxidation of alcohols (96). The addition of desferrioxamine or iron EDTA also had no effects on the oxidation of alcohols by this system (96). These results suggested that primary alcohols may be oxidized by two pathways in microsomes, one dependent on the interaction of the alcohols with hydroxyl radicals (desferrioxamine sensitive), the other appearing to be independent of these radicals (desferrioxamine insensitive).

To determine which microsomal components participate in the two pathways, the oxidation of ethanol and typical hydroxyl radical scavengers by NADPH cytochrome P450 reductase and cytochrome P450 purified from phenobarbital treated rats were studied (28,29). Ethanol and the scavengers could be oxidized by the reductase system alone. This system was inhibited by superoxide dismutase, competing hydroxyl radical scavengers and desferrioxamine, but stimulated by either EDTA or iron. These results suggested that an iron-catalyzed Haber-Weiss reaction might be involved in the mechanism by which the reductase mediates the oxidation of typical hydroxyl radical scavengers and ethanol. The addition of cytochrome P450 had no effect on the oxidation of the scavengers, whereas the oxidation of ethanol was enhanced two- to threefold over the reductase dependent rate. The oxidation of ethanol was dependent on both the amount of reductase and P450. There was no effect of competing scavengers, superoxide dismutase or desferrioxamine on the increased rate of ethanol oxidation produced by cytochrome P450 (29).

Thus, studies with desferrioxamine, organic hydroperoxides and reconstituted systems provided support for two pathways of alcohol oxidation in rat liver microsomes. One involves the reaction with hydroxyl radicals while the other appears to involve cytochrome P450.

Recent studies have shown that chronic ethanol consumption increased the microsomal oxidation of the hydroxyl radical scavengers DMSO and KTBA. This increase was about the same as the increase in MEOS after the alcohol treatment. Moreover, part of the increase in MEOS was blocked by competing hydroxyl radical scavengers. This suggested that chronic ethanol consumption increased ·OH produced by microsomes and that increased ·OH could play a role in increasing MEOS after chronic alcohol consumption.

Studies in this thesis were carried out: a) To determine the loci of MEOS in intact microsomes, as it is possible that in purified systems the reductase may become uncoupled from efficient electron transfer to cytochrome P450. In addition the ratio of reductase to cytochrome P450 used in the purified systems was different than the ratio found in intact microsomes. b) To determine the stereospecificity of the two pathways of alcohol oxidation by induced and uninduced microsomes. It might be possible to distinguish the two pathways if one were stereospecific and the other were not. Cytochrome P450 displays stereochemical preferences for various substrates and thus may display some preference for stereoisomers of alcohols. On the other hand it seems unlikely that a species such as a free ·OH in solution would react preferentially with one stereoisomer over another. It is now generally believed that in biological systems, a highly reactive species such as ·OH would not be free in solution but rather may reflect a "·OH-like" species that is bound. Therefore, stereospecificity studies could provide some insight into the nature of the oxygen radicals produced by microsomes. c) To determine whether other simple treatments can induce the alcohol preferring cytochrome P450 isozyme(s) besides chronic ethanol consumption, the latter being time consuming, expensive and apparently affected by dietary restrictions. We have shown that pyrazole treatment appears to induce an alcohol preferring cytochrome P450 isozyme(s). d) to evaluate the relative roles of cytochrome P450 and ·OH in MEOS after chronic alcohol consumption and to investigate which pathway is responsible for the increase in MEOS after chronic ethanol ingestion.

CHAPTER II MATERIALS AND METHODS

Microsomal Preparations

Liver microsomes were prepared from male Sprague-Dawley rats. The rats were killed by decapitation and the livers immediately excised. The livers were then weighed and rinsed with an ice-cold solution of 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA (STE). The livers were minced with stainless steel scissors and rinsed once again with the STE solution to remove residual blood from the tissues. The minced livers were homogenized in the STE solution and volumes were adjusted to approximately a 1:10 dilution (gm wet weight of livers/ volume STE). Microsomes were then obtained by differential centrifugation. The homogenates were first spun at 750 x g for ten minutes to remove cellular debris and nuclei. The pellets were discarded and the supernatant was spun at 19,000 x g for ten minutes to remove mitochondria and lysosomes. The pellet was discarded and the supernatant was spun at 100,000 x g for one hour. The microsomal pellet was resuspended in 125 mM KCl and washed once at 100,000 x g and finally resuspended in 125 mM KCl. Protein was determined by the method of Lowry *et al.* (97).

Assay Conditions

The oxidation of all substrates was assayed at 37°C in 25 ml Erlenmeyer flasks containing 100 mM potassium phosphate, pH 7.4, 10 mM sodium pyrophosphate, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 2.3 units of glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, 0.1 mM EDTA, 1.0 mM azide, and about 2.5 mg of microsomal protein in a final volume of 1.0 ml. The final concentration of the substrates is indicated in the

Tables or Figure legends. The reaction was initiated with glucose-6-phosphate plus glucose-6-phosphate dehydrogenase, shaken in a 37°C water bath, and terminated in the case of KTBA, benzoate and primary and secondary alcohols by the addition of hydrochloric acid (final concentration of 300 mM) and in the case of DMSO, tertiary-butyl alcohol and aminopyrine by the addition of trichloroacetic acid (final concentration of 4.5%). In cumene hydroperoxide dependent systems, the reaction was initiated with the hydroperoxide (final concentration of 1 mM) and glucose-6-phosphate plus glucose-6-phosphate dehydrogenase were omitted.

Product Measurements

The following table represents the substrates to be assayed and their reaction products.

	Substrates	Products
A. Drugs	Aminopyrine	Formaldehyde
B. Alcohols	Ethanol	Acetaldehyde
	1-Butanol	Butyraldehyde
	2-Butanol	2-Butanone
C. ·OH Scavengers	KTBA	Ethylene
	DMSO	Formaldehyde
	t-Butanol	Formaldehyde
	[7-^{14}C]-benzoate	$^{14}CO_2$

Acetaldehyde, butyraldehyde and 2-butanone were analyzed by head space gas chromatography. After termination of the reaction, the flasks were sealed with serum stoppers and incubated at 60°C for 20 minutes. A 1.0 ml aliquot of the head space was directly injected into a Hewlett-Packard Model 5750 gas chromatograph equipped with a 6 ft. long 5% carbowax 20 M HALOPORT F 30-60 column. The injection temperature and oven tem-

perature were 100°C and 50°C, respectively. Products were assayed using a flame ionization detector. The carrier gas used was nitrogen at a flow rate of 35 ml/min. Under the above conditions, the retention times (min) were as follows: ethanol = 0.95, acetaldehyde = 0.40, 1-butanol = 3.74, butyraldehyde = 0.80, 2-butanol = 1.80, 2-butanone = 0.95.

Ethylene production from KTBA was also analyzed by head space gas chromatography. Since ethylene is very volatile even at room temperature, the flasks were sealed with serum stoppers before the reaction was initiated. All subsequent additions to the flasks were carried out by syringe through the stoppers. A 1.0 ml aliquot of the head space was directly injected into a Hewlett-Packard Model 5750 gas chromatograph equipped with a 6 ft. long Porapak N. 50-80 MESH column. The injection temperature and oven temperature were 190°C and 60°C, respectively. The carrier gas used was nitrogen at a flow rate of 35 ml/min. Under the above conditions, the retention time for ethylene was 1.80 minutes. Peak areas were quantitated by comparison to the appropriate standard curve. Formaldehyde production was assayed by the method of Nash (98) after obtaining a clear supernatant by the addition of trichloroacetic acid.

Production of $^{14}CO_2$ from [7-^{14}C]benzoate was detected as follows: The reaction flasks were fitted with gas sealing, rubber center well caps, before the reaction was initiated. All subsequent additions to the flasks were carried out by syringe through the stoppers. Following cessation of the reaction, 0.3 ml of hyamine hydroxide was added to the center well by syringe through the rubber caps. The flasks were allowed to incubate for one hour to enable absorption of the $^{14}CO_2$ into the center well. The center wells were then removed and placed directly into scintillation vials containing 10 ml of Econofluor (New England Nuclear)

and shaken vigorously. The radioactivity was then counted in a Beckman LS 9000 scintillation counter. All values were corrected for "zero time" controls in which either HCl or TCA was added to the flasks before the microsomes or where microsomes were omitted from the flasks.

Pretreatments of Rats

1) Ethanol Treatment Chronic ethanol-fed rats and their pair fed controls were obtained from Dr. Charles S. Lieber at the Bronx Veterans Administration Hospital. The treatment was as follows:

Male Sprague-Dawley rats weighing approximately 135 g were fed for four weeks a nutritionally adequate liquid diet in which ethanol provided 36% of the total calories. Pair-fed littermates consumed the same diet except that carbohydrate isocalorically replaced ethanol. The ethanol containing diet consisted of 36% of calories as ethanol, 11% as carbohydrate, 18% as protein and 35% as fat (99). Prior to the day of sacrifice, the rats received 2 aliquots of diets, one in the morning and one in the evening.

2) Phenobarbital Treatment Male Sprague-Dawley rats were injected i.p. daily for 3 days with sodium phenobarbital at a dosage of 80 mg/kg body weight/day. The animals were starved overnight before sacrifice.

3) Pyrazole Treatment Male Sprague-Dawley rats were injected i.p. for 3 days with pyrazole at a dosage of 200 mg/kg body weight/day. Controls received saline injections. The animals were starved overnight before sacrifice.

4) Cobalt Protoporphyrin IX Treatment Male Sprague-Dawley rats re-

ceived one injection of cobalt protoporphyrin IX subcutaneously at a dosage of 125 µg/kg body weight. Controls received saline injections. The animals were sacrificed 72 hours later after being starved overnight. The cobalt protoporphyrin IX is not readily soluble, hence it was first dissolved in 1N potassium hydroxide and the pH was subsequently adjusted to 7.4 with 1N HCl.

All animals, except for the pyrazole treated ones and their controls, weighed between 175 g-250 g on the day of sacrifice. Pyrazole treated rats and their saline controls weighed between 50 g-100 g.

Carbon Monoxide Inhibition

In systems which involved the addition of carbon monoxide the procedure was as follows. The flasks were sealed with serum stoppers prior to initiating the reaction. 10 cc of air were removed with a syringe and 10 cc of carbon monoxide were added in its place. The reaction was then initiated and terminated by syringe through the serum stoppers. The addition of nitrogen as a control was carried out exactly in the same manner as it was for carbon monoxide. The final concentration of the added gases was 30% CO or N_2 and 70% air. This reflects a 2:1 ratio of CO or N_2 to O_2.

Measurement of Carbon Monoxide and Substrate Binding Spectra

The carbon monoxide difference spectra and the substrate binding spectra were determined with a Perkin-Elmer 554 dual beam spectrophotometer,

The carbon monoxide difference spectra of rat liver microsomes was determined by the method of Omura and Sato (100). Approximately

0.4 mg of microsomal protein suspended in 100 mM potassium phosphate buffer (pH 7.4) were placed in a sample and a reference cuvette. A few crystals of dithionite were added to both cuvettes in order to reduce the cytochrome P450. After adjusting the baseline, pure carbon monoxide was gently bubbled through the sample cuvette for 20 seconds and the carbon monoxide difference spectra were taken over the wavelength range of 500 nm to 400 nm. An extinction coefficient of 91 mM^{-1} cm^{-1} was used to calculate the concentration of cytochrome P450.

The substrate binding spectra were determined by the method of Peterson et al. (101). Approximately 0.4 mg of microsomal protein suspended in 100 mM potassium phosphate buffer (pH 7.4) were placed in a sample and reference cuvette. After adjusting the baseline, the appropriate substrate was added to the sample cuvette and the spectra were taken from 450 nm to 350 nm.

Measurement of NADPH Cytochrome c Reductase

Microsomal activity of NADPH cytochrome c reductase was carried out by the method of Strobel and Dignam (102) in a Gilford Model 240 spectrophotometer. The reductase catalyzed transfer of electrons to cytochrome c was determined by measuring the increased absorbance at 550 nm due to the appearance of reduced cytochrome c. Approximately 0.1 mg of microsomal protein was placed in a cuvette containing 0.980 ml of 5×10^{5} M cytochrome-c in 300 mM KPi buffer (pH = 7.4). The reaction was started with .01 ml of 10 mM NADPH and followed during the linear period. The activity of the reductase was calculated using an extinction coefficient of 21.5 mM^{-1} cm^{-1}.

All chemicals were of the highest grade available. The buffers

(except $MgCl_2$) were passed through a Chelex-100 column (Bio-Rad Laboratories, Richmond, CA) to remove contaminating iron. Desferrioxamine (Desferal) was obtained from CIBA Pharmaceutical Company (Summit, NJ); (+)-2-butanol and (-)-2-butanol were obtained from Fairfield Chemical Co., Inc. (Blythwood, SC) or from Pfalz & Bauer, Inc. (Stamford, CT). Racemic 2-butanol and pyrazole were also obtained from Pfalz and Bauer Inc. Cobalt protoporphyrin IX was obtained from Porphyrin Products, Inc. (Logan, UT).

All values refer to the mean ± standard error of the mean (SEM). Statistical analyses were performed by Student's t test. The number of experiments is indicated in the Table or in the Figure legends.

CHAPTER III RESULTS

A. Characterization of the Oxidation of 2-Butanol by Microsomes from Chow Fed Rats

The microsomal oxidation of 2-butanol was first characterized because this alcohol is available as the (+) and (-)stereoisomers. This was important in order to carry out stereochemical studies to be described below (see Section D). In the course of this characterization it became apparent that this alcohol displayed many interesting properties which could be of value in studying the nature of MEOS.

