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PYRAZOLE, INTERACTIONS AND METABOLISM BY HEPATIC MICROSOMES

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PYRAZOLE, INTERACTIONS AND METABOLISM BY HEPATIC MICROSOMES

by

Dennis Elchanon Feierman

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfilment of the requirements for the degree of Doctor of Philosophy from the City University of New York

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ABSTRACT

"PYRAZOLE, INTERACTIONS AND METABOLISM BY HEPATIC MICROSOMES"

by

Dennis Elchanon Feierman

Advisor: Dr. Arthur I. Cederbaum

There is current interest in the interactions of alcohol and drugs. Drugs are metabolized via the liver microsomal mixed function oxidase system, which depends on cytochrome P-450 isozymes. Different populations of cytochrome P-450 isozymes can be induced via drugs, diet and alcohol. Alcohol induces a specific isozyme which plays a role in drug-alcohol interactions. This isozyme has been difficult to purify and its induction requires long term feeding of alcohol, which also causes many other metabolic derangements. Hence there is a need for other models which can induce the alcohol cytochrome P-450. The goal of this research is to demonstrate that pyrazole and 4-methylpyrazole, potent inhibitors of alcohol dehydrogenase that are widely used in alcohol research, interact and induce an alcohol preferring cytochrome P-450 and moreover, these agents are metabolized by this P-450.

Pyrazole and 4-methylpyrazole treatment appear to result in the induction of an alcohol-preferrring cytochrome P-450 as reflected by alcohol and drug oxidation data and binding

spectra with several substrates. Pyrazole treatment does not change the content of cytochrome P-450 or the activity of the cytochrome P-450 reductase. By contrast, treatment with 4-methylpyrazole increases the content of cytochrome P-450 about two-fold. Microsomes isolated from these treated rats exhibit several properties which are similar to microsomes isolated from rats chronically fed ethanol. This suggests the possibility that pyrazole- or 4-methylpyrazole-treatment may serve as good models to study the effects that ethanol has on the hepatic mixed-function oxidase system. Pyrazole and 4-methylpyrazole can inhibit microsomal oxidation of ethanol in vitro, and the effectiveness of these agent as inhibitors is increased in microsomes isolated from rats treated with pyrazole, 4-methylpyrazole or ethanol. Furthermore, pyrazole is metabolized by microsomes in a cytochrome P-450 dependent manner and its metabolism is increased by pyrazole-, 4-methylpyrazole or ethanol-treatment. In view of the above, extreme caution would be required in the use of pyrazole or 4-methylpyrazole to assess the role of alcohol dehydrogenase dependent and independent (e.g., microsomal) pathways in contributing towards overall metabolism of ethanol, especially in induced animals.

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CO	carbon monoxide
DMSO	dimethylsulfoxide
Fe	iron
H_2O_2	hydrogen peroxide
MEOS	microsomal ethanol oxidizing system
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
O_2^-	superoxide anion radical
.OH	hydroxyl radical
pyr	pyrazole
4-MP	4-methylpyrazole
3-MC	3-methylcholanthrene

CHAPTER I Introduction and Background

Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an extensive membranous network found throughout the cytoplasm of a cell. In rat hepatocytes, electron micrographs reveal the ER as a reticulation of tubules, vesicles and lamellae (1,2). The ER is a multifunctional organelle which can synthesize and transport proteins; synthesize and breakdown cholesterol, phospholipids and triglycerides; breakdown glycogen; desaturate fatty acids; and metabolize steroids, fatty acids and xenobiotics. The latter function is catalyzed by the cytochrome P-450 system. Of particular importance and interest is the increase in volume and surface area of the ER and concomitant increase in the activity of the cytochrome P-450 drug metabolizing system after treatment of animals with phenobarbital, carcinogens and other xenobiotics, including alcohol (3-7). The adaptability and function diversity of the ER have resulted in its being the object of many investigations.

In order to study the ER *in vitro*, disruption of cell structure via homogenization is necessary. The term "microsomes" is used to designate the artifactual microsized membranous vesicles that are formed from the

disrupted ER. Microsomes isolated from rat livers are the vehicle through which the studies on the cytochrome P-450 drug metabolizing system in this thesis were carried out.

Cytochrome P-450

Hemoproteins are one the most heavily studied biological compounds. This is probably because they are colored, readily observed and hold key roles in respiratory activities. Cytochrome P-450 is the terminal hemoprotein component of the hepatic microsomal mixed-function oxidase (drug metabolism) system. It functions in the metabolism of a broad spectrum of structurally unrelated endogenous and exogenous compounds (8). Cytochrome P-450, coupled with other metabolically linked enzymes, provide an important pathway for the elimination of xenobiotics. However, these systems are not only responsible for the detoxification of these compounds, they are also responsible for the activation of environmental agents to toxic or carcinogenic forms. Therefore, induction of this system by exposure to certain chemicals, drugs or alcohol may result in an increase in the rate of inactivation of helpful drugs or in some cases to harmful activation of environmental carcinogens.

Cytochrome P-450 was first detected as a microsomal pigment that gave a characteristic difference spectrum consisting of a broad intense absorbance band at 450 nm when dithionite reduced microsomes were bubbled with carbon monoxide (9): hence it derived its name, P-450. However, elucidation of the nature of this chromaphore was impeded by the fact that cytochrome P-450 is very unstable to various solubilization techniques. Cytochrome P-450 purification was hindered until 1967 when Ichikawa and Yamano developed an efficient way to stabilize cytochrome P-450 against detergent treatment by using glycerol and glutathione (10). Until that time, treatment of microsomes with detergent quantitatively converted the pigment to another spectrally distinct solubilized form that absorbed at 420 nm when reduced with dithionite and bubbled with carbon monoxide. This pigment, which was named P-420, turned out to be the inactivated form of P-450.

In spite of these impediments, certain properties and functions were determined. In 1962, Estabrook et al discovered that cytochrome P-450 functioned as a terminal oxidase in various important oxygenation reactions (11). Using light reversal of carbon monoxide inhibition of steriod C_{21} hydroxylase activity of adrenal cortex microsomes, they were able to demonstrate a photochemical action spectrum. This work gave impetus to look for other functions of cytochrome P-450 as well as to look at its

distribution in other organs and forms of life. It is now well established that cytochrome P-450 is capable of catalyzing the oxidation of fatty acids, alkanes, steroids, drugs, polycyclic hydrocarbons and other xenobiotics (12-15), and thus is probably the most versatile catalyst known.

Although the first confirmation that cytochrome P-450 was able to function in hydroxylation reactions was in the adrenal cortex, and there are many other extrahepatic tissues that contain cytochrome P-450 e.g. lung, intestine, kidney, placenta, testis and skin, its greatest activity and concentrations are found in the liver (12). Therefore, the studies presented in this thesis were carried out with hepatic microsomes.

Induction of Cytochrome P-450

The hepatic mixed function oxidase system, of which cytochrome P-450 is the terminal oxidase, is unique among mammalian enzymes. Two of its most striking features are: (1) a large number of structurally diverse chemicals can serve as substrates and (2) its activity can be enhanced by certain chemicals called microsomal inducing agents. The magnitude of these elevations are dose and time dependent and inducer dependent, i.e. different classes of inducers induce different isozymes of cytochrome P-450. Indeed, the

term cytochrome P-450 refers to a class of structurally and functionally related hemoprotein enzymes that have slightly different molecular weights, spectral characteristics and substrate specificity profiles. The latter differences are used to group them and their inducers into different classes.

The ability of barbiturates and other drugs to alter hepatic metabolism had been reported as early as 1940, in a paper which showed that rats treated with phenobarbital displayed an increase in ascorbate synthesis (16). It was latter shown that the same treatment could stimulate the demethylation of 3-methyl-4-monomethylaminoazobeneze (a hetapocarcinogin) by hepatic microsomal enzymes (17). It had been shown previously that certain polycyclic aromatic hydrocarbons e.g. 3-methylcholanthrene, also induced demethylation of 3-methyl-4-monomethylaminoazobeneze (18).

Following the observations that various compounds could induce drug metabolism it was wondered whether treatment resulted in the activation of nascent enzymes or resulted in the induction of new proteins. The fact that inducers could not stimulate enzyme activity in vitro (in fact the are sometimes inhibitory), that the stimulatory response was dose dependent (17,18), time dependent (19) and blocked by protein systhesis inhibitors (18,20), and that it was correlated to incorporation of labeled amino acids into microsomal protein supported the concept that activation of

drug metabolism was a result of de novo synthesis of new enzyme(s) rather than activation of nascent enzyme.

The results of treating animals with various inducers are changes in the rate of drug metabolism, increases in the concentration of mixed function oxidase components e.g. de novo synthesis of cytochrome P-450, and changes in the spectral properties of cytochrome P-450 adducts. To date, hundreds of chemicals are known to induce hepatic microsomal mixed function oxidases. Of particular interest is the differential enzyme induction that occurs with various inducers i.e. different inducers induce different populations (isozymes) of P-450. These differential effects can be seen with respect to the spectral characteristics and the specificity of the altered drug metabolism. For example, although microsomes isolated from rats treated with phenobarbital and 3-methylcholanthrene resulted in increased cytochrome spectra over controls, only the latter treatment resulted in a shift of the reduced carbon monoxide difference spectrum 2 nm to the blue (21). Recently, it was shown that microsomes from rats chronically fed ethanol showed substrate binding spectrum with either DMSO or 2-butanol that was not found in the pair-fed controls (22,23). An example of differential induction of drug metabolism is that treatment of rats with 3,4-benzpyrene is ineffective in increasing the metabolism of barbital, aminopyrine and other drugs, whereas

phenobarbital treatment is very effective (19). There are numerous other examples of differential induction (24). In this regard, the use of drug metabolic profiles and microsomal binding difference spectra are useful in ascertaining the type of effects an inducing agent has on microsomal drug metabolizing enzymes.

Purification and Presence of Multiple Forms of Cytochrome P-450

Initial attempts to purify cytochrome P-450 were unsuccessful due to instability of the enzyme to various solubilizing techniques. Solubilizing agents such as detergents, lipase, proteases, organic solvents and high salt concentrations converted cytochrome P-450 to inactive cytochrome P-420 (25). Purification of cytochrome P-450 was hindered until Ichikawa and Yamano reported that polyols e.g. glycerol, stabilized the detergent-treated enzyme (10). Successful deoxycholate solubilization of rabbit hepatic microsomes in the presence of glycerol lead to the purification and ultimately to the reconstitution of a cytochrome P-450 dependent hydroxylase system (26). Subsequently, other reconstituted mixed function oxidase activities, such as steroids, drugs and alkanes were demonstrated (13-15).

The fact that unlike other enzymes, a large number of structurally diverse chemicals can serve as substrates for cytochrome P-450 and that inducers of mixed function oxidase activity showed differential effects, probably gave great impetus to look for multiple isozymes of cytochrome P-450. Spectral studies supported the hypothesis that multiple isozymes of cytochrome P-450 existed. Microsomes isolated from rats treated with 3-methylcholanthrene displayed a reduced carbon monoxide difference spectrum that was shifted from 450 nm to 446-448 nm when compared to either controls or phenobarbital induced (21). Additional support for the hypothesis comes from binding spectra with ethylisocyanide, which when added to microsomes results in Soret peaks at 430 and 455 nm. The relative heights of these peaks are inversely proportional to each other and pH dependent (27). Hence, a plot of the two peak height as a function of pH results in two intersecting curves. Microsomes isolated from either control rats or rats treated with phenobarbital have curves that intersect at pH 7.4; however, microsomes isolated from 3-methylcholanthrene result in curves that intersect at pH 6.9 (28,29).

The ultimate proof that multiple forms of cytochrome P-450 exist must come from purification and biochemical characterization of each isozyme. To date, several different P-450 isozymes have been purified and have been shown to be different isozymes by immunochemical

double-diffusion analysis or cross-reaction experiments (30-32) or by amino acid analysis (33-35).

Of particular interest is the isozyme(s) induced by chronic alcohol consumption. Ohnishi and Lieber were the first to show that alcohol induced a distinct isozyme in rats (6). The isolation of this isozyme from rats has been difficult because of its instability. Furthermore, the techniques used to induce this isozyme cause many other metabolic derangements. Hence, there exists a need for other models for the induction of this isozyme(s). One of the goals of this thesis is to characterize the inductive affects of pyrazole and 4-methylpyrazole and to demonstrate that these agents induce an isozyme(s) of cytochrome P-450 that is the same or similar to that induced by ethanol and that the former my serve as a good model for the latter.

Substrate Binding Spectra

Any enzyme that has a cofactor(s) requirement for catalysis has a unique characteristic of making a dead-end enzyme substrate complex that can be extensively studied. Cytochrome P-450 has a "reducing equivalents cofactor" (which are electrons that are transferred from NADPH via cytochrome P-450 reductase: see Mechanism of Action); hence, enzyme substrate complexes can be readily studied. A distinctive characteristic of cytochrome P-450 is the

binding spectrum (a change in absorbance) that results from the interaction of P-450 with a substrate.

Narasimhulu and coworkers were the first to demonstrate a change in absorbance as a result of the interaction between cytochrome P-450 in solubilized adrenocortical microsomes and a substrate, 17-hydroxyprogesterone (36). The optical spectrum, which was later classified as a type I binding spectrum, showed a peak at 388 nm, an isosbestic point at 407 nm and a trough at 420 nm. A short time later, similar results were found when drugs were added to rat hepatic microsomes; in addition, certain drug interactions resulted in spectra that had the peaks and troughs reversed (37). These were later classified as type II binding spectra.

For a clear understanding of the interaction between cytochrome P-450 and a substrate it is necessary to examine the coordination chemistry of the heme iron and the various spin states. For simplicity, the discussion will be limited to the ferric form, Fe^{3+}. In this form, protoporphyrin iron has five electrons in the 3d subshell which can occupy any of the five electronic orbitals (d_{xy}, d_{xz}, d_{yz}, $d_{x^2-y^2}$, and d_{z^2}) associated with the 3d subshell. In a symmetric environment all d-orbitals are degenerate, i.e. they have the identical energies; therefore, each electron will occupy a separate electronic orbital with parallel spins (unpaired), since electrons repel each other, this is the most stable

configuration. This symmetry is lost when iron is placed into a porphyrin that is bound to a protein, because this renders the d-orbital energy levels non-degenerate and splits the 3d energy levels into two subsets. Since the $d_{x^2-y^2}$ and d_{z^2} orbital point out of the heme plane (and towards the direction of the ligands when the are present), their energies are raised. If the energy barrier separating these two subsets is large enough, the electrons will tend to occupy the lower energy electronic orbitals of the 3d subshell in a manner that will be consistent with the Pauli exclusion principle: only two electrons can occupy a single electronic orbital and they must have opposite spins. The result is that one electron is left unpaired corresponding to a total spin (S=# of unpaired electrons/2) of S=1/2 (low spin). Some of the protein bound iron-porphyrins will remain in the configuration where each electron occupies a different electron orbital because the enhanced stability gained by having a half filled orbital is enough to over come the energy barrier between the two subsets. The result is that all five electrons are unpaired, corresponding to a total spin of S=5/2 (high spin).

An analogous scheme exists for the heme iron at the active site of cytochrome P-450. The amount of P-450 that will be either in the low or high spin state will depend on the environment of the heme in a given isozyme, the energy

(temperature) of the system and the ligands (substrates) present. The latter effect is the cause of binding spectra. As a ligand draws closer to the $d_{x^2-y^2}$ and d_{z^2} orbitals, the energy of these orbitals increases because of the repulsion between the electrons of the ligand and those in the d orbital; hence, the two subsets of the d orbitals are energetically very separated and the low spin state will be energetically more favorable: a high to low spin transition occurs. Other ligands interact with cytochrome P-450 such that they decrease the energy difference between the two subsets and it becomes more energetically favorable to distribute the electrons in all five d orbitals: a low to high spin transition occurs.

There exist good evidence that cytochrome P-450 exists in both high and low spin states and that these states can be correlated to different absorption peaks, 390 nm and 420 nm respectively (38). Mitani and Horie were one of the first to suggest a correlation between the observed type I and type II spectral changes and the spin state of cytochrome P-450 (39,40). They concluded that cytochrome P-450 exists as a mixture of high spin and low spin components, that the type I difference spectrum was the result of a substrate-induce increase in the high spin state of cytochrome P-450 with a concomitant decrease in the low spin state and that the type II spectrum was the result of an increase in the low spin state of cytochrome

P-450 with a concomitant decrease in the high spin state. These spectral changes had been previously categorized and described in detail (41). In rat hepatic microsomes they were classified as type I, which is characterized by a change in difference spectrum that results in the appearance of an absorption peak at 385-390 nm and a trough at 420 nm, type II, as a change in difference spectrum that results in the appearance of an absorption peak at 425-435 nm and a trough at 390-405 nm, and modified type II, which is characterized by the appearance of an absorption peak at 420 nm and a trough at 388-390 nm.

The modified type II spectrum, which appears to be the mirror image of the type I spectral change, was initially envisioned as a displacement of endogenous type I substrates from the binding site of cytochrome P-450 and was renamed reverse type I (42). Despite extraction of endogenous substrates from microsomes one could still obtain reverse type I spectrum (43). Alcohols, which typically invoke a reverse type I spectrum, are now believed to interact directly with the heme iron in cytochrome P-450 rather than to displace endogenous substrates (43). Over a hundred compounds are known to invoke binding spectra (38). These binding spectra have been shown to be reversible and dependent upon the presence and amount of added compound in typical Michaelis fashion (41). In fact, if substrate dissociation is far from rate

$$S+E \underset{k3}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} E+P;\ Ks=k2/k1;\ Km=(k2+k3)/k1$$

limiting (k3 is much less than Ks) a comparison between the concentration of substrate which gives the half-maximal spectral change (Ks) or the half-maximal enzyme activity (Km) are valid. The fact that a compound can invoke a binding spectrum is no guarantee that it will be metabolized: the addition of octyalmine to microsomes results in a type II binding spectrum but it is not metabolized. However, binding does necessitate some sort of interaction: octylamine is an inhibitor of cytochrome P-450.

Mechanism of Action

Cytochrome P-450 dependent oxidative reactions show typical mixed-function oxidase stiochiometry:

$$RH + NADPH + H^{+} + O_2 \longrightarrow ROH + H_2O + NADP^{+}$$

The cytochrome P-450 catalytic cycle are thought to involve the following steps (figure I):

1) The binding of the substrate to the ferric form of the enzyme. This step is usually rapid and stoichiometric (44). The binding of the substrate can change the spin state of the iron (see above) and can also affect the oxidation-reduction potential (45).

2) The substrate-bound ferric enzyme is reduced by the reduced form of NADPH-cytochrome P-450 reductase.

3) The addition of molecular oxygen to the substrate-bound ferrous enzyme to form an oxygenated complex.

4) The oxygenated complex can accept a second electron form NADPH-cytochrome P-450 reductase, or possibly cytochrome b_5 (46). Alternatively, without the addition of another electron, the complex can decay to release superoxide anion and the substrate bound ferric enzyme (47).

5) The release of H_2O to form the postulated substrate bound iron-oxene complex (48).

6) The addition of oxygen to the substrate. The oxygenated product dissociates and the original oxidized cytochrome P-450 is regenerated.