A-1 2-Butanol Is Not a Substrate for Catalase

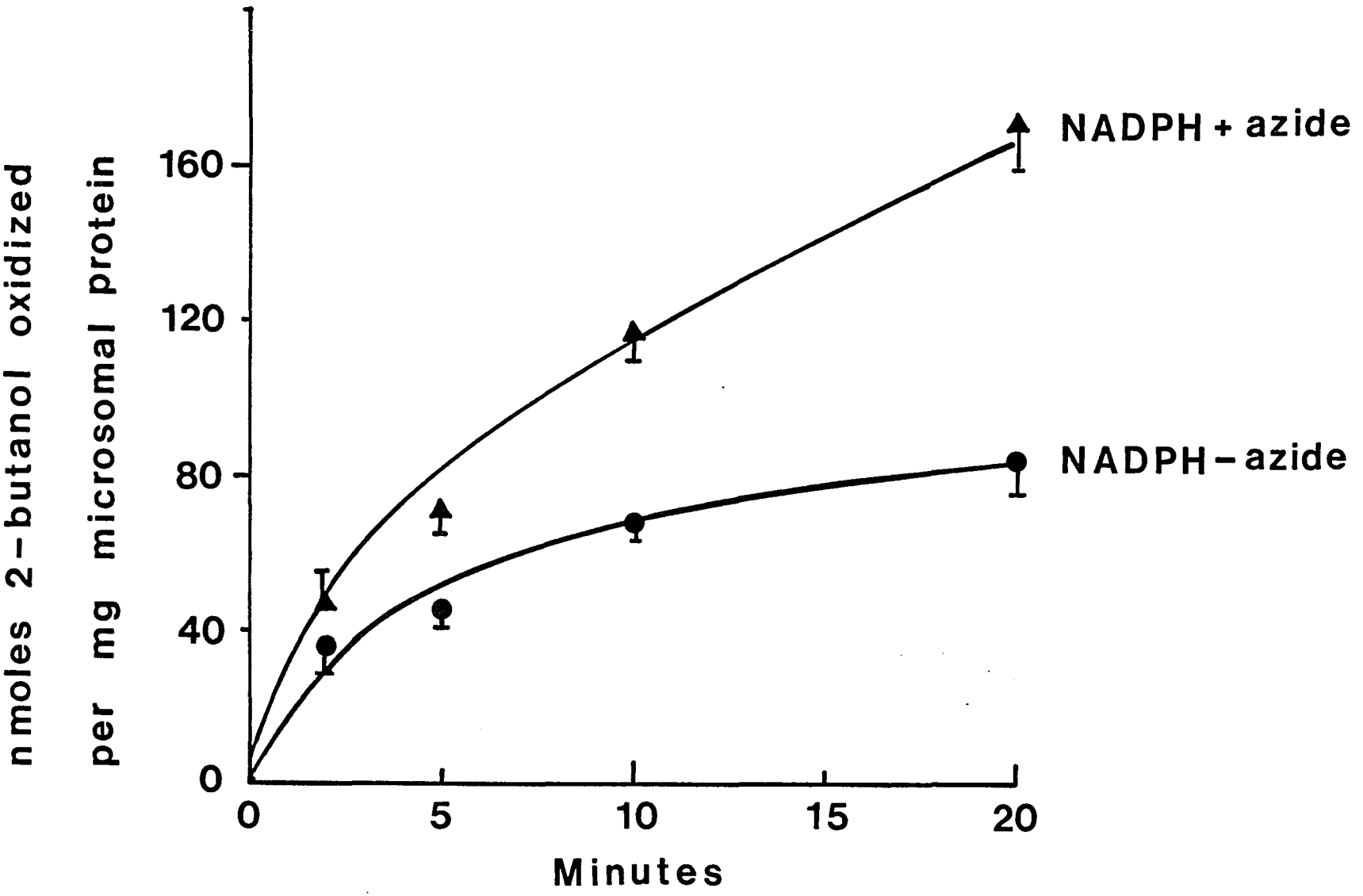
Catalase is found as a contaminant of microsomal preparations. Since some alcohols are substrates for the peroxidatic activity of catalase, the presence of this enzyme can interfere with the determination of MEOS. Studies were carried out to determine if 2-butanol was a substrate for catalase.

The oxidation of 2-butanol was assayed in the presence of an NADPH generating system. Figure 1 shows that microsomes oxidized 2-butanol to its corresponding ketone, 2-butanone. Azide, an inhibitor of catalase, has previously been shown to enhance the oxidation of several ·OH scavengers, presumably by allowing H_2O_2 to accumulate (88). Hydrogen peroxide is the precursor of ·OH. In a manner analogous to that described above, the addition of azide increased the NADPH dependent oxidation of 2-butanol by about twofold (Fig. 1). This should be contrasted to the decreased oxidation of ethanol and methanol (both of which are substrates for the peroxidatic activity of catalase) produced by azide. 2-Butanol was also not oxidized in the presence of

FIGURE 1

Time course of the oxidation of 2-butanol by rat liver microsomes.

The oxidation of 2-butanol (final concentration, 33 mM) was assayed under two reaction conditions: a) an NADPH generating system in the absence of azide; b) an NADPH generating system in the presence of azide (1.0 mM). Results are from 2 experiments.


nmoles 2-butanol oxidized
per mg microsomal protein
160
120
80
40
0
0
5
10
15
20
Minutes
NADPH + azide
NADPH - azide

purified catalase plus an H_2O_2 generating system (data not shown). Hence, unlike the shorter chain alcohols (i.e., methanol and ethanol), 2-butanol is not a substrate for the peroxidatic activity of catalase.

A-2 Time Dependent Oxidation of 2-Butanol, Ethanol and 1-Butanol in the Presence and Absence of Desferrioxamine

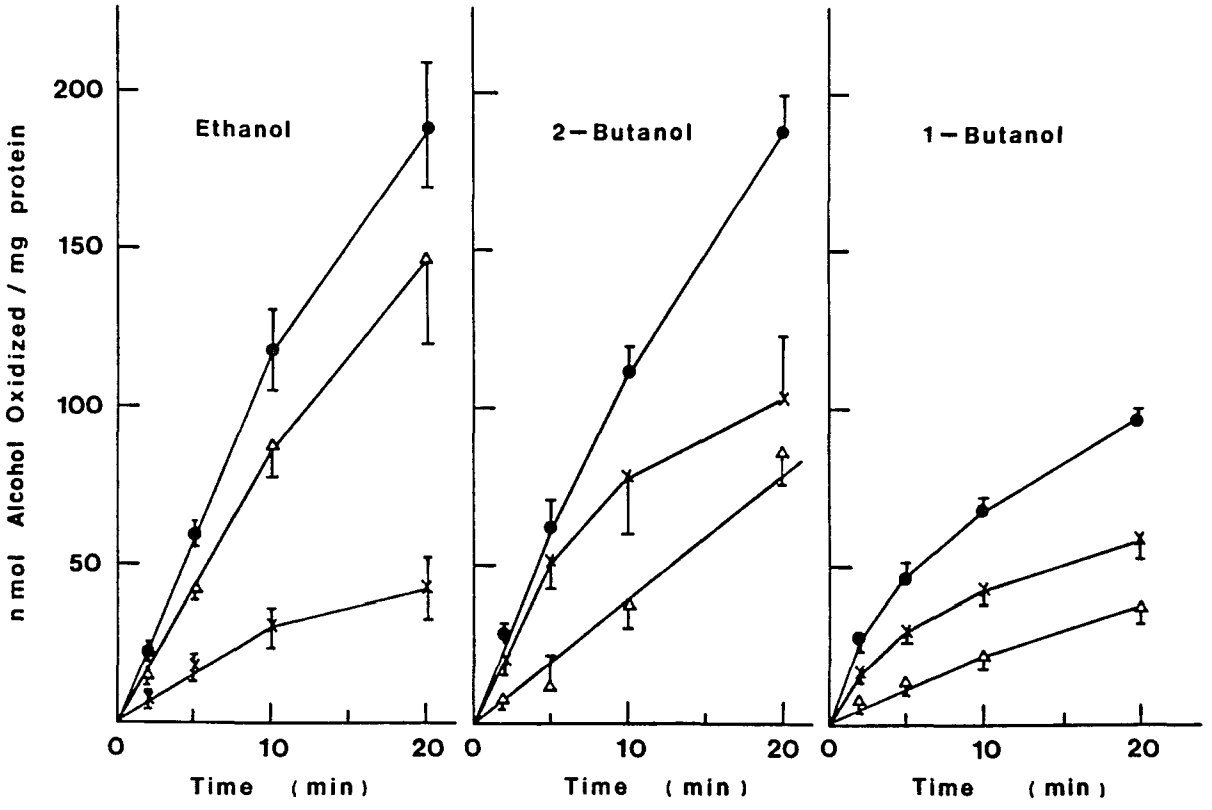
Figure 2 compares the time dependent oxidation of 2-butanol with that of ethanol and 1-butanol in the absence and presence of desferrioxamine. The rate in the absence of desferrioxamine reflects the oxidation of alcohols by two pathways, the ·OH dependent, and the cytochrome P450 dependent system. Desferrioxamine, a potent iron-chelating agent, nearly completely abolished the production of ·OH by microsomes (94). The desferrioxamine resistant rate of oxidation of alcohols appears to reflect the cytochrome P450, ·OH independent pathway of alcohol oxidation. The difference between the rates in the absence and presence of desferrioxamine is taken as the ·OH dependent rate of oxidation of alcohols. All three alcohols are oxidized in a time dependent manner, in both the absence and presence of desferrioxamine. The total rates of oxidation of ethanol and 2-butanol were similar at all time points, whereas the oxidation rate of 1-butanol was considerably less at the later time points (Fig. 2). Desferrioxamine produced a significantly greater inhibition of ethanol oxidation than of the oxidation of 2- or 1-butanol. With ethanol as the substrate, it appeared that the ·OH pathway was responsible for about 60 to 70% of the total oxidation, whereas with 2- and 1-butanol, the ·OH pathway comprised about 30% of the total oxidation. Hence, the longer chain alcohols appear to be better substrates for the cytochrome P450 pathway, which probably reflects their greater hydrophobicity. Indeed, the K_i

FIGURE 2

Time course for the oxidation of ethanol, 2-butanol and 1-butanol by microsomes from chow fed rats.

The oxidation of the alcohols was determined as described in Methods in the absence and presence of 0.25 mM desferrioxamine. The ·OH dependent rate was calculated by subtracting the rate in the presence of desferrioxamine from the total rate. Results are from 3 experiments.

- ● Rate in the absence of desferrioxamine (total rate)
- x Rate in the presence of desferrioxamine
- Δ ·OH dependent rate


n mol Alcohol Oxidized / mg protein
200
150
100
50
Ethanol
2-Butanol
1-Butanol
0
10
20
Time (min)

for inhibition of drug metabolism by alcohols correlated with their partition coefficient (103,104). Of significance is the observation that in the presence of desferrioxamine, 2-butanol was oxidized at approximately twice the rate of the other alcohols. This suggested that 2-butanol could serve as a relatively better substrate for cytochrome P450 than the other alcohols.

A-3 Kinetic Constants for the Microsomal Oxidation of 2-Butanol, Ethanol and 1-Butanol in the Presence and Absence of Desferrioxamine

The relative kinetic constants for alcohol oxidation were determined using Lineweaver-Burk plots. With ethanol and 2-butanol, the alcohol substrate concentrations used were 5,10,25,50 and 100 mM, while with 1-butanol, substrate concentrations of 2,5,10,25 and 50 mM were utilized.

Table I shows that in the absence of desferrioxamine, the K_m for ethanol and 2-butanol was the same, while the K_m for 1-butanol was much lower. The K_m values for the three alcohols were not significantly changed in the presence of desferrioxamine. The V_{max} for 2-butanol was greater that that for ethanol and 1-butanol. In particular, the V_{max} for 2-butanol oxidation in the presence of desferrioxamine was especially higher than the V_{max} for the other two alcohols (Table I). Thus the kinetic data also suggest that 2-butanol is a particularly good substrate for the cytochrome P450 pathway.

Table I. Kinetic constants for the oxidation of ethanol, 2-butanol and 1-butanol in the absence and presence of desferrioxamine.

Substrate	-Desferrioxamine		+Desferrioxamine	
	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})
Ethanol	14.9	17.7	10.4	5.9
2-Butanol	19.1	23.8	18.6	13.6
1-Butanol	4.4	6.2	5.8	4.7

K_m and V_{max} values were obtained from a least squares linear regression of Lineweaver-Burk plots of data which determined the rates of oxidation of alcohol as a function of the concentration of the alcohol. Alcohol concentrations ranged from 5 to 100 mM in the case of ethanol and 2-butanol, and from 2 to 50 mM in the case of 1-butanol. Results are from 6 experiments with ethanol or 2-butanol as substrate, and 3 experiments with 1-butanol as substrate.

A-4 Effect of Hydroxyl Radical Scavengers and Desferrioxamine on the Microsomal Oxidation of 2-Butanol

Table II shows that benzoate and dimethylsulfoxide (DMSO) produced some inhibition of 2-butanol oxidation. The extent of inhibition by the competing ·OH scavengers was about the same as that found with desferrioxamine (Table II). Previous experiments (94,105) showed that ethanol oxidation was reduced 50-70% by these additions which should be contrasted to the lower inhibition seen with 2-butanol (Table II). Taken as a whole, these results suggest some role for ·OH in the oxidation of 2-butanol, although relative to ethanol, 2-butanol appears to be oxidized preferably by the cytochrome P450 pathway. The somewhat greater extent of inhibition by 60 mM DMSO may be due, in part, to the recently demonstrated interaction of high concentrations of DMSO with cytochrome P450 (77,106).

A-5 Cumene Hydroperoxide Supported Oxidation of 2-Butanol

Organic hydroperoxides such as cumene hydroperoxide react directly with cytochrome P450 to form an oxygenated complex that catalyzes the metabolism of drugs. This reaction occurs in the absence of NADPH, cytochrome P450 reductase or O_2 (107). Similarly, cumene hydroperoxide is also capable of oxidizing ethanol to acetaldehyde in a microsomal system and by purified cytochrome P450 (95). The oxidation of typical ·OH scavengers, however, was not supported by cumene hydroperoxide, e.g., formaldehyde was not produced from DMSO and $^{14}CO_2$ was not produced from 7-^{14}C benzoate (96). Therefore, ·OH does not appear to be involved in a cumene hydroperoxide cytochrome P450 mediated oxidative reaction. Table III shows that cumene hydroperoxide supported microsomal oxidation of 2-butanol to 2-butanone. 2-Butanol was oxidized at the same rate

Table II. Effect of hydroxyl radical scavenging agents and desferrioxamine on microsomal oxidation of 2-butanol.

Addition	Concentration (mM)	2-Butanol Oxidation (nmol/min/mg protein)	Effect %	P
Control		13.7 ± 0.8		
Benzoate	30	10.5 ± 0.5	-23	<0.02
Benzoate	60	9.9 ± 0.4	-28	<0.05
DMSO	30	9.7 ± 0.8	-29	<0.01
DMSO	60	7.1 ± 0.2	-48	<0.001
Desferrioxamine	0.25	8.9 ± 0.8	-36	<0.05

The oxidation of racemic 2-butanol by microsomes from chow fed rats was assayed as described in Methods. The results represent the averages of 3 or 4 experiments.

Table III. Cumene hydroperoxide dependent oxidation of 2-butanol by microsomes.

Addition	Concentration (mM)	2-butanol oxidation {nmol.min^{-1} (mg protein)$^{-1}$}	Effect %
Control (3)		11.3 ± 1.1	
Aminopyrine (2)	5	12.3 ± 2.7	+9
Aniline (2)	2	8.3 ± 2.0	-27
Control (2)		7.8 ± 0.2	-
Desferrioxamine (2)	0.25	8.3 ± 0.1	+6

The oxidation of 33 mM 2-butanol was assayed in the presence of 1 mM azide. Reactions were initiated by the addition of cumene hydroperoxide (final concentration of 1 mM). Experiments were carried out for 10 min in the presence of 0.1 mM EDTA.

when the cumene hydroperoxide concentration was varied from 0.3 to 1.0 mM (data not shown). To test whether ·OH is involved in this oxidative mechanism, the effects of desferrioxamine were studied. The data in Table III show that desferrioxamine had no effect on the oxidation rate of 2-butanol by a cumene hydroperoxide supported system, suggesting that ·OH is not involved in this system.

On the other hand, the addition of the drug aniline (which has been shown to be a good substrate for the alcohol induced cytochrome P450 (77,108)) inhibited the oxidation of 2-butanol by 27% (Table III). Aminopyrine which is not a good substrate for the alcohol preferring cytochrome P450 had no effect on the oxidation of 2-butanol (Table III). These data support the idea that cumene hydroperoxide supported oxidation of 2-butanol involves cytochrome P450 and not ·OH.

B. Use of Carbon Monoxide and Cobalt Protoporphyrin IX to Evaluate the Two Pathways of MEOS

B-1 Inhibition by Carbon Monoxide

To determine if the cytochrome P450 pathway of oxidation of alcohols could be inhibited without affecting the ·OH pathway, experiments utilizing CO were conducted. CO is a classic inhibitor of drug metabolism and it was considered that addition of CO may inhibit the cytochrome P450 dependent oxidation of alcohols, but perhaps not the ·OH dependent pathway, since the reductase and not cytochrome P450 appears to be an important loci for ·OH production. Microsomal oxidation of a variety of alcohols was inhibited by 40-50% in an atmosphere containing $CO:O_2$ at a ratio of 10:1 (109) whereas NADPH dependent H_2O_2 was inhibited 35% at a $CO:O_2$ ratio of 9:1 (78). Because oxygen is required for the formation of oxygen radicals it was important to avoid anaerobic

conditions. Reactions were thus studied under a 30% CO/70% air atmosphere ($CO:O_2$ of 2:1). For comparative purposes some assays were carried out under a 30% N_2/70% air atmosphere. Table IV shows that all substrates tested were oxidized to the same extent whether they were incubated under 30% N_2/70% air or if they were incubated under 100% air (controls). In contrast, 30% CO/70% air caused a significant decrease in the metabolism of aminopyrine. The addition of CO had no effect on the oxidation of DMSO or KTBA, suggesting that cytochrome P450 is not involved in the oxidation of these substrates. These findings also suggest that cytochrome P450 is not involved in ·OH production, at least in the case of uninduced microsomes. A trend towards some inhibition by CO against the oxidation of alcohols was observed (Table IV). As discussed above, 2-butanol, relative to ethanol, appears to be a particularly effective substrate for the cytochrome P450 dependent oxidation of alcohols; this may contribute to the greater sensitivity of 2-butanol oxidation to CO.

Since the generation of ·OH, as reflected by the oxidation of DMSO or KTBA was not affected by CO, the small effect by CO (at this $CO:O_2$ ratio of 2) on the oxidation of alcohols could reflect the possibility that the alcohols were being oxidized to a large extent by the ·OH dependent pathway. The experiments with desferrioxamine indicated that about 60% of ethanol oxidation, and 40% of 2-butanol oxidation, appeared to be ·OH dependent (Table I). Since CO produced approximately a 45% inhibition of aminopyrine oxidation, it can be calculated that the oxidation of ethanol could be inhibited by about 18% by CO (0.4 x 0.45) while the oxidation of 2-butanol could be inhibited by about 27% by CO (0.6 x 0.45). The observed inhibition by CO was 19% for ethanol and 30% for 2-butanol (Table IV). In order to study the effect of CO

Table IV. Effect of CO on the NADPH dependent microsomal oxidation of various substrates.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein		
			Control	N_2	CO
Aminopyrine	5	6	6.5 ± 0.6	6.8 ± 0.6	3.6 ± 0.2*
Ethanol	50	5	10.3 ± 1.3	10.3 ± 0.9	8.3 ± 1.1
2-Butanol	33	7	14.2 ± 2.1	13.2 ± 2.0	9.3 ± 1.8
DMSO	33	6	2.1 ± 0.4	1.8 ± 0.3	2.1 ± 0.5
KTBA	10	5	1.7 ± 0.3	1.6 ± 0.3	1.4 ± 0.2

* $P < 0.001$

Substrate oxidations were assayed as described in Materials and Methods. The flasks containing N_2 or CO were composed of a 30% gas/70% air mixture. Controls refer to flasks under 100% air atmospheres. Statistical analyses compare flasks with N_2 or CO to controls.

on the cytochrome P450 pathway of alcohol oxidation, the experiments were repeated in the presence of desferrioxamine. Table V shows that the oxidation of ethanol and 2-butanol was now as sensitive to CO as was the oxidation of aminopyrine. Thus, the cytochrome P450, but not the ·OH dependent pathway of alcohol oxidation, was inhibited by CO. Subtraction of the desferrioxamine resistant rates of alcohol oxidation from the total rates of oxidation confirmed that the ·OH dependent oxidation of alcohols was not inhibited by CO (Table VI).

B-2 Inhibition by Cobalt Protoporphyrin IX

It has previously been reported that the metalloporphyrin cobalt protoporphyrin IX (Co IX) can markedly reduce the content of rat liver microsomal P450 (110). This compound possesses the dual biological properties of repressing δ-aminolevulinate synthetase and of potently inducing microsomal heme oxygenase. Hence, heme synthesis is inhibited while heme breakdown is enhanced. As a result, the content of cytochrome P450 decreases sharply. It seemed, therefore, that this could be a powerful method for abolishing the cytochrome P450 pathway in microsomes, possibly without affecting the production of ·OH (which in reconstituted systems appears to be dependent on cytochrome P450 reductase (28,29)).

The effect of Co IX on the NADPH dependent microsomal oxidation of various substrates is shown in Table VII. The oxidation of aminopyrine was depressed 80% after the Co IX treatment. This is consistent with the observation that an acute dose of Co IX decreased the activity of ethyl-morphine demethylase and aniline hydroxylase by 88 and 63%, respectively (110). In an analogous manner, the microsomal oxidation of alcohols was inhibited by 70% after Co IX treatment (Table VII). Interestingly, when the oxidation of three different ·OH scavengers, namely

Table V. Effect of CO on the NADPH dependent microsomal oxidation of various substrates in the presence of desferrioxamine.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein		
			Control	N_2	CO
Aminopyrine	5	6	6.3 ± 0.6	6.4 ± 0.5	3.3 ± 1.8*
Ethanol	50	5	5.3 ± 0.6	5.5 ± 0.5	3.5 ± 1.5**
2-Butanol	33	7	10.8 ± 0.2	8.3 ± 0.4	4.6 ± 0.9**

* P = 0.0005
** P < 0.05

Substrate oxidations were assayed as described in Materials and Methods. The flasks containing N_2 or CO were composed of a 30% gas/70% air mixture. Statistical analyses compare flasks with N_2 or CO to controls.

Table VI. Effect of CO on the ·OH dependent oxidation of various substrates.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein		
			Control	N_2	CO
Aminopyrine	5	6	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
Ethanol	50	5	5.0 ± 0.9	4.8 ± 0.4	4.8 ± 0.9
2-Butanol	33	7	3.4 ± 0.6	4.9 ± 0.9	4.7 ± 0.8

The data in this table were derived by subtracting the data in Table V from the data in Table IV.

Table VII. Effect of Co protoporphyrin IX on the NADPH dependent microsomal oxidation of various substrates.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein	
			Saline	Co IX
Aminopyrine	5	7	4.9 ± 0.6	0.9 ± 0.2*
Ethanol	50	7	19.1 ± 3.3	6.2 ± 0.5*
2-Butanol	33	7	20.5 ± 2.9	6.0 ± 0.7*
DMSO	33	7	2.4 ± 0.5	1.0 ± 0.1**
Benzoate	33	3	3.2 ± 0.4	1.0 ± 0.3*
KTBA	10	7	2.3 ± 0.9	1.0 ± 0.1*

* $P \leq 0.01$
** $P = 0.02$

Substrate oxidations were assayed as described in Materials and Methods.

DMSO, benzoate and KTBA were studied, it was noted that the oxidation of these substrates was also decreased by 58 to 69%.

In view of these results, the oxidation of these substrates was studied in the presence of desferrioxamine. Table VIII shows that in the presence of desferrioxamine, aminopyrine, ethanol and 2-butanol oxidation were all significantly inhibited by Co IX, implying the importance of cytochrome P450 in the metabolism of these substrates. Due to the presence of desferrioxamine, no significant rates of ·OH scavenger oxidation were seen either in the case of saline treated or Co IX treated rats. When studying the effects of Co IX on a cumene hydroperoxide supported system (Table IX) it is seen that under these conditions the results parallel the data seen in the case of an NADPH supported system. A significant decrease in the oxidation of aminopyrine, ethanol and 2-butanol in the cumene hydroperoxide supported system supports the findings that Co IX has a significant deleterious effect on cytochrome P450.

Table X represents the oxygen radical dependent oxidation of various substrates in the absence and presence of Co IX. Since aminopyrine is not oxidized by ·OH, no significant rates of formaldehyde production are seen by either set of microsomes. In contrast, the oxidation of both alcohols and the ·OH scavengers were significantly decreased by treatment with Co IX.

It has previously been suggested that the oxygen radicals produced by the mixed function oxidase system are derived from cytochrome P450 reductase (27,28). In view of the results in Table X it was believed that Co IX may not only decrease the content of cytochrome P450 but may also be affecting the activity of cytochrome P450 reductase. This possibility was tested and, as can be seen in Table XI, in addition to the reduced

Table VIII. Effect of Co IX on the NADPH dependent microsomal oxidation of various substrates in the presence of desferrioxamine.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein	
			Saline	Co IX
Aminopyrine	5	7	4.4 ± 0.5	0.7 ± 0.1*
Ethanol	50	7	6.7 ± 0.9	2.3 ± 0.2*
2-Butanol	33	7	12.3 ± 1.0	2.8 ± 0.5*
DMSO	33	6	0.2 ± 0.0	0.2 ± 0.0
KTBA	10	6	0.2 ± 0.0	0.1 ± 0.0

* $P \leq 0.0002$

Substrate oxidations were assayed as described in Materials and Methods. The final concentration of desferrioxamine was 250 μM.

Table IX. Effect of Co protoporphyrin IX on the cumene hydroperoxide supported microsomal oxidation of various substrates.

Substrate	Concentration (mM)	Product formation nmol/min/mg microsomal protein	
		Saline	Co IX
Aminopyrine	5	3.3 ± 0.8	0.8 ± 0.3*
Ethanol	50	3.3 ± 0.6	1.8 ± 0.4*
2-Butanol	33	5.9 ± 0.9	2.4 ± 1.0*

* $p \leq 0.0001$

Substrate oxidations were assayed as described in Materials and Methods. The above values represent the means of 7 experiments.

Table X. Effect of Co protoporphyrin IX on the ·OH dependent rate of microsomal oxidation of various substrates.

Substrate	Concentration (mM)	Number of experiments	Product formation nmol/min/mg microsomal protein	
			Saline	Cobalt
Aminopyrine	5	7	0.5 ± 0.2	0.2 ± 0.1
Ethanol	50	7	12.4 ± 2.6	3.9 ± 0.4*
2-Butanol	33	7	8.2 ± 2.0	3.2 ± 0.5**
DMSO	33	6	2.2 ± 0.8	0.8 ± 0.1
KTBA	10	6	2.1 ± 0.1	0.9 ± 0.1**

* $P < 0.01$
** $P < 0.05$

The results in this table reflect the subtraction of the values in Table IX from those of Table VIII.

Table XI. Effect of Co protoporphyrin IX on the microsomal content of cytochrome P450 reductase and cytochrome P450.

Treatment	Cytochrome P450 reductase (nmol cytochrome c reduced/min/mg)	Cytochrome P450 nmol/mg
Saline	104 ± 15	1.21
Co IX	46 ± 6	0.26 ± 0.02

Enzymatic assays were carried out as described in Materials and Methods. The above values represent the means of 2-5 experiments.

levels of cytochrome P450, acute treatment with Co IX greatly diminished the activity of cytochrome P450 reductase. Hence Co IX is not specific for the destruction of cytochrome P450 alone. Indeed, it is possible that other enzymes could be destroyed by this compound as well.

These experiments suggest that Co IX treatment cannot be used to differentiate the two microsomal pathways of alcohol oxidation since this treatment lowers both the content of cytochrome P450 and the activity of the reductase (and hence ·OH generation). On the other hand, CO under conditions in which anaerobiosis did not occur was valuable in distinguishing the two pathways.

These results suggest that the desferrioxamine sensitive, ·OH dependent pathway of alcohol oxidation is mediated by the reductase, in analogy to results with ·OH scavengers, while the desferrioxamine resistant pathway of alcohol oxidation is mediated by cytochrome P450, in analogy to results with aminopyrine. By the use of desferrioxamine or carbon monoxide, either of the two alcohol oxidizing pathways can be inhibited independently of each other.

C. Induction and Contribution of the Cytochrome P450 Pathway and the Hydroxyl Radical Pathway After Chronic Alcohol Consumption

C-1 Oxidation of 2-Butanol by Microsomes from Ethanol Fed Rats

Chronic ethanol consumption has been shown to increase the microsomal oxidation of ethanol (73,84). A cytochrome P450 isozyme distinct from those induced by phenobarbital or 3-methylcholanthrene was induced by ethanol (6,77) which proved to have high activity for the metabolism of ethanol and aniline (77,108). Since 2-butanol appeared to be an effective substrate for the cytochrome P450 pathway of alcohol oxidation, we compared its oxidation by microsomes from chronic ethanol

fed rats and their pair-fed controls. Chronic ethanol consumption resulted in a threefold increase in the rate of oxidation of 2-butanol (Fig. 3). The ·OH dependent oxidation of 2-butanol was slightly increased, but clearly most of the increase in the oxidation of 2-butanol reflected an increase in the cytochrome P450 pathway (compare rates in the presence of desferrioxamine, Fig. 3). Thus, 2-butanol appears to be an effective substrate for the ethanol inducible cytochrome P450 isozyme(s).

C-2 2-Butanol Binding Spectrum with Microsomes from Ethanol Fed Rats

Substrates for cytochrome P450 typically display type 1, type 2, or reverse type 1 binding spectra. As can be seen in Figure 4, 2-butanol displayed a reverse type 1 binding spectrum with microsomes from chronic alcohol fed rats. By contrast, no clear spectrum with 2-butanol was observed with microsomes from the pair fed controls or from chow fed controls (data not shown). Although the significance of binding spectra is difficult to interpret, these results may suggest some special interaction of 2-butanol with microsomes from the chronic ethanol fed rats.

C-3 Oxidation of DMSO by Microsomes from Ethanol Fed Rats

Initial experiments confirmed previous observations (111) that microsomes from chronic ethanol fed rats catalyzed the oxidation of 33 mM DMSO to a greater extent than did microsomes from the pair fed controls as the rate of formaldehyde production from DMSO was increased by 65% after ethanol consumption (Table XII). The oxidation of a higher concentration of DMSO was also studied. The rate of formaldehyde production from DMSO was slightly increased (16% for pair fed controls; 33% for ethanol treated) when the DMSO concentration was elevated from 33 mM to 100 mM. The oxidation of 100 mM DMSO by microsomes was nearly

FIGURE 3

Rate of oxidation of racemic 2-butanol by microsomes from ethanol fed rats and their pair fed controls.