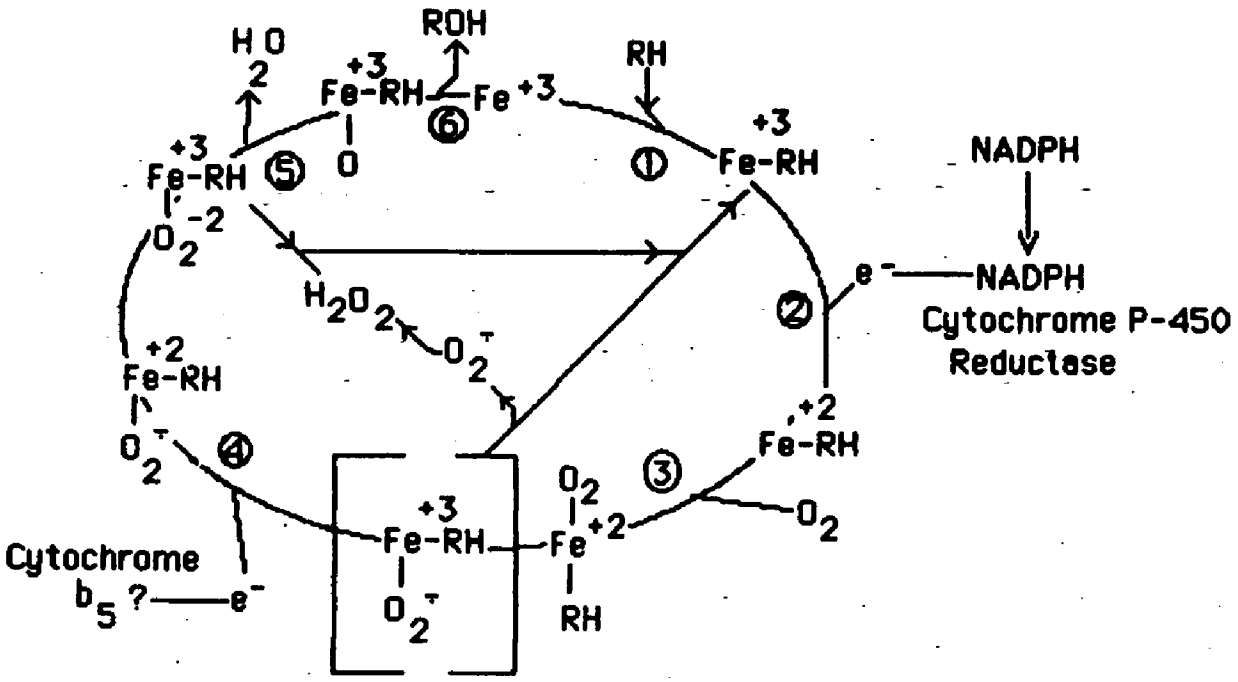

H2O2
ROH
RH
+3 Fe-RH
Fe+3
+3 Fe-RH
NADPH
+3 Fe-RH
O2-2
H2O2
O2-
e-
NADPH
Cytochrome P-450
Reductase
+2 Fe-RH
O2-
+2 Fe-RH
O2
+3 Fe-RH
O2-
Fe+2
O2
O2
RH
Cytochrome
b5 ?
e-

FIGURE 1

NADPH Cytochrome P-450 Reductase

In contrast to cytochrome P-450, which was studied and isolated by following its native ability to hydroxylate or demethylate various substrates, NADPH cytochrome P-450 reductase was first studied and isolated in a form which was incapable of catalyzing its native function. In fact, it became known by its ability to reduce cytochrome c and was named NADPH-cytochrome c reductase (49,50).

NADPH cytochrome P-450 reductase is a membrane bound flavoprotein that contains one molecule of FAD and one molecule of FMN (51,52). The preliminary evidence that the reductase functioned in the microsomal mixed-function oxidase system came from two areas of investigation. One area showed that NADPH cytochrome c reductase activity rose and fell in response to phenobarbital treatment in a manner that was similar to the drug hydroxylation activity of rat hepatic microsomes (53,54). The second area of investigation which supported the role for the reductase in drug metabolism was through the use of antibodies developed against purified NADPH cytochrome c reductase. These antibodies, which strongly inhibited the reductase activity in hepatic microsomes, also inhibited the microsomal drug hydroxylation activity (55,56). Finally, the reductase was purified from microsomes in its native form after solubilizing the enzyme with detergents

(52,57,58). This native form functions as a catalyst for the reduction of cytochrome P-450.

The Microsomal Alcohol Oxidizing System

Ethanol is metabolized predominantly by the liver, as extrahepatic oxidation of ethanol does not exceed 15% (59,60). The major pathway of ethanol metabolism is via alcohol dehydrogenase, a cytosolic nicotinamide adenine dinucleotide (NAD^+) dependent enzyme (eq.1).

$$CH_3-CH_2-OH + NAD^+ \longrightarrow CH_3-CHO + NADH \quad (1)$$

Two minor pathways of ethanol metabolism are (1) the H_2O_2 dependent peroxidatic activity of catalase (61,62, eq.2),

$$CH_3-CH_2-OH + H_2O_2 \longrightarrow CH_3-CHO + H_2O \quad (2)$$

and (2) the nicotinamide adenine dinucleotide phosphate (NADPH) dependent microsomal alcohol oxidizing system (eq.3).

$$CH_3-CH_2-OH + NADPH \longrightarrow CH_3-CHO + NADP^+ \quad (3)$$

How isolated rat liver microsomes oxidized ethanol had been a subject of much controversy (63). Microsomal oxidation of ethanol and methanol was first described by Orme-Johnson and Ziegler in 1965 (64). Subsequently, more detailed characterizations of the microsomal ethanol oxidizing system (MEOS) were worked out by Lieber and DeCarli (65-67). Many ascribed MEOS to be due to the peroxidatic activity of contaminating catalase (68,69), while others

ascribed it to contaminating "microsomal" alcohol dehydrogenase (70,71). MEOS was differentiated from alcohol dehydrogenase on the basis of cofactor requirements, pH optimum, Km and the effects of pyrazole, a potent inhibitor of alcohol dehydrogenase (66,67). However, differentiation of MEOS from the peroxidatic activity of catalase remained a subject of controversy. NADPH dependent microsomal electron transfer is known to generate hydrogen peroxide (72). Therefore, the oxidation of ethanol, a peroxidatic substrate for catalase (eq.2), can be attributed to contaminating catalase. MEOS has been differentiated from catalase by 1) physically separating MEOS from catalase (as well as alcohol dehydrogenase) via column chromatography (25,65,73,74), 2) the use of acatalasemic mice (75), 3) the use of azide, a potent inhibitor of catalase and 4) the use of alcohols that do not serve as substrates for the peroxidatic activity of catalase e.g. 1-butanol (6,76,77).

The dependence of MEOS on NADPH and oxygen and its partial inhibition by carbon monoxide resembles the mixed function oxidase system, which is responsible for the metabolism of many drugs. In fact, these requirements for ethanol oxidation, along with ethanol's ability to competitively inhibit the metabolism of drugs known to be metabolized by the mixed function oxidase system, led to the hypothesis that MEOS involved components of the mixed

function oxidase system (65). Furthermore, chronic treatment with ethanol induces components of the mixed function oxidase system (78,79). This induction was found to result in the induction of a specific cytochrome P-450 hemoprotein that was distinct from those induced by either phenobarbital or 3-methylcholanthrene (6,65,79,80).

Finally, ethanol has been shown to be metabolized by the mixed function oxidase system in studies with reconstituted systems containing purified cytochrome P-450, NADPH, cytochrome P-450 reductase and phospholipids (6,73,81). Recently, a unique ethanol induced cytochrome P-450 has been purified and characterized from ethanol treated rabbits (7,82).

In addition to catalyzing the oxidation of drugs, alcohols and other xenobiotics, isolated rat liver microsomes catalyze the oxidation of a variety of hydroxyl radical scavenging agents. These oxidations are dependent on NADPH and are sensitive to inhibition by competitive hydroxyl radical scavengers (83-85). The addition of desferrioxamine, an iron chelating agent known to block the generation of hydroxyl radicals generated by various systems, was found to inhibit the latter oxidations (86,87). Experiments utilizing NADPH-cytochrome P-450 reductase and cytochrome P-450 purified from phenobarbital-treated rats showed that, in the presence of NADPH, the reductase itself is capable of oxidizing hydroxyl radical

scavenging agents (81,88). These oxidations appear to be dependent upon the amount of reductase in the system and, with the exception of ethanol as a hydroxyl radical scavenger, show little dependence upon cytochrome P-450 being present in the reaction mixture (81,88). The addition of iron-EDTA resulted in a stimulation of the reductase-dependent oxidation oxidation of radical scavenging agents (86,87,89). Analogously, the addition of iron-EDTA to the reductase was shown to enhance the ESR signal characteristic of the DMPO-hydroxyl radical adduct that is produced during electron transfer (90,91).

The primary event in the sequence of reactions leading to the reductase-dependent oxidation of hydroxyl radical scavenging agents is believed to be due, at least in part, to the superoxide production via the autoxidation of the reductase (89,92) according to:

$$2O_2 + NADPH \longrightarrow NADP^+ + H^+ + 2O_2^- \quad (4)$$

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

$$H_2O_2 + O_2^- \longrightarrow O_2 + OH^- + OH \quad (6)$$

Reaction (6) above represents the classical Haber-Weiss reaction which, although thermodynamically favorable, is kinetically quite slow. However, the reaction can be catalyzed by transition metal cations such as iron and copper (93,94).

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2 \quad (7)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} OH^- + OH \quad (8)$$

Reaction (7) and (8) represent the iron-catalyzed Haber-Weiss reaction, in which ferric is reduced by superoxide radical to the ferrous state (reaction 4), which then reacts with H_2O_2 to produce hydroxyl radical (reaction 5, the Fenton reaction).

Recent results from our laboratory have shown that iron appears to play an important role in the reductase (microsomal)-dependent generation of hydroxyl radicals, however, the effect of iron appears to be dependent on the nature of the iron chelate e.g. unchelated iron or iron-nucleotides are poorly effective in promoting the generation of hydroxyl radicals (89,92,95).

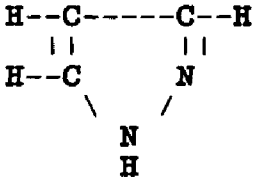
Ethanol oxidation by hydroxyl radicals produces acetaldehyde. Studies from our laboratory have indicated that there are two pathways responsible for oxidation of alcohol via MEOS (23,81,88): (1) a cytochrome P-450 dependent in which there is no role for the hydroxyl radical and (2) a hydroxyl radical dependent (cytochrome P-450 independent), that is dependent on reductase and properly chelated iron. Therefore, in order to evaluate the cytochrome P-450 dependent (hydroxyl radical independent) rate of alcohol oxidation, chelates such as EDTA were omitted from the reaction mixtures and efforts were made to scrupulously remove contaminating iron from the water and buffers.

The contribution of MEOS to the overall oxidation of ethanol can range from 10-50% depending on the concentration of ethanol (96). Although MEOS usually constitutes a minor pathway of ethanol metabolism, chronic ethanol consumption results in the induction of the mixed function oxidase system (77,78) and concomitantly, ethanol oxidation. Rats regularly fed ethanol for six weeks show significantly higher rates of microsomal ethanol oxidation compared to pair-fed controls (65). Furthermore, Petersen et al. (97) reported that an acute dose of ethanol stimulates MEOS in mice. These elevations in MEOS activity are responsible, in part, for the interactions of alcohol and drugs. It has been pointed out that ethanol-drug interactions are quite complex (98,99). Ethanol administration can have paradoxical effects with respect to metabolism, toxicity or teratogenicity of drugs and/or other foreign substances (100). In the presence of alcohol, drug metabolism is depressed because alcohol competes with drugs at the cytochrome P-450 level (101-103). However, in the absence of alcohol but after chronic ethanol intake, drug metabolism is increased because of the induction of total cytochrome P-450 by alcohol. Consequently, drug therapy for the alcoholic or alcohol-using patients must be carefully evaluated.

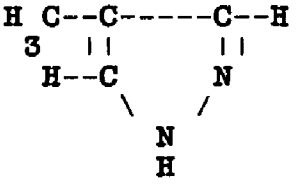
Human studies are, at best, difficult to conduct; therefore, much of the data involving MEOS and alcohol-drug interaction has been obtained from animal models. However, the cost of alcohol induced animals are high and the process is time consuming as well. Rabbits must be maintained for fourteen or more days (7). Rats must be maintained on a liquid diet for thirty days or more along with their pair fed controls (104). Primate models can even be more costly and time consuming. Moreover, the alcohol diet causes other metabolic derangements, especially nutrient variation, e.g. alcohol isocalorically replaces 36% of the carbohydrate calories and carbohydrate content is known to affect the cytochrome P-450 isozyme population. Recent reports indicate that when rats are fed ad libitum the alcohol liquid diet, they consumed 50% less than rats fed the control diet in which alcohol is replaced isocalorically with dextrins. Under these condition these liquid diets are nutritionally inadequate for growing animals (105). Hence, there is a need for other models. One goal of this thesis is to demonstrate that pyrazole and 4-methylpyrazole interact with and induce an alcohol preferring cytochrome P-450 that has the same or identical properties of the isozyme induced by alcohol. These results suggest that treatment of rats with either pyrazole or 4-methylpyrazole may serve as good animal models for the induction of the alcohol induced cytochrome P-450.

Pyrazole and 4-Methylpyrazole

In 1963, Theorell and Yonetani reported that pyrazole inhibited alcohol dehydrogenase isolated from horses (106). Subsequently, the kinetics of pyrazole inhibition was characterized by Li and Theorell using alcohol dehydrogenase isolated from humans (107). These inhibitors form slowly dissociating ternary complex with alcohol



PYRAZOLE



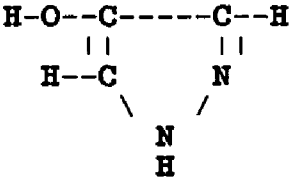
4-METHYLPYRAZOLE

dehydrogenase and nicotinamide adenine dinucleotide (oxidized form) and act as competitive inhibitors with respect to ethanol (107). In vivo, pyrazole has been shown to inhibit ethanol and methanol metabolism in the rat (108-110). Since pyrazole and particularly pyrazole derivatives with substitution on the 4-position, e.g. 4-methylpyrazole, are potent inhibitors of alcohol dehydrogenase, they are frequently utilized to block the metabolic consequences associated with the oxidation of ethanol (107, 109 and 111) and other alcohols

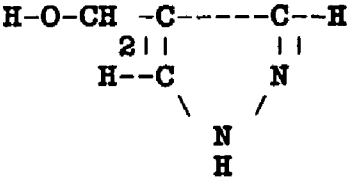
such as methanol (112) or ethylene glycol (113,114). Pyrazole can also be used to increase the half life of alcohol in experimental animals as well as to separate the direct effects of ethanol or other alcohols from those due to their metabolism. The low Ki values of pyrazole, and the even lower values of the 4-substituted pyrazole for alcohol dehydrogenase, together with their ability to effectively inhibit alcohol metabolism *in vivo*, suggest the possibility of their therapeutic use after intake of methanol or ethylene glycol to prevent the formation of toxic products. However, studies in animals have shown that the dosage required for effective inhibition of alcohol metabolism, is itself toxic and produces undesirable effects (115). Oral administration of pyrazole was found to cause diffuse hepatotoxicity (116) and thyroid necrosis (117). The administration of pyrazole, especially in combination with ethanol, was found to be hepatotoxic, whereas 4-methylpyrazole was not toxic (118-122). The reasons why pyrazole is toxic, relative to 4-methyl-pyrazole are not known. 4-Methylpyrazole has been used in studies on ethanol metabolism in human beings (123). In view of its greater effectiveness and lower toxicity, it has been suggested that 4-methylpyrazole may be useful in treatment of ethanol toxicity, as well as the toxicity associated with alcohols which need to be metabolized by alcohol dehydrogenase to toxic metabolites, e.g. methanol, ethylene glycol (112-114, 124,125).

In vivo administration of pyrazole was shown to increase the activities of aniline hydroxylase (126), alcohol oxidation by microsomes (127) and dimethylnitrosamine N-demethylase (128,129) and to induce a cytochrome P-450 isozyme with a molecular weight of 51-52,000 daltons (128,129). Pyrazole, in vitro, was shown to affect the metabolism of several drugs by the microsomal mixed-function oxidase system, including ethanol (116,130), and to bind to cytochrome P-450 to produce a type II spectral change (131). 4-Methylpyrazole, in vitro, inhibited the cytochrome P-450-dependent oxidations of drugs such as aminopyrine and ethanol (130), although the mechanism for this is not known.

The metabolic pathways for the metabolism of pyrazole and 4-methylpyrazole have not been delineated. In vivo experiments have shown that 4-hydroxypyrazole and 4-hydroxymethylpyrazole could be found in the urine of rodents given pyrazole and 4-methylpyrazole, respectively; however, the enzymes responsible for their hydroxylation



4-HYDROXYPYRAZOLE



4-HYDROXYMETHYLPYRAZOLE

have not been elucidated. When radioactive ^{14}C-4-methylpyrazole was administered to mice, 84% of the radioactivity was recovered in the urine 24 hours after treatment (132). Urine collected between 5 and 9 hours after administration of the radioactive 4-methylpyrazole contained 25% of the total administered radioactivity. The urine was then extracted into three fraction: (1) acidic or neutral metabolites, (2) basic metabolites and (3) amphoteric metabolites. Analysis of the urine revealed the presence of several metabolites including 4-hydroxymethyl pyrazole. Other secondary products, e.g. the aldehyde and the ketone of the latter metabolite, as well as N-glucuronic conjugated products, have been identified (132-134).

When radioactive pyrazole (3,4-^{14}C) was injected into rats, it was shown that its elimination from the blood was accelerated at higher doses of pyrazole or by pretreatment with pyrazole. Furthermore, pyrazole disappearance was slowed in the presence of ethanol (135). 100% of the administered radioactivity was recovered in the urine. Over 50% of the urinary radioactivity appeared to be conjugated 4-hydroxypyrazole. After acid hydrolysis, this product was purified and was shown to be 4-hydroxy-pyrazole. Only small amounts of unchanged pyrazole was found in the urine; however, if ethanol was administered simultaneously with pyrazole, 75% of the administered

pyrazole was found unchanged in the urine (135). Hence, ethanol inhibited pyrazole hydoxylation. The results to be presented in this thesis show that both pyrazole and 4-methylpyrazole interact and induce specific isozymes of cytochrome P-450. Furthermore, pyrazole is metabolized by cytochrome P-450 and that treatment with either pyrazole or 4-methylpyrazole induces this metabolism.

CHAPTER II MATERIALS AND METHODS

Microsomal Preparations

Hepatic microsomes were prepared from male Sprague-Dawley rats by differential centrifugation. The animals were sacrificed via decapitation and the livers were immediately excised and placed into ice-cold buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA (STE). The livers were trimmed of any extra-hepatic tissue, weighed, and placed into fresh STE. The tissue was then minced with stainless steel scissors and then rinsed with STE to remove residual blood. The tissue was homogenized in STE (1:5; gm liver wet weight:ml STE) with three passes at 800 rpm in a Potter-Elvehjem tissus grinder with a teflon pestle. The homogenates were centrifuged at 577 x g (2,500 rpm in a SS-34 rotor) for 10 minutes to remove unbroken cells and large subcellular particles. The supernatant was decanted and centrifuged at 13,200 x g (12,000 rpm) for an additional 10 minutes to remove mitochondria and lysosomes. The supernatant was decanted and centrifuged at 100,000 x g (37,500 rpm in a Ti-60 rotor) for 60 minutes to bring down the microsomal fraction. The microsomal pellet was rinsed and resuspended in 125 mM KCl with a Dounce homogenizer. The pellet was centrifuged again at 100,000 x g (to wash out the residual

STE), rinsed and resuspended in 125 mM KCl (1:1; gm liver wet weight:ml 125 mM KCL). Both freshly prepared microsomes and microsomes stored at -70°C were used in all experiments. There were no differences found between the fresh and frozen preparations. For the SDS-gel electrophoresis experiments, the microsomes were resuspended in 0.25 M sucrose rather than KCl. Protein was determined by the method of Lowry et al. (136).

Pretreatment of Rats

1) Pyrazole Treatment Male, Sprague-Dawley rats weighing approximately 135-150 g were injected intraperitoneally with either saline, pyrazole or 4-methylpyrazole once a day for the number of days indicated in the individual experiments. Unless otherwise specified, the usual dose of 4-methylpyrazole or pyrazole was 200 mg/kg body weight/ day. Stock solutions of 50 mg of either 4-methylpyrazole or pyrazole per ml of saline were used except in the dose response experiments where the solutions were made such that all animals (including controls) received equivalent volumes. The animals were starved overnight and sacrificed 24 hours after the last injection.

2) Ethanol Treatment Male, Sprague-Dawley rats weighing approximately 135 g were fed the Lieber-DeCarli liquid diet for 3-4 weeks (137). The ethanol diet consisted of 36% of

the calories as ethanol, 11% as carbohydrate, 18% as protein and 35% as fat. The pair-fed controls consumed the same diet except that carbohydrate isocalorically replaced ethanol. Prior to the day of sacrifice, the rats received their respective diets ad-libitum.

3) Phenobarbital Treatment Male, Sprague-Dawley rats weighing approximately 200-250 g were fed sodium phenobarbital in their drinking water (1% solution) for 10 days. The animals were starved over night before sacrifice.

4) 3-Methylcholanthrene Treatment Male, Sprague-Dawley rats weighing approximately 135-150 g were injected intraperitoneally (once a day) with a suspension of 3-methylcholanthrene in corn oil (20 mg/ml corn oil) for 3 days at a dosage of 25 mg/kg body weight/day. The animals were starved overnight and sacrificed 24 hours after the last injection. Control animals received an equivalent volume of corn oil.

Assay Conditions and Product Measurements

1) Alcohol Oxidation The oxidation of ethanol, racemic (±)-, (+)- or (-)-2-butanol was assayed in 25 ml Erlenmeyer flasks at 37 °C in a Dubnoff shaking water bath (80 rpm). The basic reaction mixture contained 100 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 1mM azide

(a potent inhibitor of catalase), varying concentrations of the alcohol substrate and about 2 mg of microsomal protein in a final volume of 1 ml. Reactions were initiated by addition of a mixture of glucose-6-phosphate (final reaction concentration was 10 mM) and 2.3 units of glucose-6-phosphate dehydrogenase (an NADPH generating system) and were terminated after 5 minutes by the addition of 0.3 ml of 1 N HCl. The production of acetaldehyde from ethanol or 2-butanone from 2-butanol was determined by a head space, gas chromatography flame ionization detection procedure. After termination of the reaction, the flasks were sealed with air tight rubber stoppers and incubated at 60 ^{o}C for 20 minutes. A 1.0 ml aliquot of head space was directly injected into a Hewlett-Packard Model 5700 gas chromatograph equipped with a 6 ft long 5% carbowax 20 M Haloport F 30-60 mesh column. The injection temperature, oven temperature and flame ionization detector temperature were 100 ^{o}C, 50 ^{o}C and 150 ^{o}C, respectively. With the carrier gas (nitrogen) set at a flow rate of 35 ml/min, acetaldehyde and and 2-butanone had retention times of 0.4 minutes and 0.95 minutes respectively. All values were corrected for with zero time controls which contained HCl added before the NADPH generating system. Since alcohols can be oxidized by hydroxyl radicals, the possible involvement of oxy-radical species was minimized by avoiding the use of EDTA and by passing all buffers (except

$MgCl_2$) and water used to prepare solutions through columns of Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA) to remove metals such as iron (95). Standard curves were prepared by adding known amounts of either acetaldehyde or 2-butanone to zero time controls.

2) Drug Oxidations The basic reaction system utilized to assay for the oxidation of various drug substrates (except pyrazole) was similar to that described above, except for the further addition of 10 mM sodium pyrophosphate and 0.1 mM EDTA. Azide was omitted from the reaction system, and the microsomal protein content was about 1 mg. Reactions were initiated by the addition of the NADPH generating system and terminated by the addition of 0.3 ml of 20% TCA.

Peak areas and absorbances were quantitated by comparison to the appropriate standard curves. All values were corrected for with "zero-time" controls in which either TCA or PCA was added to the flasks before the microsomes or where microsomes or substrates (pyrazole experiments) were omitted from the flasks.

2.1) Aminopyrine and N,N-Dimethylnitrosamine The production of formaldehyde from the N-demethylation of aminopyrine or N,N-dimethylnitrosamine was assayed by the Nash reaction (138). Aminopyrine was present at a final concentration of 10 mM, and the reactions were terminated after 10 minutes. N,N-Dimethylnitrosamine was present at final concentrations of either 0.5, 5 or 100 mM, and reactions

were terminated after 10 minutes. 1 ml of the clear TCA-supernatant fraction was mixed with 1 ml of the Nash reagent and incubated at 60 ^{o}C for 10 minutes. Nash reagent is an aqueous solution that contains 15% ammonium acetate (w:v), 0.2% acetylacetone (v:v) and 0.3% glacial acetic acid (v;v). The absorbance at 415 nm was determined immediately after the 60 ^{o}C incubation.

2.2) Aniline The production of para-aminophenol from the para-hydroxylation of aniline (1 mM final concentration except in kinetic experiments) was determined after a 10 minute reaction period by first mixing 1 ml of the clear TCA-supernatant fraction with 1 ml of 10% Na_2CO_3, followed by the addition of 1 ml of a mixture containing 2% phenol in 0.5N NaOH (prepared fresh). The absorbance at 640 nm was determined after a 30 minute incubation period.

2.3) Para-nitroanisole The production of para-nitrophenol from the O-demethylation of para-nitroanisole (1 mM final concentration) was determined after a 10 minute reaction period by mixing 1 ml of the clear TCA-supernatant fraction with 0.5 ml of 10N NaOH and measuring the absorbance at 400. The production of 4-nitrocatechol from the hydroxylation of paranitrophenol was determined in the same cuvette by measuring the absorbance at 480 nm, and using a mM extinction coefficient of 8.61 (139).

2.4) 7-Ethoxycoumarin The production of acetaldehyde and 7-hydroxycoumarin from the O-dealklation of 7-ethoxy-coumarin (0.5 mM final concentration) were determined by gas chromatography and fluorimetry, respectively. Reactions were terminated after 10 minutes with TCA. Acetaldehyde was assayed in the sealed flasks as described above. The flasks were then opened, centrifuged and the 7-hydroxycoumarin content in the supernatant was determined fluorimetrically using a Perkin-Elmer 650-10S fluorescence spectrophotometer. Excitation and emission wavelengths were set at 368 and 456 nm, respectively.

2.5) Pyrazole The basic reaction system utilized to assay for the oxidation of pyrazole was similar to that described for alcohols, except for the omission of azide, the microsomal protein content was about 4 mg and the final reaction volume was 2 ml. Reactions were initiated by the addition of the NADPH generating system and terminated by the addition of 0.1 ml of concentrated PCA. The contents were then centrifuged and the supernatant was decanted and 0.112 ml of 11.1N KOH was added to precipitate out the PCA as potassium perchlorate. The contents were centrifuged again and 1 ml of the clear supernatant was loaded onto a pre-activated (5 ml of methanol followed by 5 ml of water) SEP-PAK C_{18} cartridge (Waters Associates, Milford, MA). The metabolite, 4-hydroxypyrazole was eluted off the SEP-PAK with 1 ml of water into two-0.5 ml fractions. The

latter fraction was used for the HPLC analyses of 4-hydroxypyrazole. A 10 ul sample was applied to a 4.6-mm X 25-cm Microsorb C_{18} column (Rainin Instruments, Woburn, MA) using a Waters Universal injector (Waters Associates, Milford, MA). 4-hydroxypyrazole was separated from the remaining reaction components isocratically using a mobile phase of acetonitrile and water (1:10) containing 5 mM octanesulfonic acid and 1% glacial acetic acid (v:v). Under these conditions 4-hydroxypyrazole could be detected at 254 nm using a Waters model 400 detector or a BAS electrochemical detector with a retention time of about 6.8 minutes.

Carbon Monoxide Inhibition

In experiments that involved inhibition by carbon monoxide the following procedure was used. The flasks were sealed with serum stoppers prior to the addition of the NADPH generating system. 10 cc of air was removed with a syringe and either 10 cc of carbon monoxide or 10 cc of nitrogen (the control) was added with another syringe immediately (30% CO or added N_2 final concentration).

Measurement of Cytochrome P-450, NADPH Cytochrome c Reductase and Substrate Binding Spectra

The measurement of cytochrome P-450, NADPH cytochrome c

reductase and substrate binding spectra were determined with a Perkin-Elmer 554 dual beam spectrophotometer.

1) Cytochrome P-450 The content of cytochrome P-450 was determined by the method of Omura and Sato (25) using an extinction coefficient of 91 mM^{-1} cm^{-1}. Approximately 1.0 mg of microsomal protein was suspended in 6 ml of 100 mM potassium phosphate buffer, pH 7.4. The cytochrome P-450 was reduced with sodium dithionite and the sample was divided in half and placed into a sample and reference cuvette. A base line correction was performed to compensate for any differences between the two cuvettes before gently bubbling pure carbon monoxide (for 30 seconds) through the sample cuvette. The spectra were scanned from 500 to 400 nm and the difference in the O.D. from 490 to 450 was used to calculate the concentration of cytochrome P-450.

2) Substrate Binding Spectra Substrate binding spectra were obtained by the method of Peterson et al (140). Approximately 2.0 mg of microsomal protein was suspended in 6 ml of 100 mM potassium phosphate buffer, pH 7.4. The sample was divided in half and placed into a sample and reference cuvette. A base line correction was performed to compensate for any differences between the two cuvettes before the addition of substrate to the sample cuvette. The spectra were scanned from 450 to 350 nm.

3) NADPH Cytochrome c Reductase The activity of NADPH cytochrome c reductase was determined by the method of Strobel and Dignam (141). Approximately 0.1 mg of microsomal protein was placed in a 1 ml cuvette containing 0.98 ml of 5 x 10^{-5} M cytochrome c in 0.3 M potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of 0.01 ml of 10 mM NADPH. The initial linear velocities (change in absorbance at 550 nm, at room temperature) were used to calculate the activity of the NADPH cytochrome c reductase using an extinction coefficient of 21 mM^{-1} cm^{-1}.

SDS-Gel Electrophoresis

SDS-gel electrophoresis was performed as described by Laemmli (142) using a 7.5% acrylamide gel, 0.75 mm thick with a 4% stacking gel. Protein bands were visualized by Coomasie blue staining.

All chemicals were of the highest grade available. Pyrazole, and (+)- and (-)-2-butanol were obtained from Pfaltz and Bauer (Stamford, CT); 4-methylpyrazole was from Aldrich Chemical Co. (Milwaukee, WI) and 4-hydroxypyrazole was a gift from Lilly Pharmaceuticals (Indianapolis, IN).

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

CHAPTER III RESULTS

A. Characterization of the Effects of In Vivo Administration of Pyrazole and 4-Methylpyrazole

Pyrazole and 4-methylpyrazole, potent inhibitors of alcohol dehydrogenase, are widely used in alcohol research. These classical inhibitors were thought to be specific for alcohol dehydrogenase and were used to separate the direct effects of alcohol consumption from those due to its metabolism (see Introduction).

Previous results have suggested that there are several interactions of pyrazole and 4-methylpyrazole with hepatic microsomes (116,128-130) which indicate that these agents do not react specifically with alcohol dehydrogenase. Therefore, initial studies were carried out to characterize the time course and dose response of the interactions of pyrazole with hepatic microsomes, and to compare these results to microsomes isolated from rats treated with 4-methylpyrazole, since the latter has replaced pyrazole in many studies in view of its lower toxicity and greater inhibitory effectiveness against alcohol dehydrogenase.

A-1 The Effect of Pyrazole and 4-Methylpyrazole on Body and Liver Weight and Liver Protein

For all experiments reported in this thesis, with the exception of experiments concerning time courses and dose

responses (see below), rats were routinely treated with either 200 mg of pyrazole or 4-methylpyrazole per kg body weight per day for 2 or 3 days, respectively, and then fasted overnight. The saline controls gained weight (about 5g/day), whereas, the pyrazole and 4-methylpyrazole treated rats did not, and remained essentially at their initial starting weights (Table 1). Total liver weights were the same for the three groups, while the liver/body weight ratio was slightly increased in the pyrazole and 4-methylpyrazole treated rats, 19% and 15%, respectively (Table 1). This small increase in the liver/body weight ratio was probably due to the lack of weight gain by the treated animals. The total liver protein (mg protein/g liver wet weight) was the same for the three groups (Table 1). The content of microsomal protein was calculated by determining the ratio of the specific content of cytochrome P-450 in the homogenates and in the isolated microsomes and multiplying by the total liver protein content. Table 1 shows that neither pyrazole nor 4-methylpyrazole significantly increased the content of microsomal protein over the saline controls.

A-2 Time Course of Induction

Rats were treated with either saline, pyrazole or 4-methylpyrazole for either 1, 2 or 3 days in order to ascertain the effects that these compounds have on

Table I

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Body and Liver Weight and Liver Protein

Parameter	Treatment		
	Saline	Pyrazole	4-Methylpyrazole
Body Weight (grams)	133 ± 5.1	116 ± 2.2	121 ± 2.5
Liver Wet Weight (grams)	4.83 ± 0.10	5.01 ± 0.15	5.06 ± 0.13
Total Liver Protein (mg/g liver wet weight)	218 ± 3.7	201 ± 2.7	210 ± 10.8
Microsomal Protein (mg/g liver wet weight)	22.3 ± 2.1	23.9 ± 1.4	25.0 ± 2.7

Rats were treated with either saline or with 200 mg/kg body weight/day of either pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. Rats were fasted overnight and weighed prior to sacrificing. Protein was measured by the method of Lowry et al (136).

microsomal components and on the oxidation of ethanol, 2-butanol and aminopyrine. Because there is an increase in the total content of cytochrome P-450 when rats are treated with 4-methylpyrazole (see below), metabolic activities described below are expressed as "per mg microsomal protein" and "per nmol cytochrome P-450.

A-2.1 Content of Cytochrome P-450 and Activity of NADPH-Cytochrome P-450 Reductase

The content of cytochrome P-450 did not change significantly in the saline controls after 1, 2, or 3 days of saline treatment (Table II). Pyrazole, administered intraperitoneally at a dose of 200 mg/kg body weight/day, did not change the content of cytochrome P-450 over the saline controls, even after 3 days of treatment. By contrast, the same dose of 4-methylpyrazole resulted in an increase in the content of liver microsomal cytochrome P-450 (Table II). A 37% increase was found after a single treatment with 4-methylpyrazole. Two-day treatment was as effective as 3-day in nearly doubling the content of cytochrome P-450.

The activity of NADPH-cytochrome P-450 reductase, as measured by cytochrome c reduction, did not change after 1, 2, or 3 days of treatment with saline (Table II). Neither pyrazole nor 4-methylpyrazole treatment had any effect on the activity of the reductase (Table II).

Table II

The Effect of Pyrazole and 4-Methylpyrazole Treatment on The Activity of NADPH-Cytochrome P-450 Reductase and the Content of Cytochrome P-450

Reaction	Days of Treatment	Treatment		
		Saline	Pyrazole	4-Methylpyrazole
Cytochrome P-450 (nmol/mg microsomal protein)	1	0.73 ± 0.14	0.81 ± 0.07	1.00 ± 0.22
	2	0.86 ± 0.03	0.90 ± 0.05	1.51 ± 0.06(a)
	3	0.78 ± 0.07	0.90 ± 0.09	1.59 ± 0.10(a)
NADPH-Cytochrome P-450 Reductase (units/mg microsomal protein)	1	146 ± 13	152 ± 13	136 ± 9
	2	147 ± 14	132 ± 12	134 ± 19
	3	151 ± 8	138 ± 6	172 ± 12

The content of cytochrome P-450 and the activity of NADPH- cytochrome P-450 reductase were determined as described in Material and Methods after 1, 2, or 3 days of treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole. Results are from 4 to 6 experiments. A unit of activity for the reductase refers to 1 nmol of cytochrome c reduced/min at room temperature.

(a)-$p < 0.002$.

A-2.2 Oxidation of Ethanol, 2-butanol and Aminopyrine.

The effect of pyrazole or 4-methylpyrazole treatment on the microsomal oxidation of alcohols was compared to the effect on aminopyrine oxidation. The latter is a good substrate for the phenobarbital-, but not the alcohol-inducible isozyme of cytochrome P-450. In the saline controls, the oxidation of ethanol and 2-butanol by microsomes was constant over the 3 days of saline treatment (Fig. 2 and 3, respectively). Treatment with either pyrazole or 4-methylpyrazole resulted in a 2-3-fold increase in the microsomal oxidation of ethanol and 2-butanol when results are expressed on a "per mg of microsomal protein" basis (Fig. 2A and 3A, respectively). A significant increase in microsomal oxidation of alcohols was noted after a single treatment with pyrazole or 4-methylpyrazole (Fig. 2A and 3A). Two days of treatment with pyrazole appeared to be most effective in increasing the oxidation of alcohols, whereas with 4-methylpyrazole, the oxidation of alcohols continued to increase with successive days of treatment (Fig. 2A and 3A).

In saline controls, the oxidation of aminopyrine by microsomes did not significantly change over the 3 days of treatment (Fig. 4A). In contrast to the results with alcohols, the oxidation of aminopyrine was not increased by pyrazole treatment. The oxidation of aminopyrine was increased by 4-methylpyrazole treatment; however, the

Figure 2

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of Ethanol.

The oxidation of ethanol (final concentration, 55 mM) was determined as described in "Material and Methods" after 1, 2, or 3 days of treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole. Figure A refers to nmol of acetaldehyde/min/mg microsomal protein. Figure B refers to nmol acetaldehyde/min/nmol cytochrome P-450. Results are from 4-6 experiments.

(A)=$p<0.01$

(B)=$p<0.02$

(C)=$p<0.05$

(D)=$p<0.001$

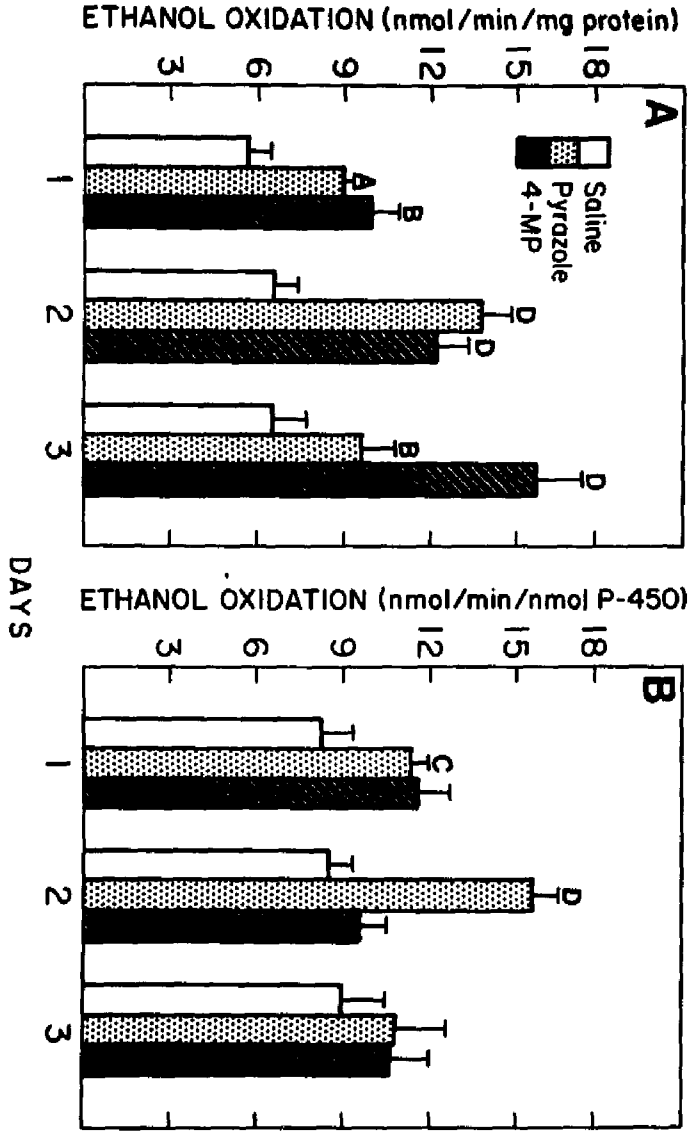

ETHANOL OXIDATION (nmol/min/mg protein)
3
6
9
12
15
18
A
Saline
Pyrazole
4-MP
1
2
3
A
B
D
D
B
D
DAYS
ETHANOL OXIDATION (nmol/min/nmol P-450)
3
6
9
12
15
18
B
1
2
3
C
D

Figure 3

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of 2-Butanol.