The oxidation of 2-butanol was determined in the absence and presence of 0.25 mM desferrioxamine. Results are from 6 pairs of animals.

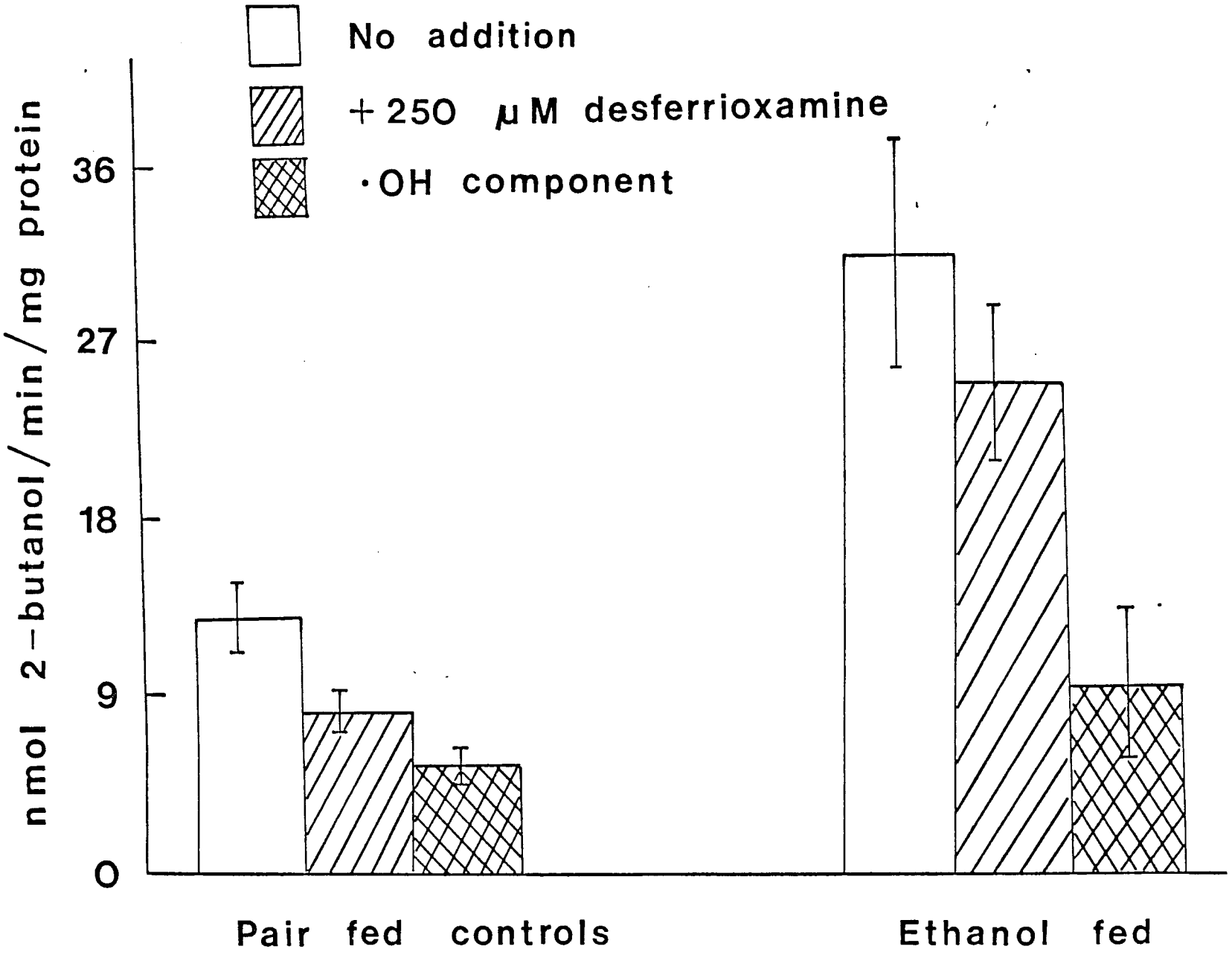

No addition
+250 μM desferrioxamine
·OH component
nmol 2-butanol/min/mg protein
36
27
18
9
0
Pair fed controls
Ethanol fed

FIGURE 4

Binding spectrum for the interaction of 2-butanol with microsomes from ethanol fed rats.

The binding spectrum was determined as described in Methods. Results from a typical spectrum are shown.

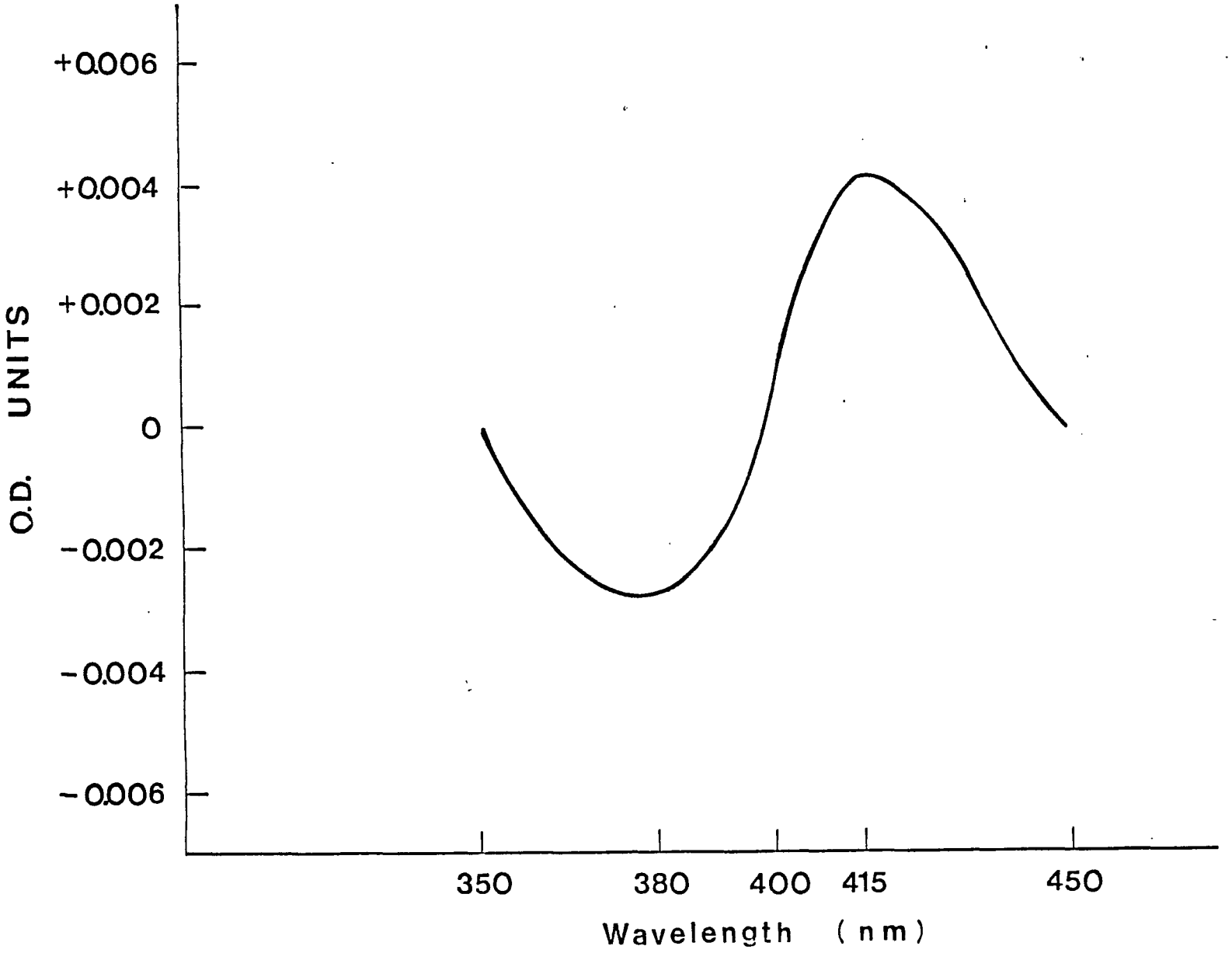

+0.006
+0.004
+0.002
0
-0.002
-0.004
-0.006
O.D. UNITS
350
380
400
415
450
Wavelength (nm)

Table XII. NADPH dependent oxidation of DMSO by microsomes from chronic ethanol fed rats and pair fed controls.[a]

Concentration of DMSO (mM)	Reaction Condition	Rate of Formaldehyde Production (nmol/min/mg microsomal protein)	
		Pair-Fed Control	Chronic Ethanol
33	control	3.1 ± 0.8	5.1 ± 1.0
	plus desferrioxamine	0.3 ± 0.1	0.2 ± 0.1
100	control	3.6 ± 1.1	6.8 ± 0.3
	plus desferrioxamine	0.4 ± 0.1	0.4 ± 0.1

[a]The production of formaldehyde from either 33 or 100 mM DMSO was assayed as described in Materials and Methods. When present, the final concentration of desferrioxamine was 0.25 mM.

Results are from 6 experiments at the 33 mM DMSO concentration, and from 3 experiments at the 100 mM DMSO concentration.

doubled after chronic ethanol consumption (Table XII).

Desferrioxamine, which had previously been shown to block the production of ·OH by microsomes from chow fed rats (94), proved to be a very effective inhibitor of the oxidation of DMSO by microsomes from both, the ethanol fed rats, and their pair fed controls (Table XII). With both microsomal preparations, and at both DMSO concentrations, desferrioxamine produced 90% or greater inhibition of DMSO oxidation. These results suggest that the increased oxidation of DMSO after chronic alcohol consumption is indeed due to the interaction of this substrate with ·OH and that the production of ·OH by both microsomal preparations could be blocked by the addition of desferrioxamine.

C-4 Oxidation of Ethanol by Microsomes from Ethanol Fed Rats

The microsomal production of acetaldehyde from ethanol was increased 70% after ethanol treatment (Table XIII). The addition of desferrioxamine produced approximately 50% inhibition of ethanol oxidation by both microsomal preparations. Three observations are especially noteworthy: first, a significant rate of ethanol oxidation persisted in the presence of a concentration of desferrioxamine which nearly completely blocked the oxidation of DMSO. This rate reflects the ·OH independent, cytochrome P450 mediated oxidation of ethanol. Second, this desferrioxamine insensitive cytochrome P450 mediated rate of ethanol oxidation was nearly twofold greater by microsomes from ethanol fed rats (Table XIII). Third, by subtracting the rate of ethanol oxidation in the presence of desferrioxamine from the total rate of ethanol oxidation, the ·OH dependent rate of ethanol oxidation could be calculated. This ·OH dependent rate of ethanol oxidation, which appears to account for about one half of the total rate of ethanol oxidation by both microsomal prepara-

Table XIII. NADPH dependent oxidation of ethanol[a] by microsomes from chronic ethanol fed rats and pair fed controls.

Reaction Condition	Rate of Acetaldehyde Production (nmol/min/mg microsomal protein)		Increase in Acetaldehyde Production
	Pair Fed Control	Chronic Ethanol	
Control	16.7 ± 2.4	28.5 ± 3.7	11.8 ± 2.2*
plus desferrioxamine	8.3 ± 0.7	14.7 ± 2.0	6.4 ± 1.5#
·OH dependent[b]	8.4 ± 2.1	13.8 ± 1.9	5.4 ± 1.1#

* $P < 0.002$
# $P < 0.01$

[a]The production of acetaldehyde from 50 mM ethanol was assayed as described in Materials and Methods. When present, the final concentration of desferrioxamine was 0.25 mM. Results are from 6 experiments.

[b]The ·OH dependent rate was calculated by subtracting the rate in the presence of desferrioxamine from the control rate.

tions, increased 65% after chronic ethanol consumption (Table XIII), a value identical to the increase in DMSO oxidation (Table XII).

C-5 Effect of Various Additions on Ethanol Oxidation by Microsomes from Ethanol Fed Rats

The addition of competing ·OH scavengers such as DMSO or benzoate resulted in an inhibition of ethanol oxidation by both, microsomes from ethanol fed rats and microsomes from their pair fed controls (Table XIV).

The extent of inhibition by the ·OH scavengers was about the same for both sets of microsomes, which is consistent with the observation that the ·OH dependent pathway of ethanol oxidation, although of greater activity in the microsomes from the ethanol fed rats, accounts for about the same percentage of the total ethanol oxidizing activity of both microsomal preparations. Next, the effects of the drug aniline were studied. Since aniline is also an effective ·OH scavenger, the concentration of aniline was kept low in order to minimize its ·OH scavenging properties. Table XIV shows that aniline produced an inhibition of ethanol oxidation with both microsomal preparations, although it appeared to be somewhat more effective in inhibiting ethanol oxidation by the microsomes from ethanol fed rats. Since 2-butanol seems to be such an effective alcohol substrate for the microsomal cytochrome P450 oxidizing pathway and in particular for the cytochrome P450 induced by chronic alcohol consumption (see Fig. 3), its effects on ethanol oxidation were studied.

Similar to results with aniline, 2-butanol inhibited the oxidation of ethanol by both microsomal preparations, but appeared to be especially effective with the microsomes from the ethanol fed rats (Table XIV).

Table XIV. Effect of various additions on the oxidation of ethanol.[a]

Addition	Rate[b] of Acetaldehyde Production: Pair-Fed Control Rate	Pair-Fed Control Effect (%)	& Effect of Addition: Chronic Ethanol Rate	Chronic Ethanol Effect (%)
Control (6)	17.3 ± 2.8	-	28.5 ± 3.7	-
DMSO 33 mM	10.7 ± 1.1	-38	16.0 ± 2.0	-44
Aniline 5 mM	13.4 ± 2.0	-23	18.5 ± 2.7	-35
Control (3)	20.5 ± 3.7	-	35.1 ± 3.7	-
DMSO 100 mM	8.7 ± 0.4	-58	14.2 ± 0.9	-60
Benzoate 33 mM	12.9 ± 1.0	-37	24.9 ± 1.9	-29
2-Butanol 33 mM	14.8 ± 0.1	-28	17.6 ± 2.2	-50

[a]The production of acetaldehyde from 50 mM ethanol was assayed as described in Materials and Methods in the absence and presence of the various additions. Results are from either 6 or 3 experiments.

[b]Rate refers to nmol acetaldehyde produced per min per mg microsomal protein.

The above experiments were repeated in the presence of desferrioxamine to wipe out the ·OH dependent pathway. Table XV shows that the desferrioxamine resistant rate of ethanol oxidation by both microsomal preparations was not especially affected by either 33 mM DMSO or benzoate, confirming a lack of a role for ·OH in ethanol oxidation in the presence of desferrioxamine. Some inhibition, which was more notable with the ethanol treated microsomes, was found at 100 mM DMSO. Aniline inhibited the desferrioxamine resistant rate of ethanol oxidation, with a somewhat greater extent of inhibition being observed with microsomes from the ethanol fed rats. Of special interest was the fact that 2-butanol proved to be a far superior inhibitor of ethanol oxidation in microsomes from the chronic ethanol fed rats than in control microsomes (Table XV). The greater extent of inhibition of the cytochrome P450 mediated oxidation of ethanol which occurs in the presence of desferrioxamine by aniline and 2-butanol with microsomes from the ethanol fed rats may be due to these agents serving as particular good substrates for the ethanol inducible cytochrome P450.