The oxidation of 2-butanol (final concentration, 55 mM) was determined as described in "Material and Methods" after 1, 2, or 3 days of treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole. Figure A refers to nmol of 2-butanone/min/mg microsomal protein. Figure B refers to nmol 2-butanone/min/nmol cytochrome P-450. Results are from 4-6 experiments.

(A)-$p<0.01$

(B)-$p<0.02$

(C)-$p<0.05$

(D)-$p<0.001$

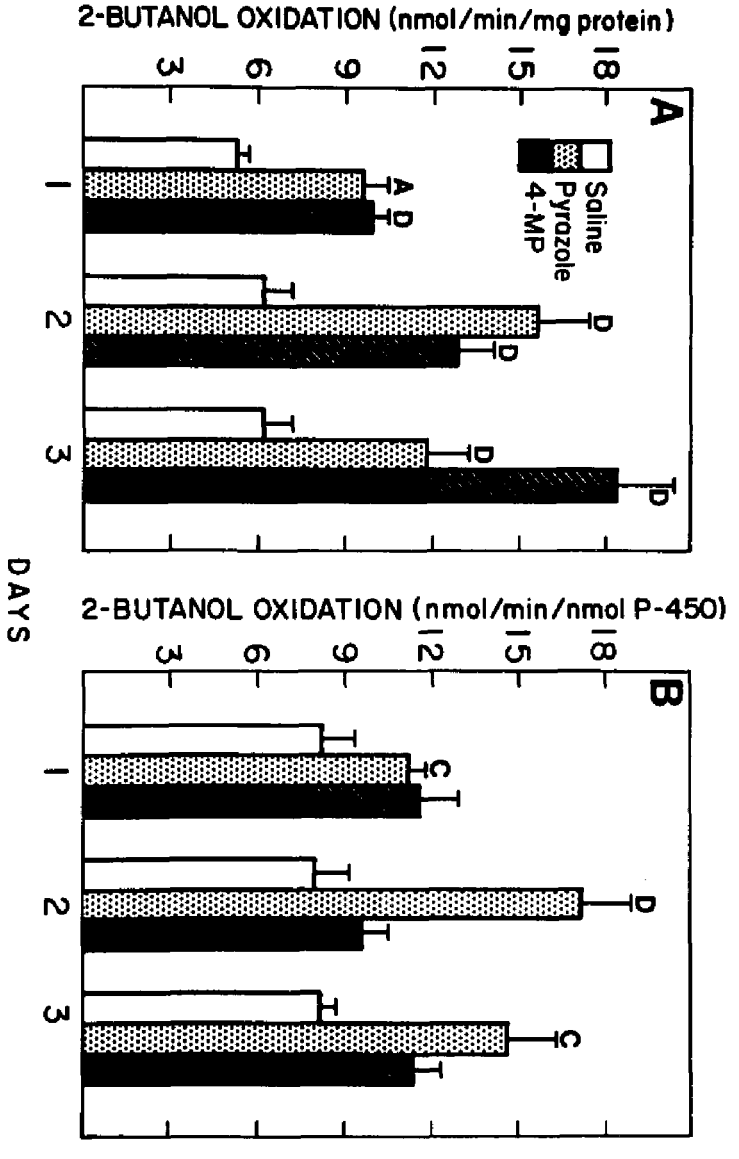

2-BUTANOL OXIDATION (nmol/min/mg protein)
3
6
9
12
15
18
A
Saline
Pyrazole
4-MP
1
2
3
DAYS
2-BUTANOL OXIDATION (nmol/min/nmol P-450)
B

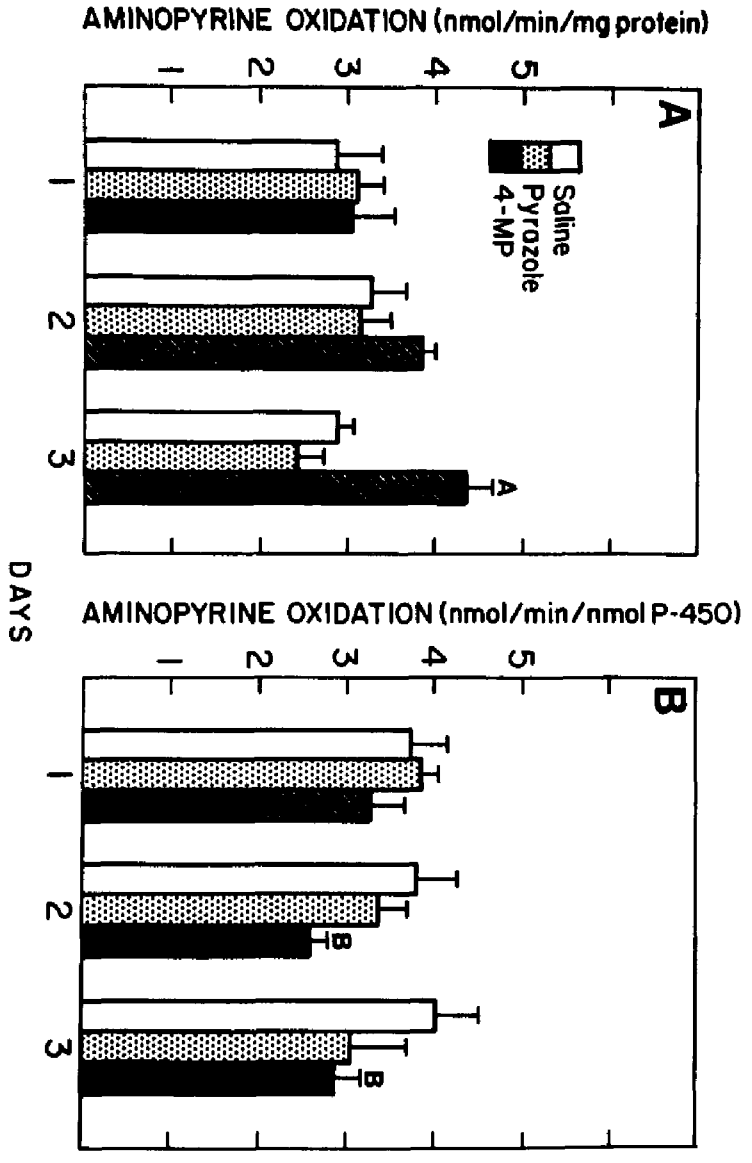
Figure 4

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of Aminopyrine.

The oxidation of aminopyrine (final concentration, 10 mM) was determined as described in "Material and Methods" after 1, 2, or 3 days of treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole. Figure A refers to nmol of formaldehyde/min/mg microsomal protein. Figure B refers to nmol formaldehyde/min/nmol cytochrome P-450. Results are from 4-6 experiments.

(A)-$p < 0.01$

(B)-$p < 0.05$


AMINOPYRINE OXIDATION (nmol/min/mg protein)
A
Saline
Pyrazole
4-MP
A
AMINOPYRINE OXIDATION (nmol/min/nmol P-450)
B
B
B
DAYS

extent of this increase (per mg microsomal protein basis) was much less than that found for the oxidation of alcohols (Fig. 4A, compare with 2A and 3A)

The results in Figures 2A, 3A, and 4A were recalculated using the levels of cytochrome P-450 determined for the various treatments (Table II) in order to express the data on a turnover basis (nmol of product/min/nmol cytochrome P-450). The turnover numbers for ethanol, 2-butanol, and aminopyrine did not change over the 3 days of saline treatment (Fig 2B, 3B and 4B, respectively). Pyrazole treatment resulted in an increased turnover number for the oxidation of ethanol and 2-butanol (Fig. 2B and 3B, respectively), whereas a slight decrease in the turnover number for the oxidation of aminopyrine was found after 2 or 3 days of treatment (Fig. 4B). A significant increase in the oxidation alcohols was found after a single treatment with pyrazole. This increase appeared to be maximal after 2 days of treatment with pyrazole (Fig 2B and 3B). The turnover number for aminopyrine oxidation was decreased by the 4-methylpyrazole treatment (Fig. 4B). A single treatment with 4-methylpyrazole produced an increase in the turnover number for the oxidation of alcohols that was similar to that found after pyrazole treatment (Fig. 2B and 3B). However, in contrast to pyrazole treatment, a second treatment with 4-methylpyrazole did not result in an enhanced turnover number for the oxidation of alcohols;

actually, some decrease in the turnover rates was found after the second day of 4-methylpyrazole treatment.

A-3 Dose Response of Induction

Two days of treatment with pyrazole appeared to be most effective in increasing the oxidation of alcohols whether the results are express "per mg microsomal protein" or "per nmol cytochrome P-450". The effects of the 4-methyl-pyrazole treatment are more complex than effects of pyrazole treatment. Three days of treatment with 4-methyl-pyrazole appeared to be most effective in increasing the oxidation of alcohols and aminopyrine when the results are expressed "per mg microsomal protein". However, when results are expressed "per nmol cytochrome P-450", 1 or 3 days of treatment with 4-methylpyrazole appeared to be equivalent in their effects (Fig. 2-4). Therefore, initial dose response curves were performed using 2 days of treatment with pyrazole and 3 days of treatment with 4-methylpyrazole.

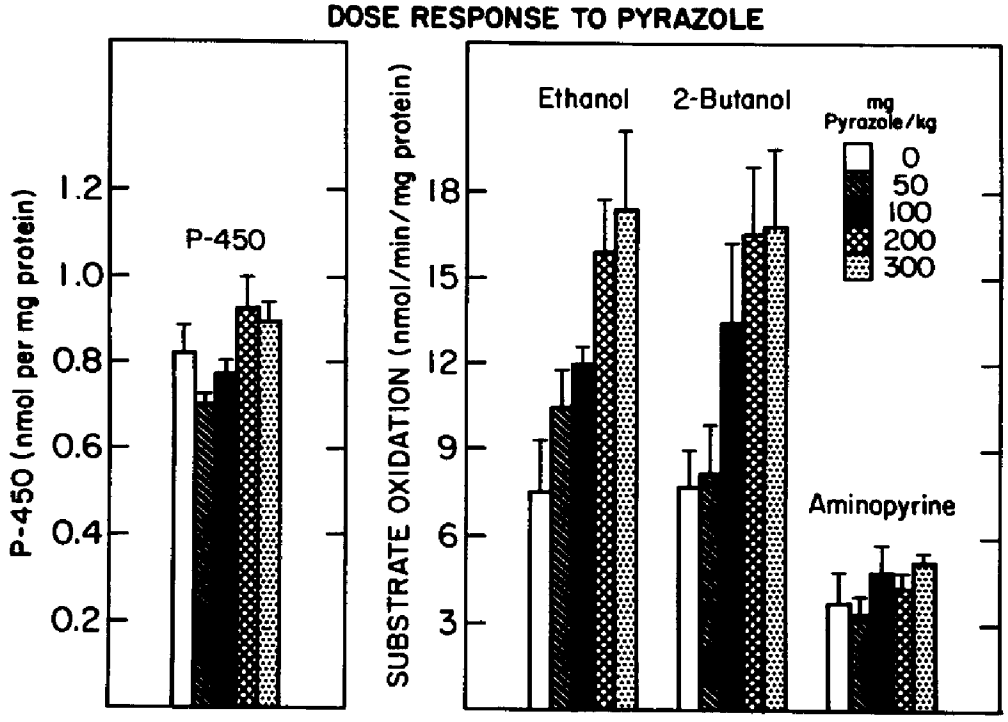
A-3.1 The Effect of Pyrazole Concentration on the Content of Cytochrome P-450 and the Microsomal Oxidation of Alcohols and Aminopyrine

Rats were treated with either saline or varying amounts of pyrazole for 2 days and the effects on the content of cytochrome P-450 and the microsomal of ethanol, 2-butanol,

Figure 5

The Effect of Pyrazole Concentration on the Content of Cytochrome P-450 and the Oxidation of Substrates.

Rats were treated for 2 days with the indicated concentrations of pyrazole and the content of cytochrome P-450 and the rate of microsomal oxidations of ethanol, 2-butanol, and aminopyrine were determined as described in "Materials and Methods". Results are from 3-4 experiments.


DOSE RESPONSE TO PYRAZOLE
P-450 (nmol per mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
P-450
SUBSTRATE OXIDATION (nmol/min/mg protein)
18
15
12
9
6
3
Ethanol
2-Butanol
Aminopyrine
mg Pyrazole/kg
0
50
100
200
300

and aminopyrine were determined. The content of cytochrome was not affected when the pyrazole dose was varied from 50-300 mg/kg body weight/day for 2 days (Fig. 5). In a similar manner, the microsomal oxidation of aminopyrine was not changed when rats were treated over this range of pyrazole concentrations. A dose-dependent increase in the microsomal oxidation of ethanol and 2-butanol was observed at pyrazole doses between 50 and 300 mg/kg body weight/day when the results are expressed "per mg microsomal protein" (Fig. 5). Since pyrazole treatment did not effect the content of cytochrome P-450, analogous results are obtained when the results are express "per nmol cytochrome P-450".

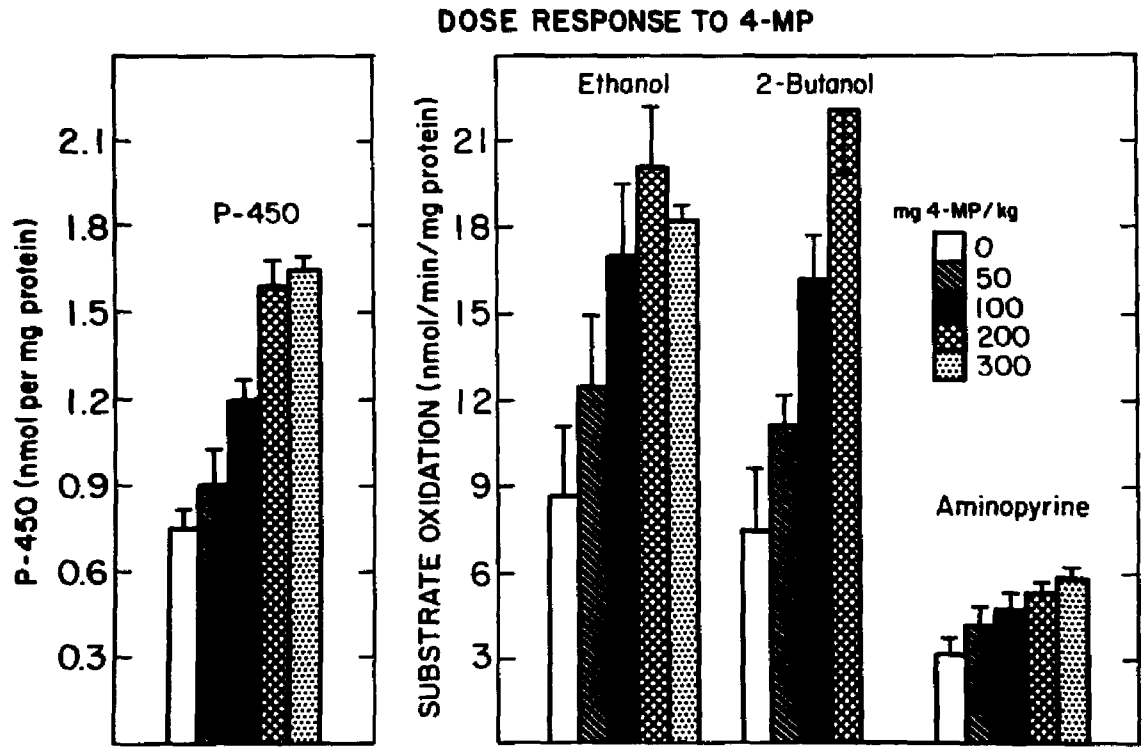
A-3.2 The Effect of 4-Methylpyrazole Concentration on the Content of Cytochrome P-450 and the Microsomal Oxidation of Alcohols and Aminopyrine

In a similar type of experiment, rats were treated with either saline or varying amounts of 4-methylpyrazole for a 3-day period. In contrast to the results with pyrazole, the content of cytochrome P-450 increased as the dose of 4-methylpyrazole injected was increased (Fig. 6). When the results are express "per mg microsomal protein", the microsomal oxidation of ethanol and 2-butanol also increased as the dose of 4-methylpyrazole injected was increased up to a level of 200 mg/kg body weight. The

Figure 6

The Effect of 4-Methylpyrazole Concentration on the Content of Cytochrome P-450 and the Oxidation of Substrates.

Rats were treated for 3 days with the indicated concentrations of 4-methylpyrazole and the content of cytochrome P-450 and the rate of microsomal oxidations of ethanol, 2-butanol, and aminopyrine were determined as described in "Materials and Methods". Results are from 3 experiments.


DOSE RESPONSE TO 4-MP
P-450
P-450 (nmol per mg protein)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
SUBSTRATE OXIDATION (nmol/min/mg protein)
Ethanol
2-Butanol
21
18
15
12
9
6
3
mg 4-MP/kg
0
50
100
200
300
Aminopyrine

microsomal oxidation of aminopyrine also increased with the dose of 4-methylpyrazole when the results are expressed "per mg microsomal protein"; however, this increase was much smaller that the increase in alcohol oxidation (Fig. 6).

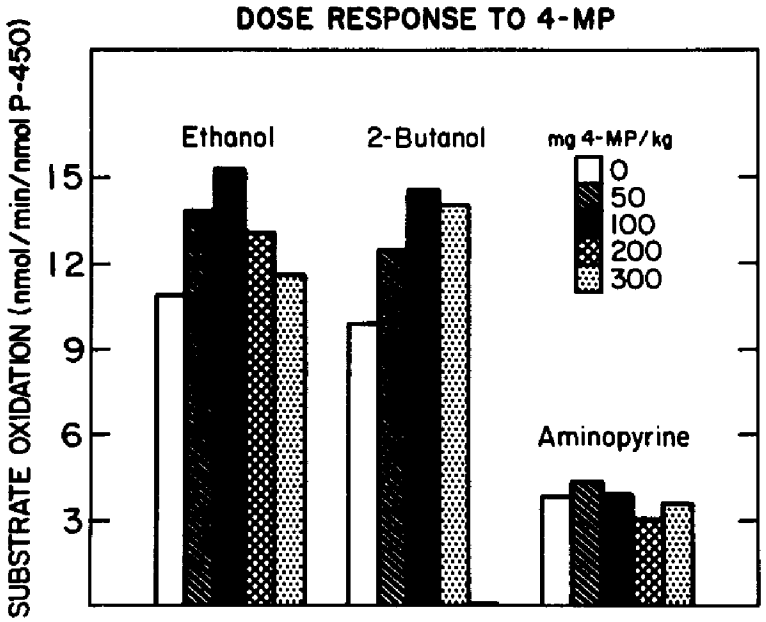
The above data was recalculated by dividing by the content of cytochrome P-450 in order to express the results as turnover numbers (per nmol cytochrome P-450, Fig. 7). Results in Fig. 7 show that the turnover number for aminopyrine oxidation was essentially not affected when the dose of 4-methylpyrazole was varied from 100 to 300 mg/kg body weight/day. When the results for microsomal oxidation of alcohols are expressed "per nmol cytochrome P-450" it appears that the increase in the oxidation of alcohols when expressed per mg microsomal protein was primarily due to the increase in total content of cytochrome P-450. There was less than a 40% increase in the turnover number for oxidation of the alcohols after 3 days of treatment with 4-methylpyrazole (Fig. 7).

The results with pyrazole suggest the possibility that pyrazole treatment may result in qualitative change in the cytochrome P-450 isozyme population, e.g., pyrazole may induce an alcohol-preferring cytochrome P-450 isozyme. The microsomal oxidation of alcohols is also increased by 4-methylpyrazole treatment; however, this increase in the oxidation of alcohols appears to be largely due to the increase in total content of cytochrome P-450 rather than

Figure 7

The Effect of 4-Methylpyrazole Concentration on the Oxidation of Substrates Expressed "per nmol cytochrome P-450".

Data were determined by dividing the individual rates for each experiment shown in Figure 6 by the content of cytochrome P-450 in the microsomes used for that experiment.


DOSE RESPONSE TO 4-MP
SUBSTRATE OXIDATION (nmol/min/nmol P-450)
15
12
9
6
3
Ethanol
2-Butanol
Aminopyrine
mg 4-MP/kg
0
50
100
200
300

to the induction of an alcohol-preferring cytochrome P-450: there was less than a 40% increase in the oxidation of these alcohols when results are expressed "per nmol cytochrome P-450" (Fig. 7). Nonetheless, there appears to be a differential affect, i.e., the increase in microsomal alcohol oxidation is greater that the increase in aminopyrine oxidation (Fig. 6). The results in Figures 2B-4B suggest the possibility that 4-methylpyrazole may cause some induction of the microsomal oxidation of alcohols after a single treatment, but that whereas a second treatment with pyrazole is especially effective in increasing the oxidation of alcohols, a second or a third treatment with 4-methylpyrazole is ineffective. Perhaps 4-methylpyrazole may initially induce an alcohol-preferring cytochrome P-450; however, further treatment may result in the induction of other isozymes of cytochrome P-450 which are not especially effective in oxidizing alcohols. The concomitant induction of nonalcohol-preferring isozymes may mask a significant contribution by an alcohol-preferring cytochrome P-450. It was therefore considered that perhaps the use of lower doses of 4-methylpyrazole for shorter periods of exposure might be useful in evaluating whether an alcohol-preferring cytochrome P-450 can be induced by 4-methylpyrazole without the concomitant induction of other isozymes which may occur with longer treatment with 4-methylpyrazole. Table III shows the results of a single

Table III

The Effect of One Day Treatment With Varying Doses of 4-Methylpyrazole on the Content of Cytochrome P-450 and the Oxidation of Ethanol, 2-Butanol and Aminopyrine

4-Methyl-Pyrazole Concentration mg kg body wt	Content of Cytochrome P-450 nmol mg protein	Specific Activity of Microsomal Substrate Oxidation nmol/min nmol cytochrome P-450		
		Ethanol	2-Butanol	Aminopyrine
0	1.03±0.04	8.1±0.8	10.0±1.1	3.9
50	1.08±0.13	12.5±1.4	15.4±1.1	4.4
100	1.33±0.10	10.5±1.5	15.4±2.2	3.8
200	1.28±0.08	10.0±0.9	12.9±2.1	4.2

Rats were given a single injection of either saline or the indicated concentration of 4-methylpyrazole. After an overnight fast, microsomes were prepared and the various activities were assayed as described in "Material and Methods". Results are from four experiments, except for the aminopyrine experiments, in which case n=2.

exposure to varying concentrations of 4-methylpyrazole on the content of cytochrome P-450 and the specific activity (nmol product/nmol cytochrome P-450) of microsomal oxidation of ethanol, 2-butanol, and aminopyrine. There was a 50% increase in the specific activity of oxidation ethanol and 2-butanol, but not aminopyrine, at the lower concentrations of 4-methylpyrazole. At 200 mg/kg body weight the increase in specific activity dropped to 25% (Table III). Thus, there appears to be an induction of an alcohol preferring cytochrome P-450 by 4-methylpyrazole treatment; however, the concomitant induction of other cytochrome P-450 isozymes complicates the interpretation.

A-4 The Specificity of Induction

The initial studies above showed that in vivo administration of pyrazole and 4-methylpyrazole can affect the mixed-function oxidase system and the microsomal oxidation of alcohols, and that there are similarities as well as differences in the interactions of these compounds with microsomes. The next set of studies were carried out to further characterize and compare the effects of pyrazole on other microsomal reactions; to compare SDS gel electrophoretic profiles of the cytochrome P-450 isozymes induced by pyrazole and 4-methylpyrazole; and to evaluate the kinetics and substrate specificities of the inducible isozymes in an effort to understand the similar as well as

the different interactions which these compounds exhibit with microsomes.

Unless otherwise specified, the usual dose of pyrazole and 4-methylpyrazole was 200 mg/kg body weight/day for 2 or 3 days, respectively.

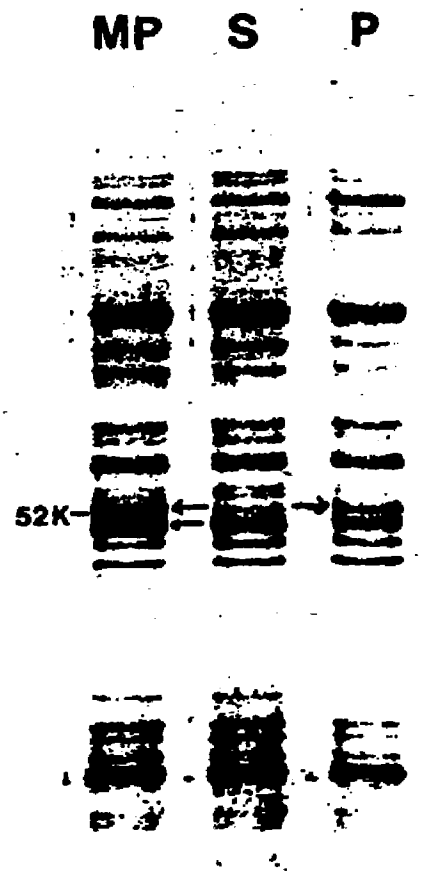
A-4.1 SDS-Gel Electrophoresis

Approximately 7.5 ug of microsomal protein was loaded onto each lane of the SDS-gel. In microsomes isolated from saline-treated rats, approximately 4 major bands were observed in the 50,000 dalton molecular weight region. Pyrazole treatment resulted in an increase of one band, with a molecular weight of about 52,000 daltons (Fig. 8). There appeared to be some loss of a 48,000-50,000 molecular weight cytochrome P-450 isozyme after the pyrazole treatment (Fig. 8) which probably explains why the total content of cytochrome P-450 remained the same despite the increase of the 52,000 molecular weight cytochrome P-450 isozyme by pyrazole. Treatment with 4-methylpyrazole also resulted in an increase of a band in the 52,000 molecular weight region. In contrast to pyrazole treatment which resulted in a decrease of a 48,000 molecular weight cytochrome P-450 isozyme, 4-methylpyrazole produced an increase in this band (Fig. 8). When gels were scanned with a densitometer both pyrazole and 4-methylpyrazole treatment resulted in a two- to three-fold increase in the

Figure 8

The Effect of Pyrazole and 4-Methylpyrazole Treatment on SDS Polyacrylamide Gel Profiles of Rat liver Microsomes

SDS polyacrylamide gel of microsomes isolated from rats that were treated with either saline or 200 mg/kg body weight pyrazole or 4-methylpyrazole for 2 and 3 days, respectively. Gels were stained with Coomasie-blue. Each lane contained 7.5 ug of microsomal protein from saline (S), pyrazole (P) or 4-methylpyrazole (MP) treated rats.


MP
S
P
52K

52,000 molecular weight band compared to saline controls. In addition, 4-methylpyrazole increased the 48,000 molecular weight band about two-fold, compared to saline controls.

A-4.2 Microsomal Drug Oxidation

The oxidation of several drugs, some of which are metabolized preferentially by certain isozyme of cytochrome P-450, was assayed to assess the effects of *in vivo* administration of pyrazole and 4-methylpyrazole. Results in Table 4 show that microsomal aniline hydroxylase activity was increased two- and three-fold by pyrazole and 4-methylpyrazole treatment, respectively, when activity was expressed "per mg microsomal protein". Similar results were found with microsomal paranitroanisole O-demethylase activity. Pyrazole treatment resulted in a two-fold increase in activity, and 4-methylpyrazole treatment resulted in a greater than three-fold increase in activity (Table IV).

7-Ethoxycoumarin O-deethylase activity is routinely assayed by the production of 7-hydroxycoumarin. The rate of production of 7-hydroxycoumarin was not significantly changed by the pyrazole or 4-methylpyrazole treatment (Table IV). During the course of these experiments, it was noticed that 7-hydroxycoumarin itself underwent further metabolism as detected by comparison of a standard

Table IV

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Drug Metabolism Expressed "per mg microsomal protein"

	Rate of Oxidation (nmol/min/mg protein)		
Substrate	Saline	Pyrazole	4-Methylpyrazole
Aniline	1.16 ± 0.0	2.34 ± 0.2(a)	3.57 ± 0.2(a)
p-Nitroanisole	0.93 ± 0.2	2.00 ± 0.3(b)	3.40 ± 0.3(a)
Aminopyrine*	2.88 ± 0.2	2.40 ± 0.3	4.37 ± 0.3(a)
7-Ethoxycoumarin			
1)7-hydroxycoumarin			
A)production	2.24 ± 0.2	2.07 ± 0.3	2.76 ± 0.3
B)disappearance	0.49 ± 0.1	1.01 ± 0.3(b)	1.13 ± 0.1(a)
C)total	2.73 ± 0.5	3.08 ± 0.5	3.89 ± 0.4(b)
2)acetaldehyde	2.52 ± 0.1	2.51 ± 0.3	3.90 ± 0.3(a)

The various drug oxidation activities were assayed as described in "Material and Methods". As described in the text, some of the 7-hydroxycoumarin produced from 7-ethoxycoumarin was found to undergo further metabolism. This was assayed and is shown in row B, 7-hydroxycoumarin disappearance. This rate was added to the rate of 7-hydroxycoumarin production (row A) to yield the total rate of 7-ethoxycoumarin O-deethylase activity (row C).

*Results with animopyrine were reported in section "A-3" and are intended for comparative purposes with other drugs.

(a)-$p < 0.01$

(b)-$p < 0.05$

containing a known amount of 7-hydroxycoumarin added to the basic reaction system which were treated with TCA either prior to the addition of 7-hydroxycoumarin or 10 minutes after. The rate of disappearance of 7-hydroxycoumarin was 20% of the rate of production of 7-hydroxycoumarin by saline microsomes. Pyrazole and 4-methylpyrazole resulted in a two-fold increase in the rate of disappearance of 7-hydroxycoumarin. Adding this rate to the rate of production of 7-hydroxycoumarin to yield the "total" rate of 7-ethoxycoumarin metabolism indicated that pyrazole treatment had no effect, whereas 4-methylpyrazole treatment produced some increase in the metabolism of 7-ethoxy-coumarin (Table IV). It was anticipated that assays of acetaldehyde production, the other product of 7-ethoxy-coumarin metabolism, would not be hindered by further metabolism as were the 7-hydroxycoumarin determinations. Results with acetaldehyde generation, which were quite similar to the total rates of 7-ethoxycoumarin metabolism, also indicated that pyrazole treatment did not induce 7-ethoxycoumarin O-deethylase activity, whereas 4-methyl-pyrazole treatment produced about a 50% increase (Table IV), similar to the increase found with aminopyrine, but much less than observed with aniline or p-nitroanisole.

The above data was recalculated by dividing by the content of cytochrome P-450 in order to express the results as turnover numbers (nmol product/min/nmol cytochrome

P-450). Results in Table V show that the turnover associated with aniline or p-nitroanisole as substrates was increased about two-fold after pyrazole or 4-methylpyrazole treatment compared to saline controls. In contrast, the turnover associated with aminopyrine or 7-ethoxycoumarin was slightly decreased by pyrazole treatment, and more significantly decreased (30%) by 4-methylpyrazole treatment (Table V).

Recent experiments have established the role of cytochrome P-450 isozymes in the metabolism of nitrosamines (143). The demethylation of N,N-dimethylnitrosamine appears to be catalyzed by two populations of cytochrome P-450, i.e., a high Km (about 37mM) and a low Km (<0.1 mM) population. Results in Table VI show that pyrazole causes an increase in the activity of the low Km N,N-dimethyl-nitrosamine demethylase. These results confirm the previous work of Yang and co-workers (143-145). A two-fold increase in activity was observed whether results were expressed on a per mg microsomal protein (Table VI,Exp.A) or per nmol cytochrome P-450 (Table VI,Exp.B). Increasing the concentration of dimethylnitrosamine ten-fold had little effect on the rates of oxidation (compare the 0.5 mM rates to the 5.0 mM rates), which indicates that the low Km isozyme(s) are probably saturated at 0.5 mM dimethyl-nitrosamine. 4-Methylpyrazole treatment produced a two-fold increase in activity at all concentrations of

Table V

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Drug Metabolism Expressed "per nmol cytochrome P-450"

Substrate	Rate of Oxidation (nmol/min/nmol P-450) Saline	Pyrazole	4-Methylpyrazole
Aniline	1.54 ± 0.2	3.20 ± 0.3(a)	2.76 ± 0.3(a)
p-Nitroanisole*	1.05 ± 0.3	2.03 ± 0.2(b)	1.98 ± 0.2(b)
Aminopyrine	4.03 ± 0.5	3.04 ± 0.7	2.86 ± 0.3(b)
7-Ethoxycoumarin			
1)7-hydroxycoumarin			
A)production	2.88 ± 0.6	2.36 ± 0.5	1.61 ± 0.3(b)
B)disappearance	0.61 ± 0.1	0.87 ± 0.2	0.65 ± 0.1
C)total	3.49 ± 0.6	3.23 ± 0.7	2.26 ± 0.4(b)
2)acetaldehyde	3.23 ± 0.6	3.19 ± 0.4	2.21 ± 0.2(b)

The various calculated enzymatic activities per mg microsomal protein for each drug oxidation experiment were divided by the content of cytochrome P-450 for that preparation of microsomes in order to express the results as a turnover number (nmol product/min/nmol cytochrome P-450). Results are from 4-7 experiments.

(a)-$p < 0.01$

(b)-$p < 0.05$

Table VI

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal N,N-Dimethylnitrosamine Demethylase Activity

Concentration of DMN		Rate of Oxidation (nmol/min/) Saline	Pyrazole	4-Methylpyrazole
A.	0.5 mM	1.90 ± 0.2	4.22 ± 0.6(a)	3.55 ± 0.2(a)
	5.0 mM	2.21 ± 0.1	4.36 ± 0.4(a)	4.49 ± 0.3(a)
	100 mM	5.70 ± 0.4	7.44 ± 0.8	9.11 ± 0.5(a)
	(100-0.5)	3.80 ± 0.4	3.22 ± 1.4	5.56 ± 0.4(b)
B.	0.5 mM	2.36 ± 0.5	4.04 ± 0.7(b)	1.90 ± 0.1
	5.0 mM	2.76 ± 0.6	4.15 ± 0.5(b)	2.42 ± 0.1
	100 mM	6.90 ± 0.9	7.06 ± 0.8	4.91 ± 0.2
	(100-0.5)	4.54 ± 0.5	3.02 ± 1.3	3.01 ± 0.1(b)

The oxidation of N,N-dimethylnitrosamine (DMN) was assayed as described in "Materials and Methods". Results in experiment A refer to activity per mg microsomal protein, while results in experiment B refer to activity per nmol cytochrome P-450. Results are from 3 experiments. The (100-0.5) concentration refers to the rates at 0.5 mM being subtracted from the rates at 100 mM to yield activity of the high Km isozyme(s).

(a)-$p < 0.01$

(b)-$p < 0.05$

dimethylnitrosamine tested. This increase appeared to reflect the increase in total cytochrome P-450 since no increase in activity occurred when results were expressed per nmol cytochrome P-450 (Table VI,Exp.B). The activity at 100 mM dimethylnitrosamine reflects both low Km and high Km activities; therefore, subtracting the rates at 0.5 mM dimethylnitrosamine from the rates at 100 mM should yield the high Km activity. When results are expressed per nmol cytochrome P-450 both pyrazole and 4-methylpyrazole treatments resulted in a decrease in the activity of the high Km dimethylnitrosamine demethylase(s) (Table VI,Exp.B).

A-4.3 Kinetics of Ethanol and Aniline Oxidation

Initial studies in this thesis have shown that the oxidation of ethanol by microsomes was increased about two-fold after treatment with pyrazole or 4-methylpyrazole (A-3.1 and A-3.2), when results are expressed per mg microsomal protein. When the data is expressed per nmol cytochrome P-450 the results were more complex: pyrazole treatment resulted in a two-fold increase in ethanol oxidation, whereas there was less than a 40% increase after 4-methylpyrazole treatment. Only one concentration of ethanol was utilized in these studies which does not supply any kinetic information; therefore, the effect of pyrazole

and 4-methylpyrazole treatment on the kinetics associated with microsomal ethanol oxidation was evaluated. Since ethanol can be oxidized by hydroxyl radicals, the possible involvement of oxy-radical species in the oxidation of ethanol by microsomes was minimized in these reactions as described in "Material and Methods". Therefore, these rates and kinetic values represent the cytochrome P-450 dependent pathway of ethanol oxidation. Figure 9 shows plots of the rate of microsomal ethanol oxidation as a function of the concentration of ethanol over the range of 5.5 to 110 mM. Typical saturation kinetics were observed for the 3 preparations. Pyrazole and 4-methylpyrazole treatment increased microsomal ethanol oxidation at all concentrations of ethanol tested (Fig. 9). When plots of the rate of microsomal ethanol oxidation as a function of ethanol concentration are expressed per nmol cytochrome P-450, only pyrazole treatment resulted in an increase in ethanol oxidation (Fig. 10). The relative kinetic constants for ethanol oxidation were determined by linear regression analysis of Lineweaver-Burk plots of the data shown in Figure 9. The values for Km and Vmax are apparent values for intact microsomes since they reflect contributions made by several populations of cytochrome P-450 isozymes. Nevertheless, Lineweaver-Burk plots were linear, with correlation coefficients ranging from 0.93 to greater than 0.99. Pyrazole treatment did not alter the Km

Figure 9

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of Ethanol Expressed "per mg microsomal protein" as a Function of the Concentration of Ethanol.

The oxidation of ethanol was determined as described in "Material and Methods" after treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. The concentrations of ethanol utilized were 5.5, 11, 27.5, 55 and 110 mM, and the reactions were carried out for 5 minutes. Results are from 4-8 experiments and are expressed as nmol acetaldehyde/min/mg microsomal protein.