Table XVI shows the effects of the various additions on the ·OH dependent rate of ethanol oxidation. The data in this table were obtained by subtracting the results for each experiment shown in Table XV from results in Table XIV. It is clear that the ·OH scavengers are very effective in inhibiting the ·OH dependent oxidation of ethanol by both microsomal preparations. Inhibition of ethanol oxidation by 2-butanol was also found, since this alcohol can also scavenge ·OH. The slight inhibition seen with aniline probably also reflects some scavenging of ·OH which may occur even at these low concentrations of aniline.

Table XV. Effect of various additives on the oxidation of ethanol in the presence of desferrioxamine.[a]

Addition	Rate of Acetaldehyde Production & Effect of Addition			
	Pair-Fed Control		Chronic Ethanol	
	Rate	Effect (%)	Rate	Effect (%)
Control (6)	8.6 ± 0.7	-	14.7 ± 2.0	
DMSO 33 mM	7.6 ± 0.5	-12	12.3 ± 1.5	-16
Aniline 5 mM	6.2 ± 0.3	-28	8.2 ± 0.6	-44
Control (3)	9.4 ± 0.9	-	17.5 ± 3.1	
DMSO 100 mM	7.3 ± 0.5	-22	11.6 ± 0.9	-34
Benzoate 33 mM	8.3 ± 0.3	-12	17.5 ± 1.6	0
2-Butanol 33 mM	7.1 ± 0.0	-24	7.9 ± 0.8	-55

[a]The production of acetaldehyde in the presence of 0.25 mM desferrioxamine was assayed as described in Materials and Methods in the absence and presence of the various additives. Results are from either 6 or 3 experiments.

Table XVI. Effect of various additives on the ·OH dependent oxidation of ethanol.[a]

Addition	Rate of Acetaldehyde Production & Effect of Addition			
	Pair Fed Control		Chronic Ethanol	
	Rate	Effect (%)	Rate	Effect (%)
Control (6)	8.8 ± 2.1	-	13.8 ± 1.9	-
DMSO 33 mM	3.1 ± 0.6	-65	3.7 ± 0.9	-73
Aniline 5 mM	7.2 ± 1.8	-18	10.3 ± 2.3	-25
Control (3)	11.1 ± 2.9	-	17.6 ± 1.1	-
DMSO 100 mM	1.4 ± 0.4	-87	2.6 ± 0.4	-85
Benzoate 33 mM	4.6 ± 1.4	-59	7.4 ± 0.6	-58
2-Butanol 33 mM	7.2 ± 0.1	-35	10.0 ± 1.2	-43

[a]The ·OH dependent rate of ethanol oxidation was obtained by subtracting the rate in the presence of 0.25 mM desferrioxamine (Table IV) from the total rate (Table III), for each experiment. Results are from either 6 or 3 experiments.

C-6 DMSO Binding Spectrum with Microsomes from Ethanol Fed Rats

It has recently been shown that DMSO displayed either a reversed type 1 or a type 2 binding spectrum with microsomes from alcohol induced rats or with purified cytochrome P450 3-a isolated from ethanol treated rabbits, respectively (106,77). Rather high concentrations of DMSO were required to display these binding spectra. The results in Table XV which suggested the possibility that 100 mM DMSO was somewhat more effective in blocking the desferrioxamine resistant rate of ethanol oxidation with microsomes from the ethanol treated rats prompted a study of the binding spectra of DMSO with rat liver microsomes. In agreement with the results of others (106,77), DMSO produced a reversed type 1 binding spectrum with microsomes from the ethanol fed rats (Fig. 5). No binding spectrum with microsomes from the pair fed controls could be discerned (Fig. 5). The ability of several other ·OH scavengers to produce binding spectra with microsomes was also studied. Neither benzoate (150 mM), mannitol (150 mM) nor t-butyl alcohol (180 mM) produced binding spectra with microsomes from ethanol fed, or pair fed, control rats (data not shown). Thus, the alcohol-inducible cytochrome P450 of rat liver appears to display an interaction with DMSO which is not shared with other ·OH scavenging agents.

C-7 Cumene Hydroperoxide Supported Oxidation of Ethanol and ·OH Scavengers by Microsomes from Ethanol Fed Rats

Cumene hydroperoxide has been shown to be capable of oxidizing ethanol to acetaldehyde in a microsomal system from normal chow fed rats (95). Table XVII shows that in the absence of NADPH, cumene hydroperoxide supported the oxidation of ethanol by microsomal preparations from alcohol treated animals and their pair fed controls. However, cumene

FIGURE 5

DMSO binding spectrum with microsomes isolated from ethanol fed rats and pair fed controls.

Binding spectra were carried out as described in Materials and Methods, using a final DMSO concentration of 140 mM. Results shown are from a typical experiment. The baseline depicts the results with microsomes from pair fed controls whereas the spectrum depicts the results with microsomes from ethanol fed rats.

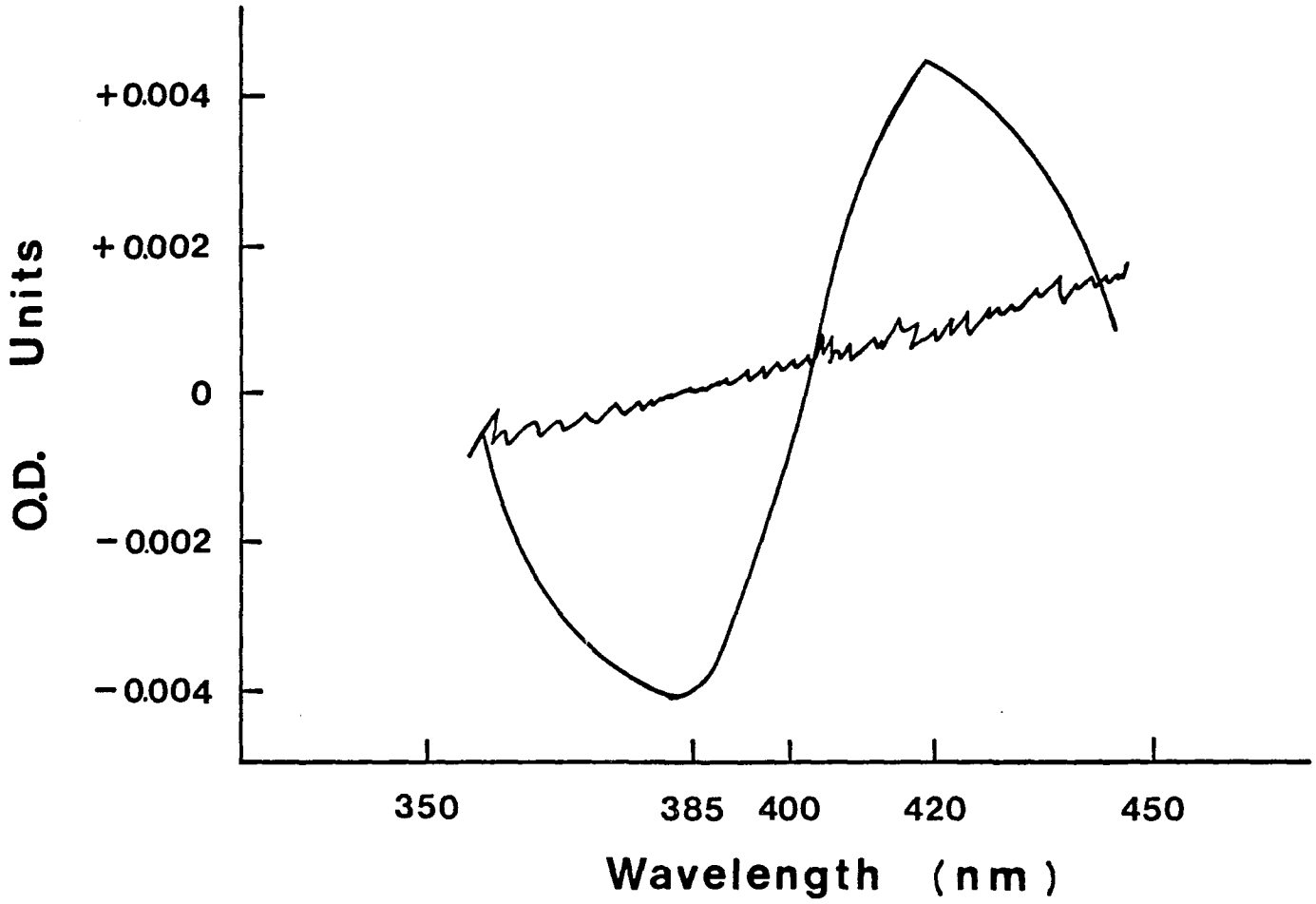

+0.004
+0.002
0
−0.002
−0.004
O.D. Units
350
385
400
420
450
Wavelength (nm)

Table XVII. Cumene hydroperoxide supported oxidation of ethanol and •OH scavengers.[a]

Substrate	Addition	Rate of Product Formation (nmol/min/mg microsomal protein)	
		Pair Fed Control	Chronic Ethanol
Ethanol	-	8.9 ± 0.3	9.6 ± 0.6
"	33 mM DMSO	9.2 ± 0.4	9.6 ± 0.7
"	100 mM DMSO	9.5 ± 0.4	9.7 ± 0.7
"	33 mM Benzoate	9.3 ± 0.3	10.4 ± 0.8
"	0.25 mM Desferrioxamine	8.9 ± 0.4	10.0 ± 0.8
DMSO	-	0.4 ± 0.1	0.4 ± 0.2
KTBA	-	0.2 ± 0.0	0.1 ± 0.0

[a]The rate of oxidation of ethanol, DMSO and KTBA by microsomes from pair fed, and chronic ethanol fed, rats in the presence of 1 mM cumene hydroperoxide was determined as described in Materials and Methods. Results are from 4 experiments.

hydroperoxide did not effectively support the oxidation of ·OH scavenging agents by these microsomes; rates of formaldehyde production from DMSO, or of ethylene production from KTBA were less than 10% of that found with an NADPH generating system (Table XVII).

Cumene hydroperoxide was previously shown to be ineffective in catalyzing the oxidation of ·OH scavengers by cytochrome P450 purified from phenobarbital treated rats (28). These results suggest that ·OH was not generated by microsomes in the presence of cumene hydroperoxide. In further support for a lack of a role for ·OH in cumene hydroperoxide supported oxidation reactions was the inability of either competing ·OH scavengers such as DMSO and benzoate or desferrioxamine to affect the rate of ethanol oxidation by both microsomal preparations (Table XVII).

The above results indicate that ethanol oxidation in the presence of cumene hydroperoxide reflects a ·OH independent, cytochrome P450 mediated reaction. However, rates of cumene hydroperoxide supported oxidation of ethanol were identical in both microsomal preparations. These identical rates of ethanol oxidation were also found when the cumene hydroperoxide concentration was either doubled or reduced in half (data not shown). A time course for the oxidation of ethanol in a cumene hydroperoxide system showed that the oxidation increased with time and began to level off at about 10 minutes (data not shown). At all time points tested, rates of ethanol oxidation were the same for both microsomal preparations.

C-8 Content of Cytochrome P450 and Activity of NADPH Cytochrome c Reductase in Microsomes from Ethanol Fed Rats

To determine if the increase in ethanol, 2-butanol and DMSO oxidation correlates with changes in the microsomal electron transfer system,

the content of cytochrome P450 and the activity of NADPH cytochrome c reductase were measured. The content of cytochrome P450 (nmol per mg protein) was 0.754 ± 0.037 for microsomes from pair fed controls, and 1.180 ± 0.096 for microsomes from the ethanol fed rats (+57%, n = 10). The activity of NADPH cytochrome c reductase (nmol cytochrome c reduced per min per mg microsomal protein) was 152 ± 29 for pair fed controls and 182 ± 13 for microsomes from ethanol fed rats (+20%, n = 5).

In summary, based upon experiments with desferrioxamine, and the effects of competing ·OH scavengers, aniline and 2-butanol, it appears that both the ·OH dependent and cytochrome P450 dependent pathways are increased in microsomes from ethanol fed rats and that each pathway seems to account for about one half of the total oxidizing activity of the microsomes. The increase in the ·OH dependent pathway of ethanol oxidation is about the same as the increase in the oxidation of DMSO. Moreover, 2-butanol appears to be an especially effective substrate for the cytochrome P450 pathway with microsomes from chronic alcohol treated rats.

D. Stereospecificity Studies with (+)- and (-)-2-Butanol.

Having characterized the oxidation of racemic 2-butanol by liver microsomes from control and alcohol treated rats, the oxidation of (+)-2-butanol and (-)-2-butanol in the absence and presence of desferrioxamine was compared. Any stereoselectivity with either pathway could serve as a tool to investigate the nature of these systems.

While cytochrome P450 is known to display stereoselectivity towards several drugs (112-117), it is unlikely that a potent oxidant such as a free ·OH would display stereospecificity. It is now generally believed that in biological systems, a highly reactive oxygen species

such as ·OH would not be free in solution but, rather, may reflect a "·OH-like" species that is bound. Therefore, stereospecificity studies could provide some insight into the nature of the ·OH or ·OH-like species generated by the microsomes.

D-1 Stereospecificity Studies with Model ·OH Generating Systems.

Prior to initiating studies of the stereospecific nature of 2-butanol oxidation in the more complex microsomal system, experiments were carried out in two well characterized model ·OH generating systems, the iron catalyzed oxidation of ascorbate (59) (eqn. 1) and the Fenton reaction (64) (eqn. 2).

eqn. 1 a) $3\ Fe^{+3} + 3\ \text{Ascorbate} \rightarrow 3\ Fe^{+2} + 3\ \text{Dehydroascorbate}$

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

c) $Fe^{+2} + H_2O_2 \rightarrow {\cdot}OH + OH^- + Fe^{+3}$

Net: $3\ \text{Ascorbate} + O_2 + 2H^+ \rightarrow 3\ \text{Dehydroascorbate} + {\cdot}OH + OH^-$

eqn. 2 $Fe^{+2} + H_2O_2 \rightarrow {\cdot}OH + OH^- + Fe^{+3}$

Both systems oxidized the (+)- and (-)-2-butanol at similar rates (Table XVIII). Thus, as expected, ·OH generated by chemical systems does not display stereospecificity with 2-butanol.

D-2 Stereospecificity Studies with Differently Induced Microsomes

Results shown in Table XIX indicate that microsomes isolated from chow fed control rats oxidized (+)-, (-)-, and racemic 2-butanol at similar rates, both in the absence as well as in the presence of desferrioxamine. Subtraction of the rates in the presence of desferrioxamine from the total rates to yield the ·OH dependent rates indicated that the ·OH dependent rates were also the same for (+)-, (-)-, and

Table XVIII. Oxidation of (-)-2-butanol and (+)-2-butanol by an ascorbic system and a Fenton system.