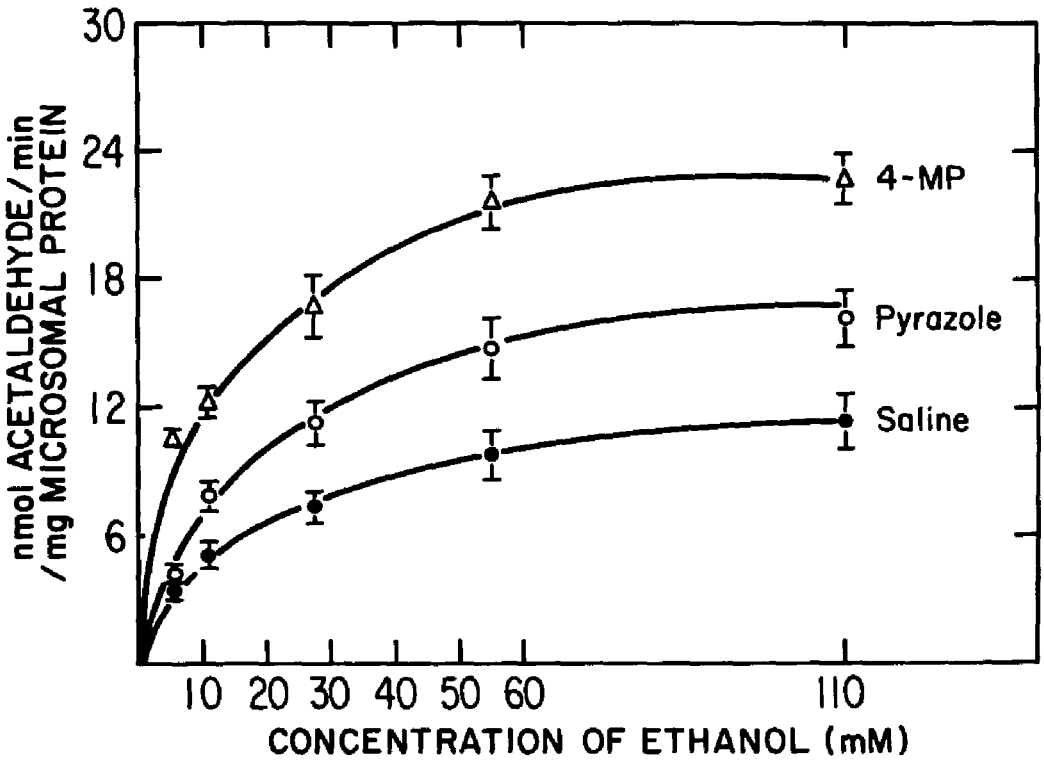
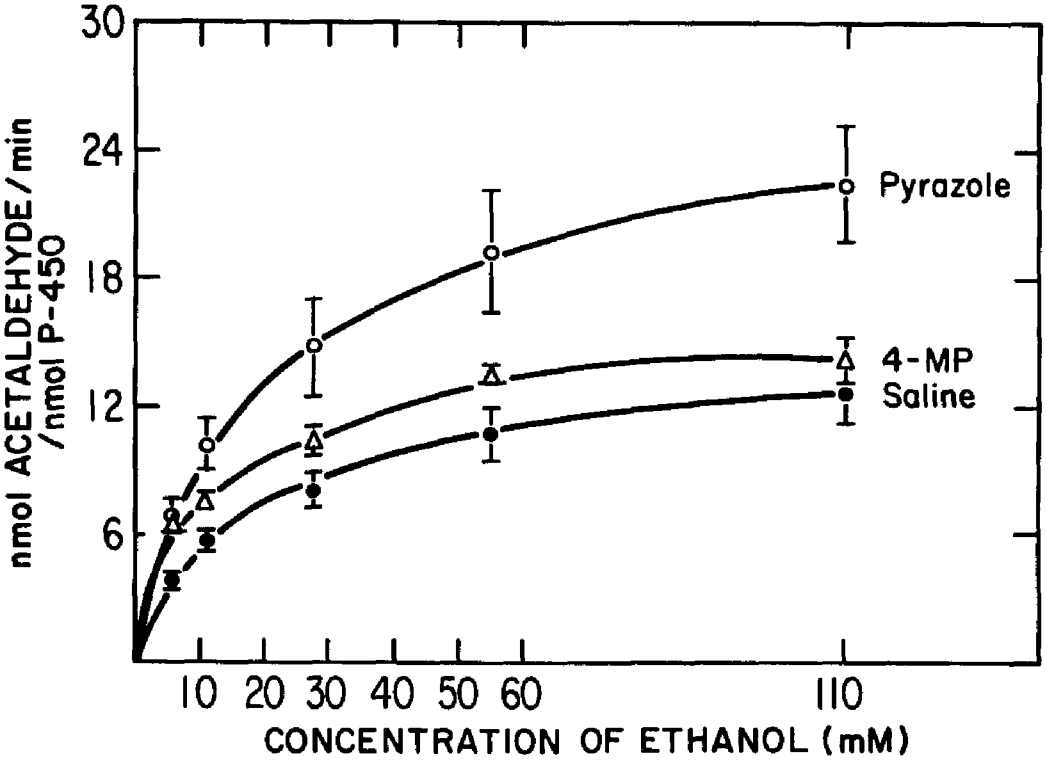

30
24
18
12
6
nmol ACETALDEHYDE/min
/mg MICROSOMAL PROTEIN
4-MP
Pyrazole
Saline
10 20 30 40 50 60
110
CONCENTRATION OF ETHANOL (mM)

Figure 10

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of Ethanol Expressed "per nmol cytochrome P-450" as a Function of the Concentration of Ethanol.

The oxidation of ethanol was determined as described in "Material and Methods" after treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. The concentrations of ethanol utilized were 5.5, 11, 27.5, 55 and 110 mM, and the reactions were carried out for 5 minutes. Results are from 4-8 experiments and are expressed as nmol acetaldehyde/min/nmol cytochrome P-450.


30
24
18
12
6
nmol ACETALDEHYDE / min
/nmol P-450
Pyrazole
4-MP
Saline
10 20 30 40 50 60
110
CONCENTRATION OF ETHANOL (mM)

for ethanol, whereas, Vmax values were increased whether expressed per mg microsomal protein or per nmol cytochrome P-450 (Table VII). The effects of 4-methylpyrazole treatment were more complex, as the Km for ethanol was decreased about 50% (Table VII). The Vmax for ethanol oxidation was increased two-fold when results were expressed per mg microsomal protein, but no increase was observed per nmol cytochrome P-450 (Table VII).

Similar experiments were conducted with aniline as the substrate. Pyrazole and 4-methylpyrazole treatment resulted in increased aniline hydroxylase activity when results are expressed per mg microsomal protein at all concentrations of aniline tested (Fig. 11). When the data are expressed per nmol cytochrome P-450, pyrazole treatment resulted in increased aniline hyroxylase activity at all concentrations of aniline tested, whereas 4-methylpyrazole treatment resulted in increased activity only at the higher concentrations of aniline (Fig. 12). Kinetic analysis of the data in Figure 11 and 12 indicated the pyrazole treatment lowered the Km for aniline and raised the Vmax whether results are expressed per mg microsomal protein or per nmol cytochrome P-450 (Table VIII). On the other hand, 4-methylpyrazole treatment raised the Km for aniline while increasing the Vmax three fold when results are express per mg microsomal protein and greater than 50% when results are expressed per nmol cytochrome P-450. Hence, it appears

Table VII

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Kinetics of Microsomal Oxidation of Ethanol

Kinetic parameter	Saline	Pyrazole	4-Methylpyrazole
Km (mM)	14.0 ± 1.1	13.9 ± 0.7	6.6 ± 0.3(a)
Vmax (per mg protein)	11.8 ± 1.2	18.1 ± 1.8(b)	21.9 ± 0.9(a)
Vmax (per nmol P-450)	13.0 ± 1.2	23.6 ± 3.2(a)	13.6 ± 0.4

The kinetic parameters were calculated from linear regressions of Lineweaver-Burk plots of the data shown in Figures 9 and 10.

(a)-$p < 0.001$

(b)-$p < 0.01$

Figure 11

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Aniline Hydroxylation Expressed "per mg microsomal protein" as a Function of the Concentration of Aniline.

The hydroxylation of aniline was determined as described in "Material and Methods" after treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. The concentrations of aniline utilized were 10, 25, 50, 100 and 500 uM, and the reactions were carried out for 10 minutes. Results are from 5 experiments and are expressed as nmol p-aminophenol/min/mg microsomal protein.

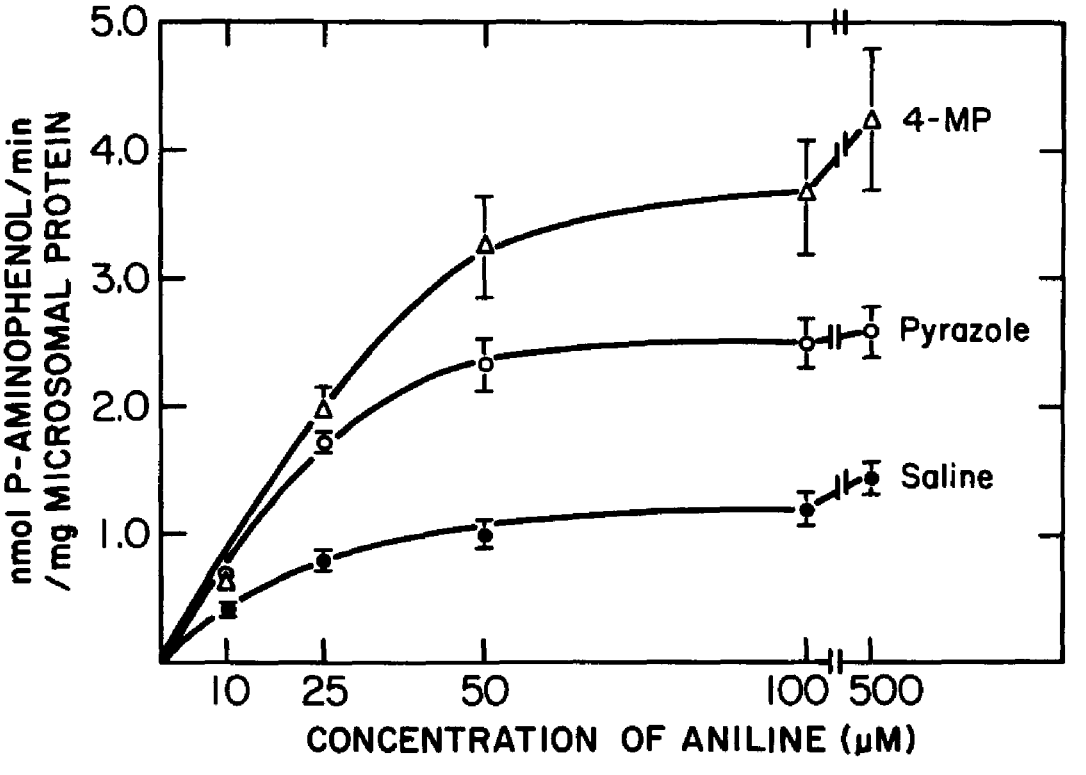

5.0
4.0
3.0
2.0
1.0
nmol P-AMINOPHENOL/min
/mg MICROSOMAL PROTEIN
4-MP
Pyrazole
Saline
10
25
50
100
500
CONCENTRATION OF ANILINE (μM)

Figure 12

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Aniline Hydroxylation Expressed "per nmol cytochrome P-450" as a Function of the Concentration of Aniline.

The hydroxylation of aniline was determined as described in "Material and Methods" after treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. The concentrations of aniline utilized were 10, 25, 50, 100 and 500 uM, and the reactions were carried out for 10 minutes. Results are from 5 experiments and are expressed as nmol p-aminophenol/min/nmol cytochrome P-450.

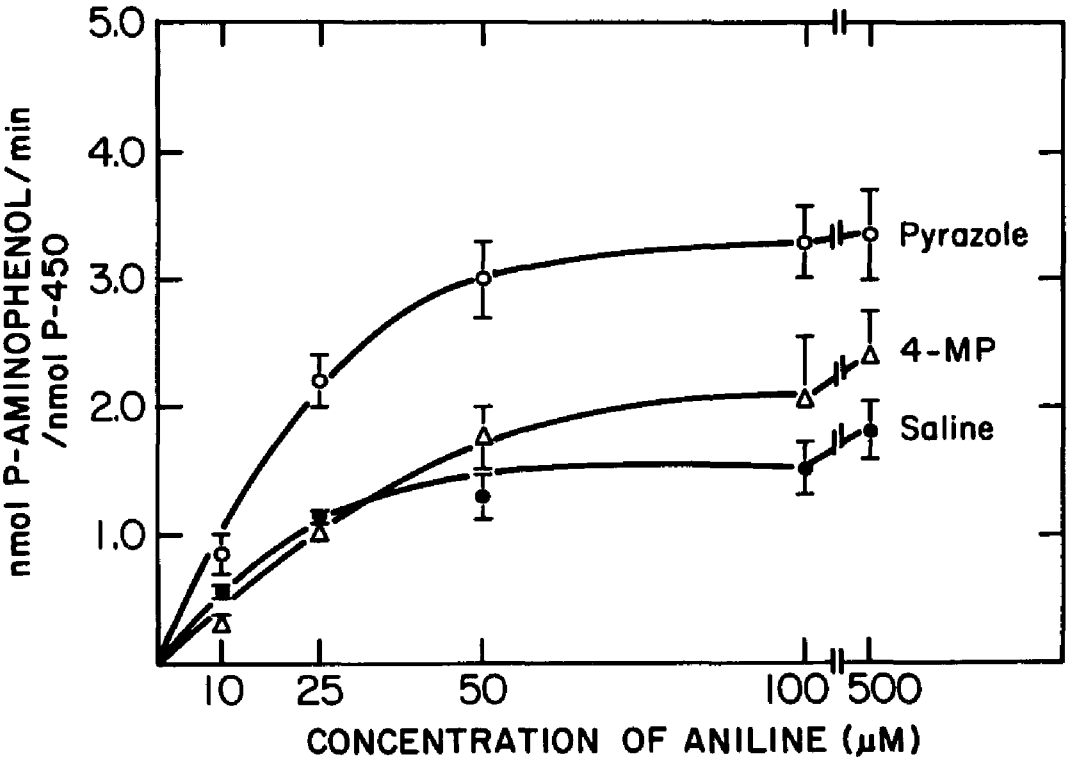

5.0
4.0
3.0
2.0
1.0
nmol P-AMINOPHENOL/min
/nmol P-450
Pyrazole
4-MP
Saline
10
25
50
100
500
CONCENTRATION OF ANILINE (μM)

Table VIII

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Kinetics of Microsomal Oxidation of Aniline

Kinetic parameter	Saline	Pyrazole	4-Methylpyrazole
Km (uM)	26 ± 6	13 ± 2(c)	50 ± 5(a)
Vmax (per mg protein)	1.51 ± 0.09	2.66 ± 0.20(a)	4.75 ± 0.60(a)
Vmax (per nmol P-450)	1.90 ± 0.24	3.41 ± 0.36(b)	2.90 ± 0.34(c)

The kinetic parameters were calculated from linear regressions of Lineweaver-Burk plots of the data shown in Figures 11 and 12.

(a)-$p < 0.001$

(b)-$p < 0.01$

(c)-$p < 0.05$

that the increase in aniline hydroxylase is due, in part, to the two-fold elevation in the content of cytochrome P-450 caused by 4-methylpyrazole treatment although a clear increase in turnover number also occurs (Table VIII).

A-4.4 Kinetic Studies on the Stereochemical Preference for (+)-2-Butanol

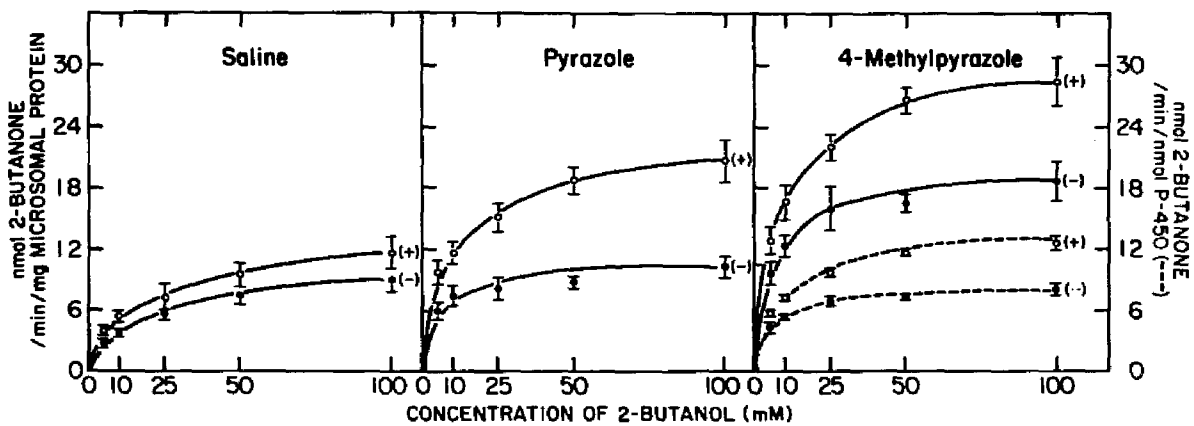
Previous studies have shown that microsomes isolated from rats chronically fed ethanol or from rats treated with pyrazole catalyzed the oxidation of (+)-2-butanol at rates two- to three-fold greater than rates found with the (-)-2-butanol isomer (23,127). This stereopreference, which was not found with microsomes isolated from chow-fed or pair-fed controls, or with microsomes isolated from phenobarbital-treated rats, probably reflects qualitative changes in the cytochrome P-450 isozyme population produced after pyrazole treatment or ethanol consumption. Experiments were carried out to evaluate the kinetics for this stereochemical preference by microsomes from the pyrazole-treated rats and to ascertain whether 4-methylpyrazole treatment also induced a stereochemical preference for the (+)-2-butanol isomer.

The oxidation of (+)-2-butanol by microsomes isolated from saline treated rats was slightly, but not significantly higher than the rates found with the (-)-2-butanol isomer (Fig. 13). By contrast, both pyrazole and

Figure 13

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation (+)- and (-)-2-Butanol as a Function of the Concentration of each Isomer.

The oxidation of ethanol was determined as described in "Material and Methods" after treatment with either saline or 200 mg/kg body weight/day pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. The concentrations of each respective isomer utilized were 5.0, 10, 25, 50 and 100 mM, and the reactions were carried out for 5 minutes. Results are from 4 experiments and are expressed as nmol 2-butanone /min/mg microsomal protein (solid lines), and nmol 2-butanone/min/nmol cytochrome P-450 (dashed lines).


Saline
Pyrazole
4-Methylpyrazole
nmol 2-BUTANONE /min/mg MICROSOMAL PROTEIN
nmol 2-BUTANONE /min/nmol P-450 (---)
CONCENTRATION OF 2-BUTANOL (mM)
0
6
12
18
24
30
0 10 25 50 100
(+)
(-)

4-methylpyrazole treatment produced a stereochemical preference for the (+)-2-butanol isomer (Fig. 13). The rate of oxidation of the (+)-isomer, but not the (-)-isomer, was increased about two-fold by pyrazole treatment whether results were expressed per mg microsomal protein (Fig. 13) or per nmol cytochrome P-450, since pyrazole treatment had no effect of the total content of cytochrome P-450. The oxidation of both isomers was increased by 4-methylpyrazole treatment when results were expressed per mg microsomal protein (Fig. 13); however, the rate for the (+)-isomer remained higher than that for the (-)-isomer. When results were expressed per nmol cytochrome P-450 no increase compared to the saline controls was found for the (+)-isomer (Fig. 13, dashed lines), whereas, there was some decrease in the rates of oxidation for the (-)-isomer (Fig. 13, dashed lines).

The relative kinetic constants for (+)- and (-)-2-butanol oxidation were determined by linear regression analysis of Lineweaver-Burk plots of the data shown in Fig. 13. In microsomes isolated from saline controls, the Km and Vmax for the (+)- and (-)-2-butanol were about the same (Table IX). Little or no significant stereochemical preference was observed. The pyrazole treatment lowered the Km values for both isomers; however, the Vmax was elevated only for the (+)-isomer (per mg microsomal protein or per nmol cytochrome P-450) (Table IX). It is for

Table IX

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Kinetics of Microsomal Oxidation of 2-Butanol

Kinetic Parameter	2-Butanol isomer	Treatment		
		Saline	Pyrazole	4-Methylpyrazole
Km (mM)	+	9.0 ± 1.0	5.7 ± 0.9(a,c)	6.6 ± 0.9(a)
	-	13.3 ± 1.0	3.2 ± 0.4(b)	5.1 ± 1.0(a)
Vmax (per mg protein)	+	11.0 ± 1.7	19.7 ± 1.6(a,d)	28.7 ± 1.3(b,d)
	-	9.1 ± 0.8	9.5 ± 0.8	18.9 ± 2.0(a)
Vmax (per nmol P-450)	+	13.1 ± 1.8	27.5 ± 1.2(b,e)	13.7 ± 0.4(e)
	-	11.0 ± 1.5	13.3 ± 0.7	8.3 ± 0.5

The kinetic parameters were calculated from linear regressions of Lineweaver-Burk plots of the data shown in Figure 13.

(a)-$p<0.01$

(b)-$p<0.001$

with respect to saline controls

(c)-$p<0.05$

(d)-$p<0.01$

(e)-$p<0.001$

with respect to the (-)-isomer

interest that although pyrazole treatment resulted in an even higher affinity for the (-)-isomer, the Vmax for the (-)-isomer is the same as in the saline controls. The 4-methylpyrazole treatment also lowered the Km values for both isomers, while Vmax per mg microsomal protein was increased about three-fold for the (+)-isomer, and two-fold for the (-)-isomer (Table IX). The difference in the Vmax between the (+)- and (-)-isomer was significant, indicating stereochemical preference for the (+)-isomer. When results were expressed per nmol cytochrome P-450 this preference was also observed, although it was not as impressive as the stereochemical preference found with the microsomes isolated from the pyrazole treated rats (Table IX).