Alcohol	Oxidation of 2-Butanol (nmol) Ascorbate System	Fenton System
(-) 2-butanol	630	110
(+) 2-butanol	610	130

The oxidation of 2-butanol by the ascorbate or by the Fenton system was carried out as described in Methods. Results from a typical experiment are shown.

Table XIX. Oxidation of racemic, (+) and (-)-2-butanol by liver microsomes from chow fed and phenobarbital treated rats.

Microsomes	Substrate	Rate of Oxidation (nmol/min/mg protein) Total	plus Desferrioxamine	·OH-Component
Chow fed (n = 4-6)	Racemic 2-butanol	12.6 ± 1.9	8.5 ± 1.1	4.1 ± 0.8
	(+)-2-butanol	10.2 ± 1.0	6.6 ± 0.6	3.6 ± 0.7
	(-)-2-butanol	11.1 ± 1.8	7.6 ± 1.2	3.5 ± 0.7
Phenobarbital (n = 3)	Racemic 2-butanol	11.3 ± 1.3	7.8 ± 1.0	3.5 ± 0.9
	(+)-2-butanol	10.8 ± 1.6	6.8 ± 0.9	4.0 ± 1.1
	(-)-2-butanol	9.6 ± 0.8	7.2 ± 1.3	2.4 ± 0.6

The oxidation of racemic, (+)-, and (-)-2-butanol by microsomes from chow fed controls and from phenobarbital treated rats was assayed in the absence (Total) and presence of 0.25 mM desferrioxamine.

racemic 2-butanol. These results suggest that neither the cytochrome P450 pathway nor the ·OH pathway of alcohol oxidation by microsomes from chow fed animals is stereospecific with respect to 2-butanol.

In view of the multiplicity of cytochrome P450 in normal microsomes, it was considered that perhaps an induced system might display different results. Rats were treated with phenobarbital, a well known inducer of certain cytochrome P450 isozymes. The rate of oxidation of 2-butanol by microsomes from phenobarbital treated rats was similar to rates found with microsomes from controls (Table XIX). No stereospecificity was observed either in the absence or in the presence of desferrioxamine for microsomes isolated from phenobarbital treated rats. The possibility that the (+) or (-) isomers could be interconverted by a "racemase" present in the microsomes was considered. To rule this out, the following experiment was performed. Microsomes were washed extensively to remove contaminating enzymes, primarily alcohol dehydrogenase. The microsomes were then incubated with the individual isomers under standard experimental conditions. Yeast alcohol dehydrogenase plus $NAD^{(+)}$ was next added to the system. We had previously seen that this enzyme displayed absolute stereospecificity for the (+) over the (-) isomer of 2-butanol. Production of 2-butanone or of NADH was observed only with (+)-2-butanol. Thus, no interconversion of the isomers occurred when they were incubated with microsomes.

Experiments were next carried out with microsomes isolated from the chronic ethanol fed rats. As shown in Table XX, whereas the oxidation of the (+)-2-butanol stereoisomer was the same as that of racemic 2-buanol, the (-)-2-butanol stereoisomer was oxidized at a significantly lower rate. This difference was especially notable in the presence of desferrioxamine, where the (+)-isomer was oxidized at a twofold greater

Table XX. Oxidation of racemic, (+) and (-)-2-butanol by liver microsomes from ethanol fed rats and pair fed controls.

Microsomes	Substrate	Rate of Oxidation (nmol/min/mg protein) Total	plus Desferrioxamine	·OH-Component
Chronic ethanol (n = 3)	Racemic 2-butanol	37.2 ± 1.8	29.5 ± 3.2	7.7 ± 1.7
	(+)-2-butanol	34.0 ± 2.8	27.9 ± 2.5	6.1 ± 0.6
	(-)-2-butanol	20.7 ± 2.4*	14.0 ± 1.3*	6.7 ± 1.2
Pair fed (n = 3)	Racemic 2-butanol	15.2 ± 2.0	9.4 ± 0.3	5.8 ± 1.8
	(+)-2-butanol	14.0 ± 1.7	8.3 ± 0.6	5.7 ± 1.6
	(-)-2-butanol	11.6 ± 2.1	6.5 ± 0.2*	5.1 ± 2.0

*P < 0.01 with respect to the (+)-isomer or the racemic 2-butanol

The oxidation of racemic, (+)-, and (-)-2-butanol by microsomes from ethanol fed rats, and their pair fed controls, was assayed in the absence and presence of 0.25 mM desferrioxamine.

rate than the (-)-isomer (Table XX). Thus, in these microsomes, the cytochrome P450 dependent pathway of alcohol oxidation displays some stereospecificity. The ·OH dependent pathway of alcohol oxidation was, however, not stereospecific.

It was also interesting to note that there was a trend toward some discrimination against the (-)-isomer by microsomes from the pair fed animals although the differences were not as striking as those seen after chronic ethanol treatment (Table XX). No such discrimination was noted for the ·OH dependent pathway. Thus, the stereoselectivity displayed by the microsomal alcohol oxidizing system after chronic alcohol consumption seems to be due to the cytochrome P450 component.

E. Effects of Pyrazole on the Microsomal Alcohol Oxidizing System.

Pyrazole, a classic inhibitor of alcohol dehydrogenase, has previously been shown to be a potent inducer of the activity of dimethylnitrosamine demethylase (118,119) and was found to bind to cytochrome P450, producing a type 2 binding spectrum (120). Pyrazole treatment induced a protein with a molecular weight of 50,000-51,000 daltons in rat liver (118,119). These effects are similar to those seen by chronic ethanol consumption. Thus, chronic ethanol consumption by rats has also been shown to enhance the activity of dimethylnitrosamine demethylase (121,122) and to induce a protein with properties of cytochrome P450 with a molecular weight of 50,000 to 52,000 daltons (6,121). In addition, the magnitude of spectral changes produced when either ethanol or pyrazole bound to cytochrome P450 was increased by ethanol consumption (123). In view of these results, it appeared that pyrazole treatment may induce a cytochrome P450 isozyme with properties similar to those induced by chronic alcohol consumption.

E-1 Pyrazole Induces Microsomal Alcohol Oxidation.

Table XXI shows that microsomes from pyrazole induced rats, in the presence of desferrioxamine, catalyzed the oxidation of 2-butanol and ethanol at rates that were twofold greater than microsomes from saline controls. This induction is comparable to that seen with chronic ethanol consumption (Fig. 3, Table XIII). Because the increase is seen especially in the presence of desferrioxamine it seems that the cytochrome P450 component and not the ·OH component is responsible for this induction.

While alcohol oxidation is increased, the oxidation of aminopyrine was not changed by pyrazole treatment. The content of cytochrome P450 per mg microsomal protein was not increased by the pyrazole treatment: 0.81 ± 0.13 nmol/mg protein for salines vs. 0.71 ± 0.07 nmol/mg protein for pyrazole treated. Hence, the oxidation of ethanol and 2-butanol was also enhanced when results were expressed as turnover numbers (nmol/min/mg cytochrome P450) (Table XXII).

E-2 Binding Spectra with Pyrazole Induced Microsomes.

Another similarity between alcohol induction and pyrazole induction in rat liver microsomes concerned the binding spectra of DMSO and 2-butanol. As was the case after chronic alcohol treatment (Figs. 4 and 5), DMSO and 2-butanol produced a reversed type 1 binding spectrum with microsomes isolated from pyrazole treated rats (Figs. 6 and 7). No spectra could be observed when DMSO or 2-butanol was added to microsomes from the saline controls. These results are yet another indication that chronic alcohol consumption and pyrazole treatment may induce the same cytochrome P450 isozyme(s).

Table XXI. Effect of pyrazole induction on the oxidation of alcohols and aminopyrine as expressed per mg protein.

Substrate	Concentration (mM)	Treatment	Product formation (nmol/min/mg protein) - desferrioxamine	+ desferrioxamine	
Ethanol	50	Saline	43.6 ± 6	14.9 ± 2	
"	50	Pyrazole	52.9 ± 3	25.1 ± 2	P = .005
2-Butanol	33	Saline	48.6 ± 6	21.5 ± 3	
" "	33	Pyrazole	73.5 ± 14	38.6 ± 4	P = .02
Aminopyrine	5	Saline	N.D.	7.0 ± 1.1	
"	5	Pyrazole	N.D.	6.5 ± 0.6	

Substrate oxidations were assayed as described in Materials and Methods. When present, the final concentration of desferrioxamine was 250 μM. The above values represent the means of 4 experiments. Rats used in these experiments weighed 50 grams.

Table XXII. Effect of pyrazole induction on the oxidation of alcohols and aminopyrine as expressed per nmol - cytochrome P450.

Substrate	Concentration (mM)	Treatment	Product formation nmol/min/nmol P450
Ethanol	50	Saline	18.4
"	50	Pyrazole	35.3
2-Butanol	33	Saline	26.5
" "	33	Pyrazole	54.4
Aminopyrine	5	Saline	8.6
"	5	Pyrazole	9.2

Substrates were assayed as described in Materials and Methods. Desferrioxamine was present in all reaction mixtures at a concentration of 250 μM. The above values represent the means of 4 experiments. Rats used in these experiments weighed 50 grams.

FIGURE 6

DMSO binding spectra with saline and pyrazole induced rat liver microsomes

The binding spectra were determined as described in Methods. Results from a typical spectrum are shown.

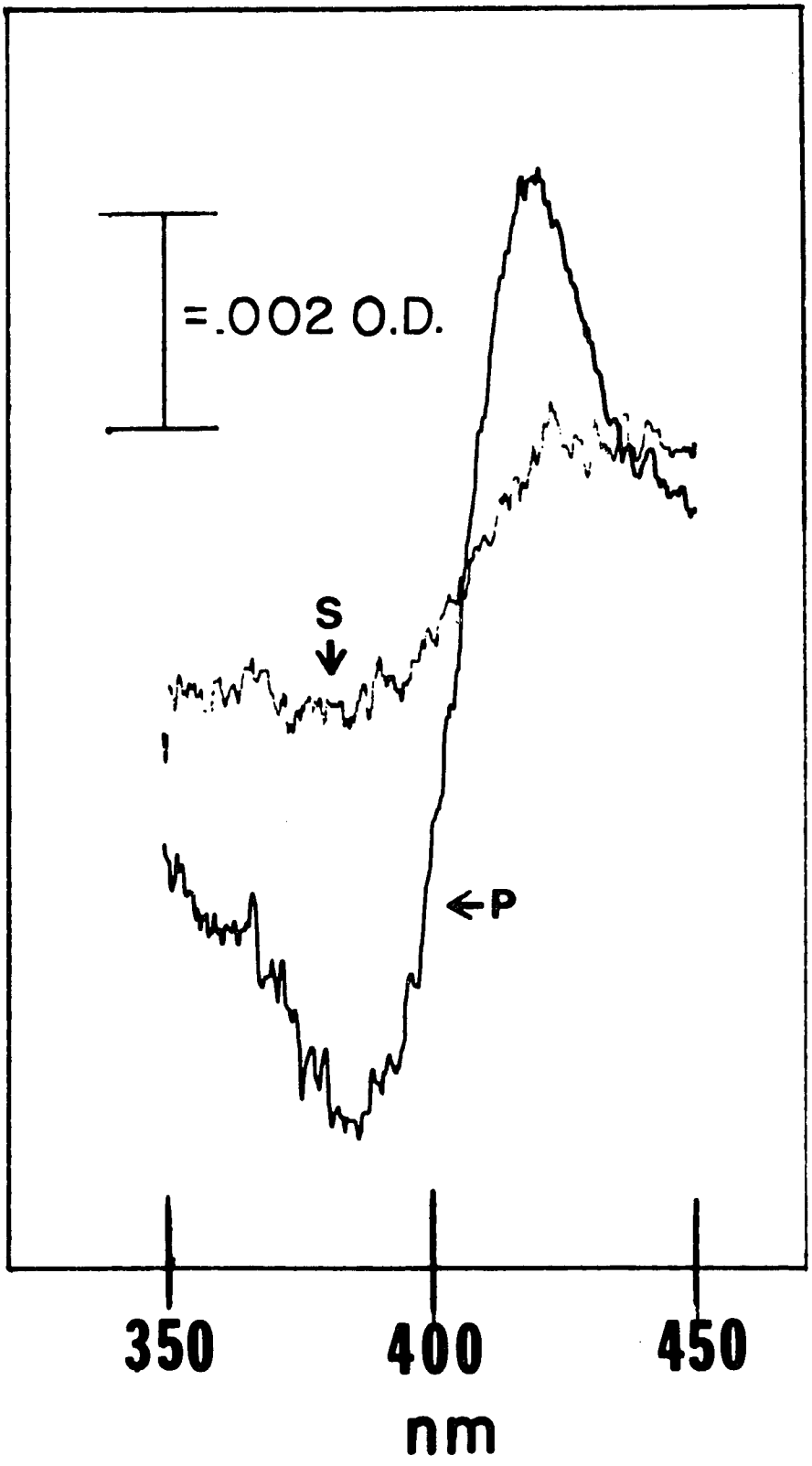
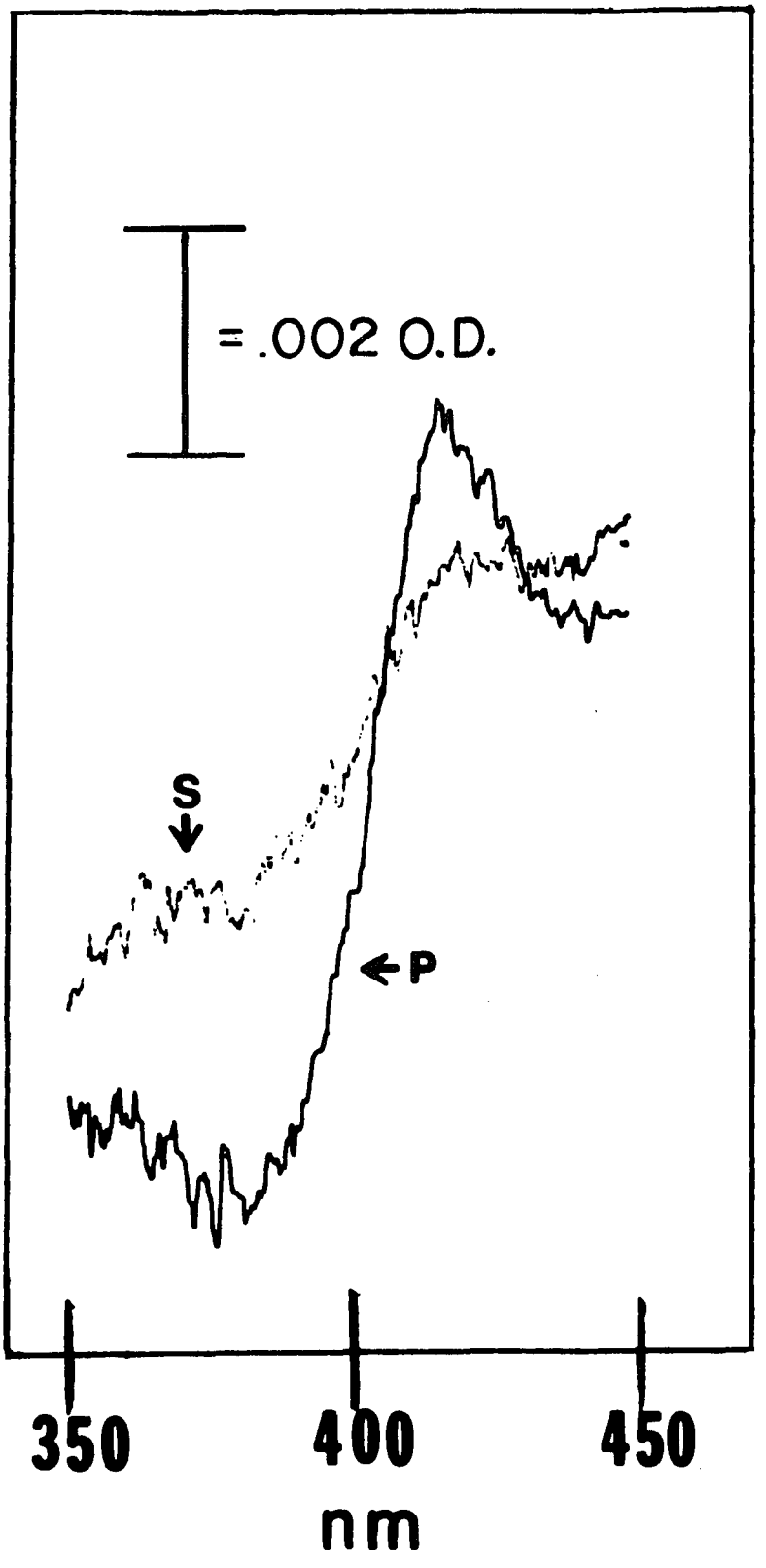

=.002 O.D.
S
P
350
400
450
nm

FIGURE 7

2-Butanol binding spectra with saline and pyrazole induced rat liver microsomes

The binding spectra were determined as described in Materials and Methods. Results from a typical spectrum are shown.


= .002 O.D.
S
P
350
400
450
nm

E-3 Stereospecificity of the Pyrazole Induced Cytochrome P450.

As previously noted (Section D), a characteristic of the alcohol induced cytochrome P450 is that unlike others tested (cytochrome P450 from pair fed controls, chow fed or phenobarbital treated rats), the alcohol induced cytochrome P450 displayed stereospecificity towards (+)- versus (-)-2-butanol. It was therefore of interest to see if pyrazole treatment would display similar results. Indeed stereopreference of the (+) isomer was observed in the presence of desferrioxamine (Table XXIII). It was, however, noted that the 50 g saline controls also were displaying stereospecificity. This was in contrast to previous results observed with larger control rats (250 grams) in which no stereodiscrimination was observed (Table XIX). It seemed that in addition to the effects of induction by pyrazole, animal size was also playing a role in stereochemical preference. An intermediate sized animal (100 g) was therefore studied.

As can be seen in Table XXIII, the animals treated with pyrazole still displayed a large stereopreference for the (+) alcohol while the saline controls displayed some stereospecificity although considerably less than that seen for the younger animals. Thus, like ethanol, pyrazole seems to induce a cytochrome P450 that displays stereochemical preferences towards (+)- over (-)-2-butanol.

E-4 Effect of Carbon Monoxide on the Microsomal Oxidation of Alcohols by Pyrazole Induced Rats.

Experiments with carbon monoxide were carried out to demonstrate that the oxidation of the alcohols was dependent on the catalytic activity of cytochrome P450. The effect of a 30% CO:70% air mixture on the oxidation of the alcohols and aminopyrine was compared to the effects

Table XXIII. Effects of pyrazole induction on the stereospecificity of 2-butanol oxidation.

Rat weight	50 grams		100 grams	
Treatment	Saline	Pyrazole	Saline	Pyrazole
(+) 2-Butanol	19.4 ± 2	42.4 ± 5	11.6 ± 3	30.7 ± 7
(-) 2-Butanol	10.2 ± 2*	17.6 ± 2**	7.8 ± 3	11.3 ± 3**

* $.01 < P < .02$
** $.001 < P < .002$

Substrate oxidations were assayed as described in Materials and Methods. Desferrioxamine was present at a final concentration of 250 μM. (n = 3)

of a 30% N_2:70% air mixture. The N_2:air mixture had no effect on the oxidation of any one of the substrates as compared to the controls (100% air) (Table XXIV). In contrast, addition of CO caused a significant decrease in the oxidation of alcohols and aminopyrine with microsomes from pyrazole induced rats and the saline controls.

The oxidation of the alcohols appeared to be somewhat more depressed by the CO treatment in the pyrazole treated than the saline group. In both groups, however, the inhibitory effects of CO were larger for racemic and (+)-2-butanol than they were for (-)-2-butanol.

In summary, microsomes from pyrazole treated rats show several properties with regard to the microsomal alcohol oxidizing system which are similar to those found after chronic ethanol consumption. Since pyrazole and its derivatives are widely used to inhibit the metabolism of ethanol via alcohol dehydrogenase, induction of the microsomal metabolism of alcohols by the same treatment may not be desirable.

Table XXIV. Effect of carbon monoxide on pyrazole induced microsomal substrate oxidation.

Substrate	Concentration (mM)	Product formation (nmol/min/mg protein							
		Saline				Pyrazole			
		Control	N_2	CO	% Inh.	Control	N_2	CO	% Inh.
Ethanol	50	11.8 ± 1.1	12.5 ± 0.6	6.8 ± 0.6*	-42	19.9 ± 1.7	21.2 ± 2.3	8.7 ± 1.1*	-56
Racemic-2-Butanol	33	14.7 ± 1.1	15.7 ± 0.6	7.7 ± 0.6*	-48	36.1 ± 5.2	33.0 ± 4.6	10.3 ± 0.6*	-71
(+)-2-Butanol	33	14.5 ± 0.6	15.2 ± 0.6	6.3 ± 0.0*	-57	32.9 ± 3.5	29.8 ± 2.3	8.7 ± 0.6*	-74
(-)-2-Butanol	33	8.2 ± 1.7	9.0 ± 1.7	6.0 ± 1.1	-27	13.4 ± 1.1	13.6 ± 1.7	6.8 ± 0.6*	-49
Aminopyrine	5	7.0 ± 1.1	6.7 ± 1.1	2.8 ± 0.6*	-60	6.5 ± 0.6	5.7 ± 0.0	2.6 ± 0.6*	-60

*$P \leq 0.02$

Substrate oxidations were assayed as described in Materials and Methods. Desferrioxamine was present in all reaction mixtures at a final concentration of 250 μM. The above values represent the means of 3 experiments.

CHAPTER IV SUMMARY AND DISCUSSION

Initial studies characterizing the oxidation of the secondary alcohol, 2-butanol, by rat liver microsomes have shown that this alcohol is metabolized to its corresponding ketone, 2-butanone (Fig. 1). Unlike ethanol and methanol, 2-butanol was not a substrate for the enzyme catalase (Fig. 1). This enzyme is usually found as a contaminant in isolated microsomes and it had been originally argued that the activity of the microsomal alcohol oxidizing system was due to contamination by this enzyme (124). It was later shown by Lieber and coworkers that in fact this was not the case (73,84). Since 2-butanol turned out not to be a substrate for catalase, results involving its oxidation could not be attributed to contamination of that enzyme. In addition, azide, a potent inhibitor of catalase, was routinely added to the reaction mixture further avoiding any participation of the enzyme.

Previous studies (28,29,94) have suggested that isolated rat liver microsomes oxidize alcohols by two pathways; one depends on the interaction of the alcohol with ·OH, the other involves cytochrome P450, and appears to be independent of ·OH. The inhibition of 2-butanol oxidation by desferrioxamine and by competing ·OH scavengers (Table II) suggests some role for ·OH in the oxidation of 2-butanol. However, the contribution of the ·OH dependent pathway towards the overall oxidation of 2-butanol appears to be considerably less than when ethanol is the substrate for the microsomal alcohol oxidizing system. The kinetic studies shown in Figure 2 and Table I indicate that 2-butanol is a particularly effective alcohol substrate for the cytochrome P450 pathway of oxidation of alcohols. The V_{max} for oxidation of 2-butanol is

significantly greater than that for ethanol, whereas sensitivity of 2-butanol oxidation to desferrioxamine and to competitive ·OH scavengers is considerably less than that found with ethanol. 1-Butanol also appears to be oxidized primarily by the cytochrome P450 pathway; however, the V_{max} for 1-butanol oxidation is much smaller than that for 2-butanol oxidation. This lower rate of 1-butanol oxidation occurs despite the fact that the K_m for 1-butanol is lower than that of 2-butanol (Table I). It appears that the position of the oxidizable hydroxyl group may be important in determining the overall turnover of the alcohol substrate. In this context, tertiary butyl alcohol is oxidized to acetone and formaldehyde at much lower rates than either 1- or 2-butanol and t-butyl alcohol does not appear to be a substrate for cytochrome P450 (125). Instead, its oxidation by microsomes seems to occur entirely via the ·OH pathway (125).

Organic hydroperoxides can catalyze the oxidation of drugs and alcohols but not ·OH scavengers in microsomal systems (126,127,95,96).

The finding that the oxidation of 2-butanol can be catalyzed by cumene hydroperoxide in place of the NADPH generating system in a reaction insensitive to desferrioxamine (Table III) suggests the presence of a cytochrome P450 dependent pathway which is independent of ·OH in the mechanism of microsomal oxidation of 2-butanol. Additional evidence for the involvement of cytochrome P450 in the metabolism of 2-butanol came from studies utilizing CO. A rather low $CO:O_2$ ratio (2:1) was employed in these experiments rather than the 5:1 or 10:1 ratio usually employed in order to avoid anaerobiosis, which would interfere with oxygen radical production. These studies showed that while CO did not inhibit the oxidation of ·OH scavengers, CO partially

blocked the oxidation of 2-butanol. The lack of inhibition by CO on DMSO oxidation is particularly interesting in view of a report by others which states that DMSO appears to be metabolized by cytochrome P450 and not ·OH (77). The latter studies, however, were conducted with cytochrome P450 isolated from alcohol induced rabbits and species differences may account for this discrepancy.

The present results suggest that cytochrome P450 is not involved in the production of ·OH, at least with uninduced microsomes. Indeed, it has previously been shown that in reconstituted systems from phenobarbital treated rats, oxidation of ·OH scavengers was the same whether the system was composed of NADPH cytochrome P450 reductase and phospholipids alone or if cytochrome P450 was added to the reductase system (28). Because the above experiments were conducted with a reconstituted system it was possible that purification procedures could cause uncoupling of the reductase from efficient electron transfer to cytochrome P450. In addition, the ratio of reductase to cytochrome P450 used in the purified systems was different than the ratio found in intact microsomes.

The present studies have shown that, in intact microsomes, MEOS is composed of a cytochrome P450 component which does not appear to involve the production of ·OH (at least in the case of uninduced microsomes) and a ·OH producing component for which the reductase appears to be responsible. While ·OH scavengers are oxidized solely by the latter component and drugs solely by the former, alcohols seem to be oxidized by both. Table V supports this theory. While the addition of desferrioxamine (which blocks production of ·OH) did not change the per cent inhibition of CO on the metabolism of aminopyrine, it greatly increased the effect of CO on ethanol and 2-butanol oxidation. Under

these conditions, the percent inhibition of CO on alcohol oxidation becomes the same as it is for the drug aminopyrine. Thus, by the use of desferrioxamine or CO, each of the two alcohol oxidizing pathways can be blocked independently of one another.

To further discern the cytochrome P450 pathway from the ·OH pathway, rats were treated with the synthetic metalloporphyrin Co IX. Previous studies showed that a single injection of Co IX greatly decreased the microsomal content of cytochrome P450 (110). In our studies it was noted that in additon the activity of cytochrome P450 reductase was also significantly diminished (Table XI). Consistent with these observations was the fact that not only was drug metabolism decreased, but the oxidation of ·OH scavengers and the ·OH dependent oxidation of alcohols was also decreased. Hence, Co IX is not a specific inhibitor of cytochrome P450 function as previously suggested (110) and inhibition of drug and alcohol metabolism by Co IX could reflect effects on the reductase as well as on cytochrome P450.

The experiments described above were conducted with microsomes from normal "untreated" rats. It was of interest to see what the effects of chronic alcohol consumption would be under conditions similar to those described above.

Chronic consumption of ethanol increases the rate of oxidation of ethanol by microsomes (73,41). This increase is due to the increase in total content of cytochrome P450, as well as the induction of a specific cytochrome P450 isozyme(s) which is more active in oxidizing ethanol and certain substrates (e.g., aniline) than control cytochrome P450 isozymes (77,108). Microsomes from ethanol fed rats also catalyzed the oxidation of ·OH scavengers at rates which were higher than control rates (111).

Experiments were carried out to evaluate the relative contributions of the two pathways in oxidizing ethanol by microsomes from ethanol fed rats and their pair fed controls, and to determine which of the two pathways is responsible for the increased rate of ethanol oxidation. Results in the current studies show that both the cytochrome P450 pathway and the ·OH pathway are increased after chronic alcohol consumption and that both of these pathways appear to contribute equally towards the overall rate of ethanol metabolism by both microsomal preparations. Thus, although both pathways are increased, their percent contribution, in the oxidation of ethanol is the same for microsomes from alcoholic rats or their pair fed controls. The addition of desferrioxamine or competing ·OH scavengers results in a decrease of about 40 to 50% in the rate of ethanol oxidation by both microsomal preparations (Tables XIV,XV). The rate of formaldehyde production from DMSO is decreased by 90% or more by desferrioxamine (Table XII) under conditions in which ethanol oxidation is decreased by 40 to 50%. Thus, part of the ethanol oxidizing activity of both microsomal preparations (about one half) is ·OH dependent, while the other part is independent of ·OH. Results summarized in Table XIII demonstrate that of the 12 nmol/min/mg protein increase in the rate of ethanol oxidation found after chronic ethanol consumption, there is still an increase of about 6 nmol/min/mg protein when the ·OH dependent pathway of ethanol oxidation is essentially abolished. About 50 to 60% of the increase in the rate of ethanol oxidation can be attributed to the cytochrome P450 pathway, whereas the remainder is due to an increase in the ·OH dependent pathway. The calculated percent increase in the ·OH dependent pathway of ethanol oxidation (about 60% over control rates) is in excellent agreement with the percent increase of DMSO oxidation found after chronic ethanol consump-

tion (Table XII, 65%).

Further support for two pathways in ethanol oxidation, and for increases in both of these pathways after chronic ethanol consumption is provided by the inhibition studies by various additives. Whereas the overall rate of ethanol oxidation by both microsomal preparations is inhibited by competing ·OH scavengers, aniline, and 2-butanol (Table XIV), the inhibition by the ·OH scavengers is much more pronounced when examining the ·OH dependent pathway of ethanol oxidation (Table XVI). On the other hand, the inhibition by aniline and 2-butanol, at least with microsomes from the ethanol fed rats, appears to be greater in the ·OH independent pathway of ethanol oxidation (Table XV). This probably reflects the ability of aniline and 2-butanol to act as good substrates for the alcohol preferring cytochrome P450.