B. Binding Spectra

As previously mentioned in the "Introduction and Background", microsomal binding difference spectra can be useful in ascertaining the type of effects an inducing agent has on microsomal drug metabolizing enzymes. Therefore, characterization of the effect that pyrazole and 4-methylpyrazole treatment had on microsomal binding difference spectra was carried out.

B-1 Binding Spectra with Dimethyl Sulfoxide, 2-Butanol and Ethanol

The preceding studies that have been presented showed that pyrazole and 4-methylpyrazole treatment can affect the mixed-function oxidase system and the microsomal oxidation of alcohols and drugs e.g., aniline and p-nitroanisole. Furthermore, microsomes isolated from pyrazole- of 4-methylpyrazole-treated rats exhibit several properties which are similar to each other, as well as with microsomes isolated from rats chronically fed ethanol.

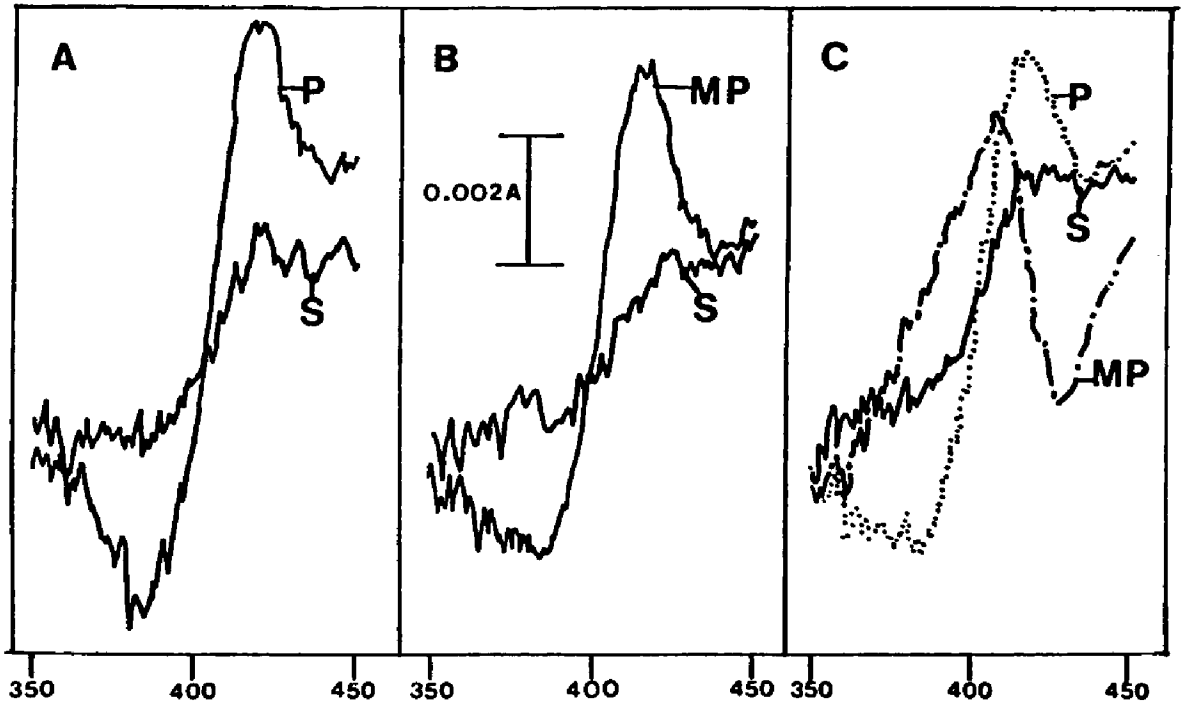
Dimethyl sulfoxide (DMSO) was shown to produce an inverse type I binding spectrum with microsomes isolated from ethanol-treated rats (22,146) or a type II binding spectrum with cytochrome P-450 3a (the rabbit ethanol induce isozyme) (72). 2-Butanol was shown to produce a type II binding spectrum with microsomes isolated from chronic ethanol-fed rats but not with microsomes from the pair-fed controls (23). The magnitude of the binding spectral change produced when ethanol binds to microsomes was enhanced after ethanol consumption (147). Therefore, binding studies were carried out with DMSO, 2-butanol and ethanol after treatment with pyrazole or 4-methylpyrazole.

Figure 14, A and C, shows that type II binding spectra with DMSO (peak at 418-420 nm and trough at 380-385 nm) and

Figure 14

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Binding of DMSO and 2-Butanol

Binding spectrum of DMSO (final concentration 110 mM) and 2-butanol (final concentration 120 mM) with microsomes (approximately 2 mg/6 ml) from saline (S), pyrazole (P), or 4-methylpyrazole (MP)-treated rats. Binding spectra were obtained as described in "Material and Methods". Experiment A refers to binding spectrum of DMSO after 2 days of treatment with either saline or pyrazole (200 mg/kg body weight/day). Experiment B refers to binding spectrum of DMSO after 3 days of treatment with either saline or 4-methylpyrazole (200 mg/kg body weight/day). Experiment C refers to binding spectrum with 2-butanol after either 2 (pyrazole experiment) or 3 days (4-methylpyrazole experiment) of drug treatment. Identical spectra were obtained from microsomes for the 2- and 3-day saline treatment.


A
P
S
B
MP
0.002A
S
C
P
S
MP
350
400
450
350
400
450
350
400
450

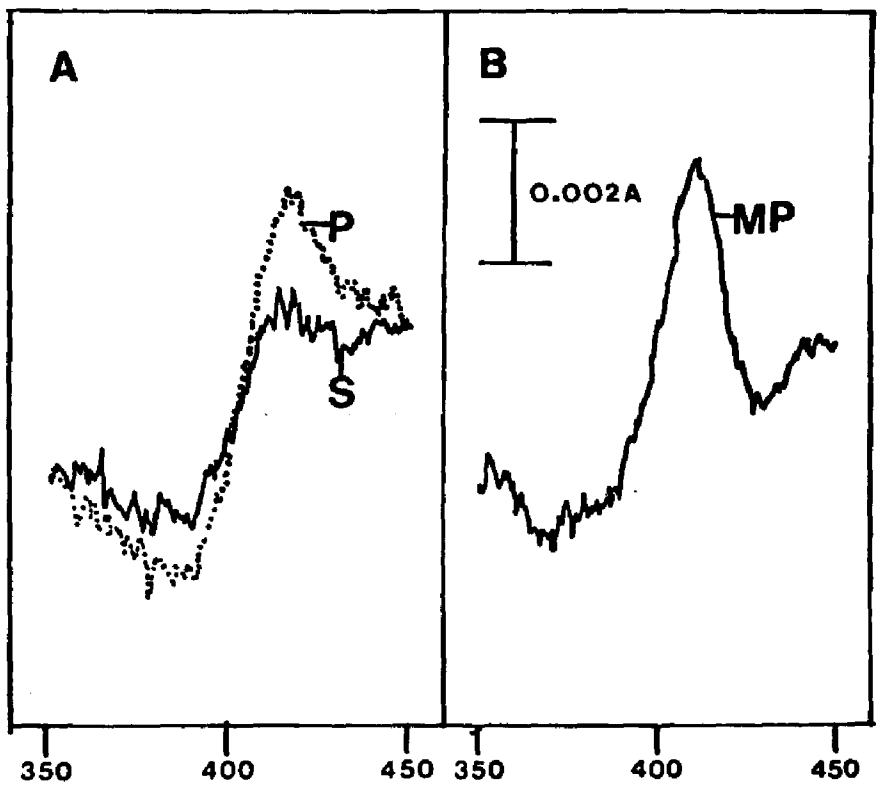
2-butanol (peak at 415-417 nm and trough at 380-385 nm), respectively, were observed with microsomes from rats treated with pyrazole, 200 mg/kg body weight/day for 2 days. No distinct spectra were observed with saline controls with DMSO or 2-butanol. With microsomes isolated from 4-methlypyrazole-treated rats (200 mg/kg body weight/day for 3 days), DMSO produced a type II binding spectrum (Fig. 14B) that appeared to be identical to that which was observed with microsomes isolated from pyrazole-treated rats (peak at 419-420 nm and trough at 380-385 nm). However, the interaction of 2-butanol with microsomes from 4-methylpyrazole-treated rats was quite different than that found with microsomes from the pyrazole-treated rats (Fig. 14C). The binding spectrum appeared to be type I, not type II; however, the spectrum was shifted towards longer wavelengths (peak at 408-410 nm and trough at 426-428 nm) than that usually observed with drugs such as hexobarbital which give rise to the typical type I binding spectrum (peak at 385 nm and trough at 420 nm).

Ethanol reacted with microsomes from saline treated rats to produce a type II binding spectrum, with a peak at 416-420 nm and a trough at 386-390 nm (Fig. 15A). Treatment with either pyrazole or 4-methylpyrazole resulted in an increase in the ethanol-binding spectrum (Fig. 15 A and B, respectively). The pyrazole treatment increased the magnitude of the change in absorbance of the peak to trough

Figure 15

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Binding of Ethanol

Binding spectrum of ethanol (final concentration 154 mM) with microsomes (approximately 2 mg/6 ml) from saline (S), pyrazole (P), or 4-methylpyrazole (MP)-treated rats. Binding spectra were obtained as described in "Material and Methods". Experiment A refers to binding spectrum of ethanol after 2 days of treatment with either saline or pyrazole (200 mg/kg body weight/day). Experiment B refers to binding spectrum of ethanol after 3 days of treatment 4-methylpyrazole (200 mg/kg body weight/day). Identical spectra were obtained from microsomes for the 2- and 3-day saline treatment.


A
P
S
350
400
450
B
0.002A
MP
350
400
450

absorbance difference (measured as A418nm minus A388nm) but did not change the spectrum from that observed with microsomes from saline controls (Fig. 15A). One the other hand, not only did the 4-methylpyrazole treatment produce an increase in the change in absorbance, but it also caused the ethanol-binding spectrum to shift, with the peak now being at 412-414 nm, and it also resulted in the production of an additional trough at 426-428 nm (Fig. 15B). In this regard, the ethanol binding spectrum with microsomes from the 4-methylpyrazole treated rats resembles that observed with 2-butanol (Fig. 14C and 15B).

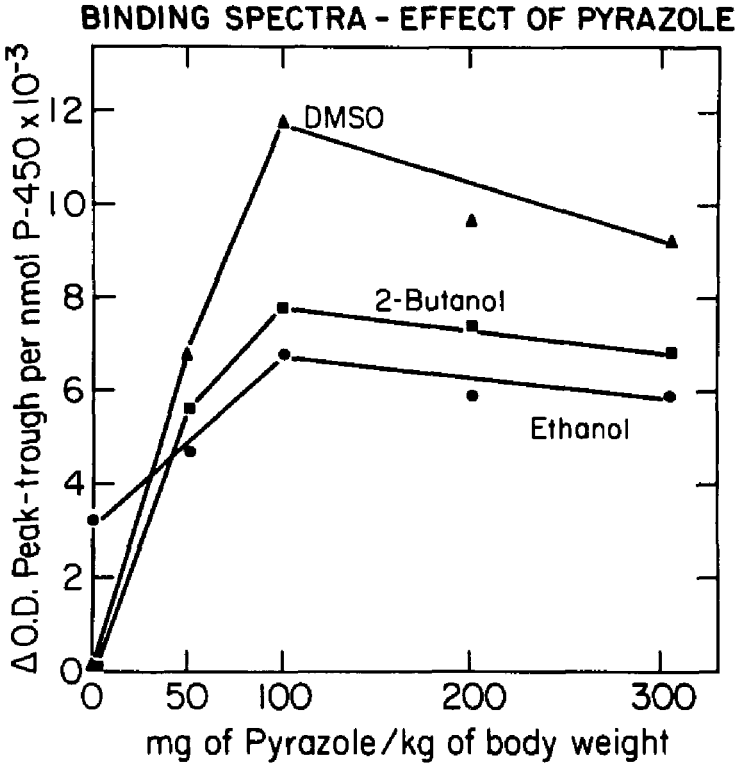
B-2 Dose-Response of Pyrazole on the Magnitude of Binding Spectra with Dimethyl Sulfoxide, 2-Butanol and Ethanol

Dose-responce curves with respect to the binding spectrum associated with the addition of DMSO, 2-butanol or ethanol to microsomes from saline-, pyrazole- or 4-methyl-pyrazole-treated rats were performed. Whereas, the saline controls exhibited no distinct binding spectrum DMSO or 2-butanol, 2 days of treatment with 50 mg of pyrazole/kg body weight was sufficient to result in binding spectra with these two compounds (Fig. 16). Maximum increase in the binding spectrum occurred at about 100 mg of pyrazole/ kg body weight. With ethanol as the substrate, the microsomes from the saline controls exhibit a binding spectrum (Fig. 15). Pyrazole increased this binding

Figure 16

The Effect of Pyrazole Concentration on the Magnitude of Substrate Binding Spectra in Microsomes.

Rats were treated for 2 days with the indicated concentrations of pyrazole and binding spectra were obtained as described in "Materials and Methods". Results are expressed as the change in O.D./nmol cytochrome P-450. Wavelengths utilized were: ethanol, 418-388; DMSO, 419-385; 2-butanol, 416-385. Results are the mean of 3-4 experiments.


BINDING SPECTRA - EFFECT OF PYRAZOLE
ΔO.D. Peak-trough per nmol P-450 x 10-3
0
2
4
6
8
10
12
DMSO
2-Butanol
Ethanol
0
50
100
200
300
mg of Pyrazole/kg of body weight

spectrum, with the maximum increase again occurring at a dose of 100 mg pyrazole/kg body weight (Fig. 16).

B-2.1 Dose-Response of 4-Methylpyrazole on the Magnitude of Binding Spectra with Dimethyl Sulfoxide, 2-Butanol and Ethanol

Similar experiments were performed with microsomes from 4-methylpyrazole treated rats. When rats were treated for 3 days, there was a dose-dependent increase in the binding spectrum with DMSO, 2-butanol and ethanol when results were expressed as change in O.D. (measured from peak to trough)/ mg microsomal protein (Fig. 17). When results were expressed as the change in O.D./nmol cytochrome P-450, there was no increase in the ethanol-binding spectrum as the dose of 4-methylpyrazole was elevated as compared to saline controls (Fig. 18). It is interesting to note that these results are similar to results found with ethanol and 2-butanol metabolism, i.e., there was an increase in alcohol metabolism when rates are expressed per mg protein but not when results are expressed per nmol cytochrome P-450 (Fig. 6 and 7). In addition, the DMSO and 2-butanol binding spectrum displayed different responses to the increasing concentrations of 4-methylpyrazole, with the former showing a maximal increase at lower doses of 4-methylpyrazole, e.g., 100 mg per kg body weight, whereas the latter binding spectrum increased as the concentration of 4-methylpyrazole increased (Fig. 18).

Figure 17

The Effect of 4-Methylpyrazole Concentration on the Magnitude of Substrate Binding Spectra in Microsomes.

Rats were treated for 3 days with the indicated concentrations of 4-methylpyrazole and binding spectra were obtained as described in "Materials and Methods". Results are expressed as the change in O.D./mg microsomal protein. Wavelengths utilized were: ethanol, 413-388; DMSO, 419-385; 2-butanol, 430-410. Results are the mean of 3-4 experiments.

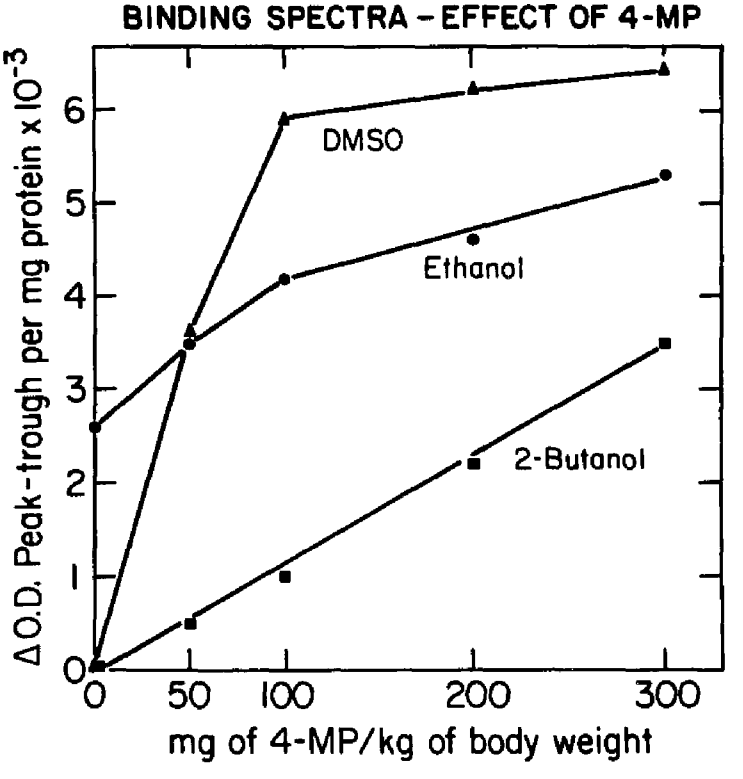
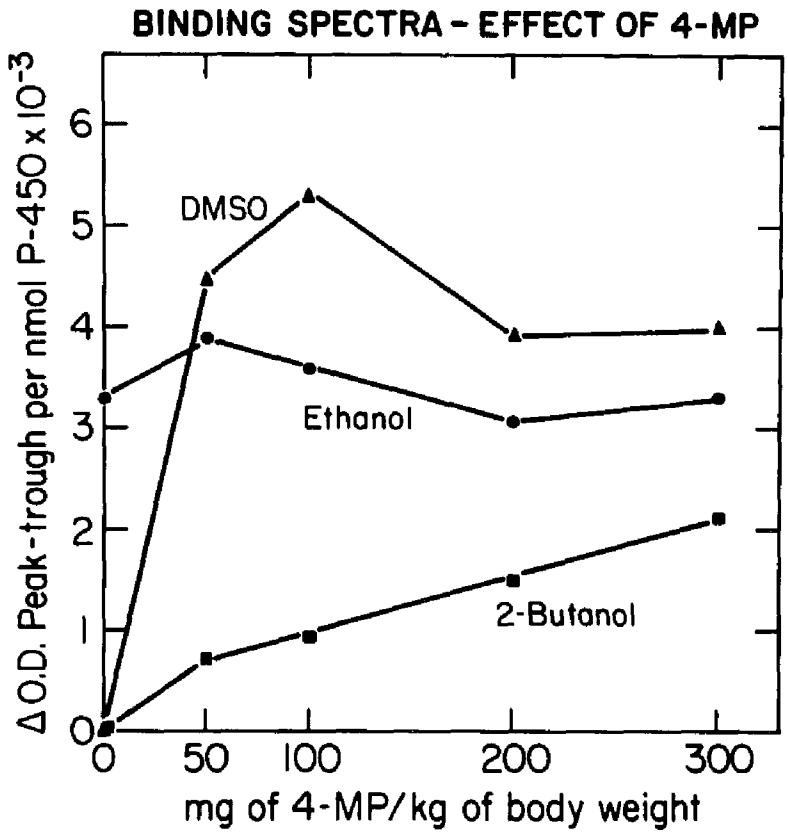

BINDING SPECTRA - EFFECT OF 4-MP
ΔO.D. Peak-trough per mg protein x 10^{-3}
0
1
2
3
4
5
6
DMSO
Ethanol
2-Butanol
0
50
100
200
300
mg of 4-MP/kg of body weight

Figure 18

The Effect of 4-Methylpyrazole Concentration on the Magnitude of Substrate Binding Spectra in Microsomes.

Rats were treated for 3 days with the indicated concentrations of 4-methylpyrazole and binding spectra were obtained as described in "Materials and Methods". Results are expressed as the change in O.D./nmol cytochrome P-450. Wavelengths utilized were: ethanol, 413-388; DMSO, 419-385; 2-butanol, 430-410. Results are the mean of 3-4 experiments.