Since results with uninduced microsomes suggested that 2-butanol, relative to ethanol, was a better substrate for the cytochrome P450 pathway than for the ·OH pathway, the effects of chronic alcohol consumption on the microsomal oxidation of 2-butanol was evaluated. As can be seen in Figure 3 the oxidation of 2-butanol is also increased after ethanol consumption. This increase is largely insensitive to desferrioxamine, which suggests that the increase is primarily due to enhanced metabolism of 2-butanol by the cytochrome P450 pathway, and that 2-butanol, as compared to ethanol, may be a more effective substrate for the alcohol inducible cytochrome P450. This may prove to be of value in studies concerning the mechanism of alcohol oxidation, and the stoichiometry between utilization of NADPH and O_2 and product formation.

Another point of interest was the ability of 2-butanol to yield an inverse type 1 binding spectrum with microsomes from chronic alcoholic

rats but not with microsomes from pair fed, chow fed or phenobarbital treated rats. This may prove to be a quick and easy technique for the identification of the induction of a particular cytochrome P450 isozyme(s). As will be discussed below, DMSO binding spectra may also be an additional way to identify alcohol preferring P450 isozymes.

The cytochrome P450 dependent pathway could be enhanced because of an increase either in the content or a greater activity of an ethanol inducible isozyme. The 60% increase in content of cytochrome P450 can account for a large part of the increase in the rate of ethanol oxidation by the cytochrome P450 pathway. When the cytochrome P450 dependent rate of ethanol oxidation is expressed as turnover number (nmol acetaldehyde/min/nmol P450), rates are: for the pair fed control, 10, and for chronic ethanol, 12.7. Thus, the increase in content, as well as enhanced activity contribute to the increase in the rate of ethanol oxidation, although the former appears to make the major contribution. This probably reflects the possibility that even in the induced microsomes the ethanol preferring cytochrome P450 isozyme(s) can account for only a small percentage of the total P450 population. Partial purification of alcohol induced isozyme(s) from rats showed higher turnover numbers for ethanol and n-butanol than other P450 isozymes (6). Additionally, purified reconstituted systems from alcohol treated rabbits showed high turnover rates with alcohols as compared to other cytochrome P450 isozymes (7).

Since the reductase is an important loci for the production of ·OH (27-29), changes in reductase activity could be responsible for the increase in the ·OH dependent pathway of ethanol oxidation. The 20% increase in reductase activity does not appear to account fully for the 65% increase in the ·OH dependent pathway of ethanol oxidation.

H_2O_2 is the precursor of ·OH, and the ·OH dependent pathway requires the presence of iron, or actually, an appropriate iron chelate, e.g., iron EDTA. It is of interest that there are reports that the iron content of the liver cells (128,129), and the rate of microsomal production of H_2O_2 (124,130) are increased after chronic ethanol consumption These changes may contribute to the increase in the ·OH dependent pathway of ethanol oxidation.

Many toxins, e.g., acetaminophen (131) or carbon tetrachloride (132) must first be activated by cytochrome P450, therefore changes in the amount and forms of cytochrome P450 may play an important role in the increased hepatotoxicity of these agents. In addition, since ·OH is such a powerful reactant, increased production of ·OH after chronic ethanol consumption may also contribute to liver cell damage in alcoholics. In this respect, it is interesting to note that hydroxyl radicals may be the most important free radicals initiating lipid peroxidation in microsomes (91,133) and some investigators have found evidence that lipid peroxidation is increased after chronic alcohol consumption (134). However, others have found that in rat microsomes lipid peroxidation was actually decreased after chronic alcohol consumption (135). Thus, this area remains controversial and further experiments will be required to see if indeed chronic alcoholism causes liver damage via increased levels of ·OH and hence lipid peroxidation.

DMSO is a potent ·OH scavenger and has been shown to be oxidized to formaldehyde by a variety of ·OH generating systems (136). Previous experiments by Morgan et al. (77,106) demonstrated an interaction between DMSO and the ethanol inducible cytochrome P450 as reflected by a binding spectrum upon the addition of DMSO to the cytochrome

P450 from rabbits or to microsomes from ethanol treated rats. This interaction was also observed in the present report. In contrast to DMSO, other ·OH scavengers did not show any binding spectra with microsomes from alcohol treated rats. It has recently been suggested that DMSO serves as a substrate for the alcohol induced cytochrome P450 isolated from rabbits (77) rather than just behaving as a ·OH scavenger. Our experiments show that with rat liver microsomes, DMSO does not serve as a substrate for cytochrome P450. In this case, no significant levels of formaldehyde, methane or ethane were observed when desferrioxamine was added to the microsomal system. Recent experiments from our laboratory (Winston and Cedarbaum, manuscript in preparation) have shown that it is very difficult to remove iron and/or EDTA from purified preparations of P450 so that addition of excess P450 could also be a source of added iron-EDTA to the reaction system. It was observed that although added P450 doubled the oxidation ·OH scavengers, boiled P450 was as effective as the native enzyme. Moreover, the increase produced by added cytochrome P450 was sensitive to desferrioxamine. Perhaps the interaction which causes a binding spectrum by DMSO may be due to solvent-like, hydrophobic interactions between this particular cytochrome P450 isozyme and DMSO, rather than a true substrate binding. It is recognized that the above results with DMSO apply to the rat liver microsomal system, and it is possible that microsomes from other species may yield different results.

Results of Table XVII show that cumene hydroperoxide supports a ·OH independent pathway of ethanol oxidation by both microsomal preparations. Interestingly, there are no differences in the cumene hydroperoxide supported rates of ethanol oxidation by microsomes isolated from ethanol fed rats or their pair fed controls. This suggests the possibility that the ethanol inducible cytochrome P450 may not function as an effective peroxy-

genase with organic hydroperoxides. In addition, since organic hydroperoxides destroy cytochrome P450, it is also possible that the ethanol inducible cytochrome P450 may be especially labile to destruction by strong oxidants such as organic hydroperoxides. This seems unlikely, however, since it was noted that similar rates of ethanol oxidation were observed for both microsomal preparations at shorter reaction periods and at a lower concentration of cumene hydroperoxide. There are differences between NADPH supported and organic hydroperoxide supported mixed function oxidase activities, e.g., with respect to the pattern of hydroxylated metabolites produced (137,138) or in sensitivity to various inhibitors (139); hence, the mechanisms of the two systems appear to differ. Indeed, in contrast to NADPH, cumene hydroperoxide supported the induction of some drugs, but not others (140). Comparisons of ethanol oxidation supported either by NADPH or cumene hydroperoxide may perhaps be of value in understanding the interaction of ethanol with control and with the ethanol inducible cytochrome P450.

With the above characteristics in mind, studies involving the stereospecificity of the microsomal alcohol oxidizing system were carried out. These studies were conducted with 2-butanol. Of importance in the choice to study 2-butanol was the fact that this alcohol exists and is readily available as two stereochemical isomers, the (+) and (-) enantiomers, and 2-butanol is not a substrate for catalase. Hence, a comparison of the oxidation of (+)-2-butanol and (-)-2-butanol may provide information concerning the mechanism of microsomal oxidation of alcohols and on the nature of the ·OH generated during microsomal electron transfer. It would be anticipated that a "·OH free in solution" would not demonstrate stereochemical discrimination, whereas a "bound ·OH" might preferentially oxidize one of the stereoisomers. On the other hand, an enzymatic reac-

tion such as that catalyzed by cytochrome P450 might display stereospecificity.

Cytochrome P450 has been shown to display sterospecificity towards certain drugs (112-117) and 2-butanol turned out to be a good alcohol substrate for the cytochrome P450 mediated pathway of MEOS. Previous experiments showed that the microsomal alcohol oxidizing system was stereospecific in that the pro-R hydrogen, but not the pro-S hydrogen, was removed when the microsomes were incubated with either R-1-^{3}H ethanol or S-1-^{3}H ethanol (141,142). This is the same stereospecificity which catalase exhibits with regard to ethanol (141,142), and since inhibitors of catalase such as azide were not used in these experiments, the question as to whether or not the microsomal alcohol oxidizing system (independent of catalase) is indeed stereospecific still remained to be determined.

It was observed that the rates of oxidation of (+), (-) and racemic 2-butanol were not significantly different from each other when microsomes from chow fed rats or phenobarbital treated rats were utilized. No stereospecificity was observed in the absence or presence of desferrioxamine with either microsomal preparation. This suggests that neither the total oxidation rate, nor the cytochrome P450 dependent rate nor the ·OH dependent rate are stereospecific for 2-butanol with these microsomes. The lack of stereospecificity by the ·OH dependent mechanism suggests that either the active oxygen species is similar to that found in the model chemical systems (e.g., free in solution) or perhaps is bound in such a manner that it can still react equally well with either isomer of 2-butanol.

The rate of oxidation of 2-butanol by microsomes from the phenobarbital treated rats is the same on a per mg microsomal protein basis as that found with uninduced control microsomes. This suggests that the

phenobarbital induced cytochrome P450 may not be an especially effective catalyst for the oxidation of alcohols. By contrast, chronic ethanol consumption results in a threefold increase in the rate of 2-butanol oxidation by the microsomes. Hence, the stereospecific nature of this inducible microsomal alcohol oxidizing system was studied. In this case, it was noted that (+)-2-butanol was preferentially oxidized in comparison with its enantiomer. The difference in the rate of oxidation is approximately a twofold increase for the (+) isomer, both in the absence and presence of desferrioxamine. The difference between the desferrioxamine inhibited rate and the total rate (which yields the ·OH rate) shows no stereospecificity. Hence, the stereochemical effects seen in microsomes from alcohol treated rats appears to be due to the induction of a distinct cytochrome P450 isozyme after chronic consumption of ethanol. The fact that complete stereospecificity is not observed in these experiments may be due to the existence of various populations of cytochromes P450 in isolated microsomal preparations, some of which may display stereospecificity with regard to the oxidation of 2-butanol, and some which may not. It would, therefore, be of interest to determine the stereospecific nature of the microsomal alcohol oxidizing pathway with purified cytochrome P450 preparations, especially the alcohol inducible cytochrome P450. To date, however, it has not been possible to purify this enzyme to homogeneity from rat liver.

It was interesting to note that microsomes from pair fed rats displayed some preference for (+)-2-butanol oxidation of the (-) isomer by the cytochrome P450 dependent pathway. While the discrimination for the (+) isomer was not as sharp as that seen with microsomes from ethanol fed rats, there was nonetheless a difference which is

not observed with microsomes from chow fed rats. This suggests the possibility that some differences in the cytochrome P450 isozyme composition may exist between microsomes isolated from rats fed the pair fed control ciet, and chow fed controls.

The sharp discrimination for (+)-2-butanol by microsomes from alcohol induced animals compared to the other systems tested, along with the binding spectra seen for 2-butanol and DMSO with these microsomes, may prove to be of value in the identification of a distinct cytochrome P450 induced by ethanol.

The above findings can be further extended to other inducers as well. Indeed pyrazole, the potent inhibitor of alcohol dehydrogenase, seems to share these characteristics.

Present studies have shown that treating rats for 3 days with pyrazole induces the microsomal alcohol oxidizing system in a similar fashion to chronic alcohol consumption. It appears that the induction by pyrazole and by chronic ethanol treatment may be due to the induction of a similar cytochrome P450 isozyme(s). In support of this idea are the following observations: Both treatments increase the desferrioxamine insensitive pathway of microsomal alcohol oxidation. Likewise, in both cases, there was stereochemical preference for (+)-2-butanol over the (-) isomer. In this respect, however, it became apparent that in addition to pyrazole treatment, animal size also appeared to cause some stereopreference (Table XXIII) although the effects were less pronounced. The effect of animal size on stereospecificity is an interesting finding in view of the changing nature of cytochrome P450 with age (143-145). It is also possible that different amounts of the same isozyme may be present in microsomes from the various sized animals.

Another similarity between induction by pyrazole and ethanol concerned binding spectra. Unlike the controls, microsomes isolated from pyrazole treated rats or ethanol treated rats displayed inverse type 1 binding spectra with DMSO or 2-butanol. It is noteworthy to see that whereas some stereospecificity was observed for untreated younger animals, no spectra were observed with these microsomes. This further supports the idea that ethanol and pyrazole induce a distinct P450 isozyme(s). In addition, the increase in the oxidation of alcohols found after pyrazole treatment is blocked by CO and is not associated with an increase in content of cytochrome P450 or in the oxidation of aminopyrine, suggesting induction of an alsohol preferring P450(s). Results of others have shown that both alcohol and pyrazole treatments induce the activity of dimethylnitrosamine N-demethylase (118,119,121) and induce a cytochrome P450 isozyme with a molecular weight of 50,000-52,000 (118-121). Further characterization of the properties of microsomes from pyrazole treated rats and eventual purification of the cytochrome P450 isozyme(s) induced by pyrazole and ethanol will be required to evaluate the possibility that both treatments induce the same proteins. In this respect it is of interest that a recent report had shown that in rabbits, imidazole, which differs from pyrazole in having a 1,3 diazole ring instead of a 1,2 diazole ring, also increases the microsomal oxidation of alcohols (146). In addition, a comparison of the amino terminal sequence, spectral and catalytic properties and the HPLC peptide maps following trypsinolysis of the cytochrome P450's induced by ethanol or imidazole in rabbits suggested that the proteins were identical (147).

The use of pyrazole for several days for the inhibition of alcohol metabolism via alcohol dehydrogenase may have to be reevaluated, since

this treatment inhibits one pathway of alcohol oxidation (alcohol dehydrogenase) but induces another (MEOS). Of particular significance are cases in which pyrazole is administered in conjunction with ethanol. The prolonged administration of both ethanol and pyrazole is considerably more toxic to the liver than administration of ethanol or pyrazole alone (148). Possible synergistic effects of pyrazole and ethanol at the microsomal level may play a role in this enhanced toxicity.

One difference between the effects of pyrazole and chronic ethanol consumption is that the former does not significantly increase the content of cytochrome P450, whereas the latter treatment usually results in a 50 to 100% increase in the content of cytochrome P450 (6,73). Teschke and coworkers (149) have recently shown that the lowering of carbohydrate content of the diet fed to the ethanol consuming rats (to allow adequate pair feeding with isocaloric carbohydrate controls) played a role in the induction of MEOS. Such dietary considerations as well as long induction times and expense would not play a role if pyrazole proves to be successful in inducing the same isozyme(s) as chronic ethanol consumption.

It is possible that many other compounds may induce the cytochrome P450(s) which ethanol and pyrazole seem to induce in rats. This may be of clinical significance in the treatment of alcoholics. The results in the work presented in this thesis suggest that studies involving stereospecificity in 2-butanol oxidation as well as the use of binding spectra may be a quick and simple technique in the identification of rat liver cytochrome P450 isozyme composition.

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