BINDING SPECTRA - EFFECT OF 4-MP
ΔO.D. Peak-trough per nmol P-450 x 10-3
0
1
2
3
4
5
6
DMSO
Ethanol
2-Butanol
0
50
100
200
300
mg of 4-MP/kg of body weight

B-3 Pyrazole and 4-Methylpyrazole Binding Spectrum

Pyrazole *in vitro*, was shown to bind to microsomes from control rats to produce a type II binding spectrum, with a peak at 430 nm and a trough at 394 nm (131). Furthermore, the magnitude of the binding spectral change produced when pyrazole binds to microsomes was enhanced in microsomes isolated from rats chronically fed ethanol (147). Considerably less is known about the *in vitro* interactions of 4-methylpyrazole with hepatic microsomes. In view of the various interactions of pyrazole with hepatic microsomes, a study was carried out to evaluate whether 4-methylpyrazole can interact with hepatic microsomes and if there is an increase in the interaction of pyrazole or 4-methylpyrazole with microsomes isolated from rats treated with either pyrazole or 4-methylpyrazole.

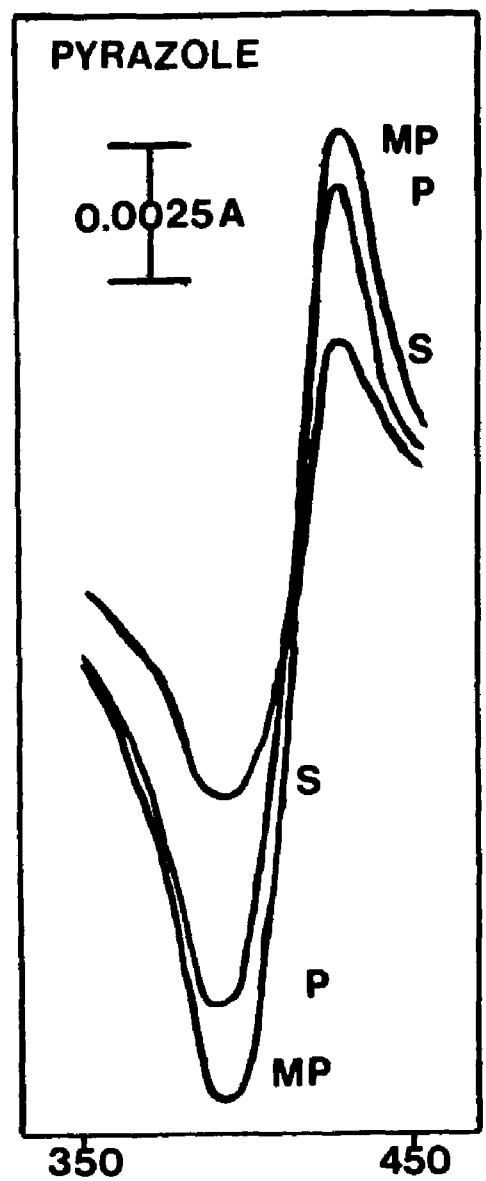
B-3.1 The Effect of Pyrazole and 4-Methylpyrazole Treatment on Pyrazole Binding Spectrum in Hepatic Microsomes

A typical spectrum for pyrazole interacting with microsomes from saline controls is shown in Fig. 19, and confirms the previous results of Rubin et al (131). Figure 19 shows that the magnitude of this spectral change is increased in microsomes isolated from rats treated with either pyrazole of 4-methylpyrazole.

Figure 19

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Binding of Pyrazole

Binding spectrum of pyrazole (final concentration, 2.67 mM) with microsomes (approximately 2 mg/6 ml) from saline (S), pyrazole (P) (200 mg/kg body weight/day for 2 days), or 4-methylpyrazole (MP) (200 mg/kg body weight/day for 3 days)-treated rats. Binding spectra were obtained as described in "Material and Methods". Results from a typical spectrum are shown.


PYRAZOLE
0.0025A
MP
P
S
S
P
MP
350
450

B-3.2 The Effect of Pyrazole and 4-Methylpyrazole Treatment on 4-Methylpyrazole Binding Spectrum in Hepatic Microsomes

Similar experiments were carried out with 4-methylpyrazole. Figure 20 shows that 4-methlypyrazole reacts with microsomes from control (saline-treated) rats to produce a type II binding spectrum with a peak at 429 nm and a trough at 392 nm. These wavelengths are similar to those for pyrazole binding to microsomes. The magnitude of the spectral change associated with 4-methylpyrazole interacting with cytochrome P-450 was about 3-fold greater than that found with pyrazole (note the difference in the scales in Fig. 19 and 20). The greater extent of 4-methyl-pyrazole binding probably reflects its greater hydro-phobicity properties. Analogous to results with pyrazole, the magnitude of the 4-methylpyrazole binding spectrum was also increased in microsomes isolated from rats treated with either pyrazole or 4-methylpyrazole (Fig. 20).

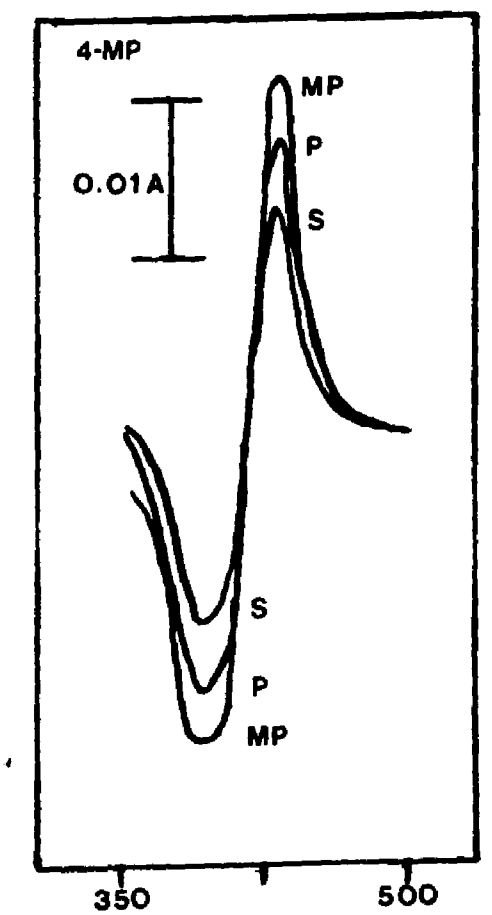
B-3.2 The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Kinetics of Binding of Pyrazole and 4-Methylpyrazole to Microsomes.

Varying concentrations of pyrazole or 4-methylpyrazole were added to microsomes from the saline-, pyrazole-, or 4-methylpyrazole treated rats and the magnitude of the binding spectrum, as reflected by the change in absorbance (429-492 nm), was determined. With all three preparations

Figure 20

The Effect of Pyrazole and 4-Methylpyrazole Treatment on Microsomal Binding of 4-Methylpyrazole

Binding spectrum of 4-methylpyrazole (final concentration, 2.67 mM) with microsomes (approximately 2 mg/6 ml) from saline (S), pyrazole (P) (200 mg/kg body weight/day for 2 days), or 4-methylpyrazole (MP) (200 mg/kg body weight/day for 3 days)-treated rats. Binding spectra were obtained as described in "Material and Methods". Results from a typical spectrum are shown.


4-MP
MP
P
S
0.01A
S
P
MP
350
500

of microsomes, the magnitude of the spectral change increased as the concentration of added pyrazole or 4-methylpyrazole was increased over the range of 0.33 mM to 2.67 mM. A Hanes-Wolfe plot of these data was linear with both pyrazole and 4-methylpyrazole as the substrates (Fig. 21 and 22, respectively) and the kinetic constants were calculated from linear regression analyses. Spectral dissociation constants (Ks) and maximal spectral changes (Vs) from the change in O.D. (429-392 nm) were calculated from these plots and are summarized in Table X. The Ks values for pyrazole and 4-methylpyrazole binding to microsomes from saline-treated rats were the same, 0.32 mM, whereas maximal binding values per mg microsomal protein of per nmol P-450 were two-fold higher with 4-methylpyrazole as the substrate compared to pyrazole. Treatment with pyrazole caused a lowering of the Ks values for both pyrazole and 4-methylpyrazole, as well as an almost two-fold increase in the maximal binding values for both substrates (Table X). Treatment with 4-methylpyrazole lowered the Ks value for 4-methylpyrazole but not for pyrazole as the substrate. Maximal binding values for both substrates were doubled when results were expressed as change in O.D./mg microsomal protein, but no such changes were observed when the spectral changes were expressed per nmol cytochrome P-450 (Table X).

Figure 21

Hanes-Wolfe Plot of the Effect of Pyrazole and 4-Methylpyrazole Treatment on Hanes-Wolfe Plots of Pyrazole Binding to Hepatic Microsomes

Hanes-Wolfe plots were constructed from the spectral changes associated with the binding of pyrazole to microsomes isolated from rats treated with either saline (S), pyrazole (P), or 4-methylpyrazole (MP). Varying concentration of pyrazole (0.33-1.67 mM) were added to microsomes, and the change in absorbance (429-392 nm) was determined. Results are from 3 experiments.

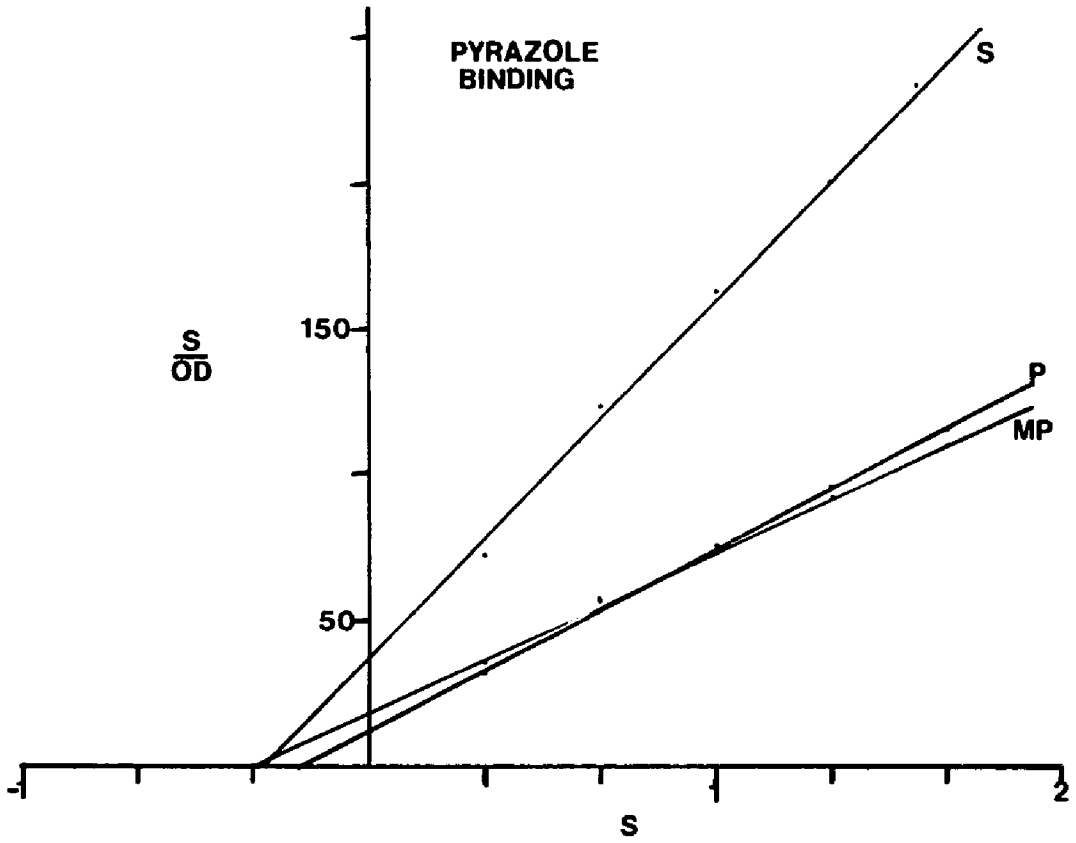

PYRAZOLE
BINDING
S
P
MP
150
50
S
OD
S
2

Figure 22

Hanes-Wolfe Plot of the Effect of Pyrazole and 4-Methylpyrazole Treatment on Hanes-Wolfe Plots of 4-Methylpyrazole Binding to Hepatic Microsomes

Hanes-Wolfe plots were constructed from the spectral changes associated with the binding of 4-methylpyrazole to microsomes isolated from rats treated with either saline (S), pyrazole (P), or 4-methylpyrazole (MP). Varying concentration of 4-methylpyrazole (0.33-2.67 mM) were added to microsomes, and the change in absorbance (429-392 nm) was determined. Results are from 3 experiments.

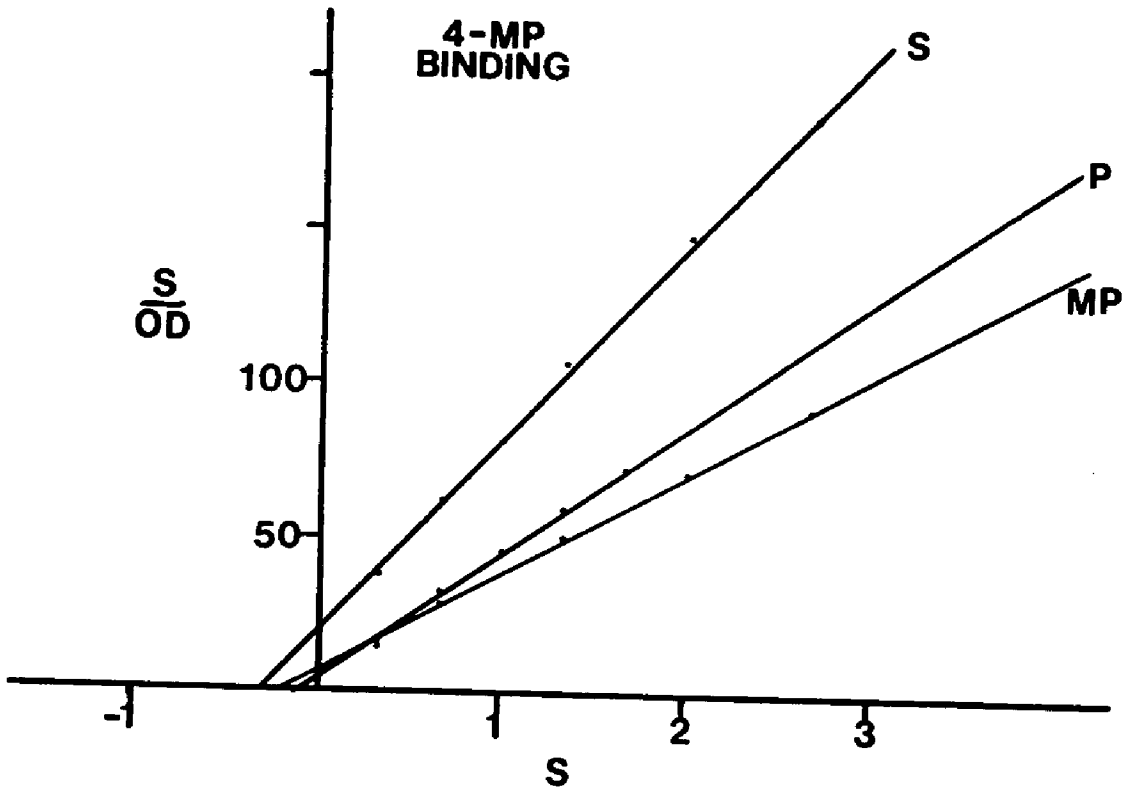

4-MP
BINDING
S
P
MP
S/OD
100
50
-1
1
2
3
S

Table X

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Binding Constants for the Binding of Pyrazole and 4-Methylpyrazole to Microsomes

parameter	Saline	Pyrazole	4-Methylpyrazole
PYRAZOLE BINDING			
Ks (mM)	0.320±0.024	0.202±0.023(a)	0.333±0.031
Vs (per mg protein)	0.0083±0.000	0.0169±0.002(b)	0.0179±0.001(b)
Vs (per nmol P-450)	0.0123±0.004	0.0244±0.002(b)	0.0099±0.002
4-METHYLPYRAZOLE BINDING			
Ks (mM)	0.319±0.019	0.116±0.009(b)	0.223±0.009(c)
Vs (per mg protein)	0.0165±0.001	0.0244±0.000(a)	0.0310±0.001(b)
Vs (per nmol P-450)	0.0219±0.002	0.0372±0.002(b)	0.0187±0.0005

The parameters were calculated from linear regressions of Hanes-Wolfe plots of the data shown in Figures 21 and 22. The content of cytochrome P-450 was about 0.70 nmol/mg microsomal protein for the saline and pyrazole treated, and about 1.7 nmol/mg microsomal protein for the 4-methylpyrazole treated

(a)-$p < 0.01$

(b)-$p < 0.001$

(c)-$p < 0.005$

C. Inhibition of Microsomal Ethanol Oxidation by Pyrazole, 4-Methylpyrazole and DMSO

The *in vitro* interactions of pyrazole and 4-methylpyrazole with liver microsomal alcohol and drug oxidations, especially after induction with inducers, have not been well characterized. *In vitro*, pyrazole and 4-methylpyrazole were shown to inhibit the metabolism of ethanol and aminopyrine (116,130). The current experiments were carried out to evaluate the *in vitro* effects of pyrazole and 4-methylpyrazole on the oxidation of ethanol by microsomes from control rats as well as rats treated with pyrazole or 4-methylpyrazole, to characterize the kinetics of inhibition by these agents, and to attempt to correlate their inhibitory effectiveness with the efficiency of binding to cytochrome P-450 in the microsomes.

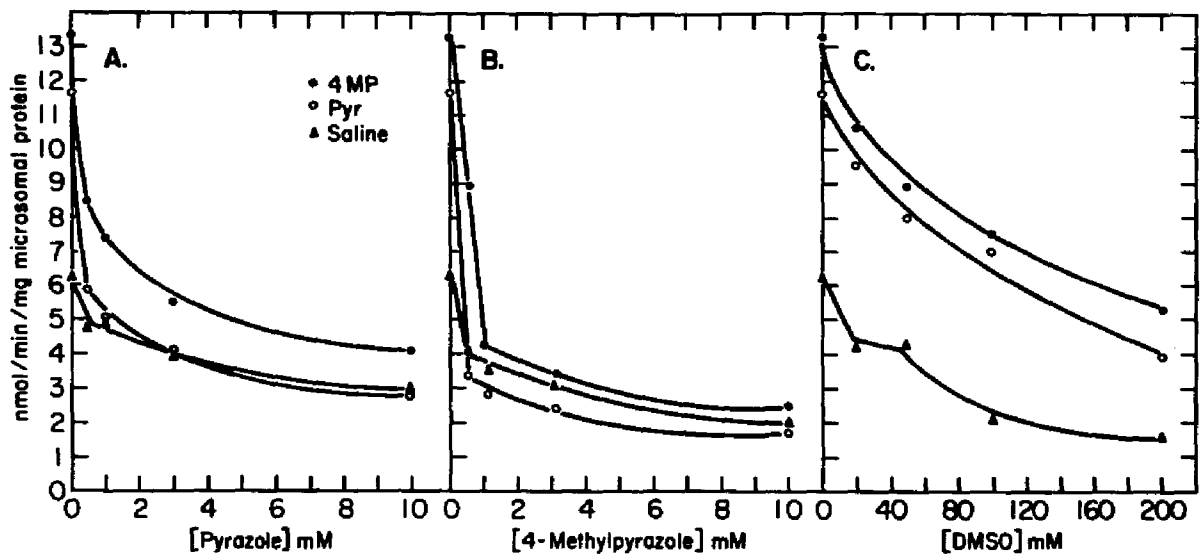
C-1 Dose Response of Inhibition of Microsomal Ethanol Oxidation by the In-Vitro Addition of Pyrazole, 4-Methylpyrazole or DMSO

At a substrate concentration of 55 mM, ethanol was oxidized at a rate of six to seven nmol per min per mg microsomal protein by control microsomes and as described above (Sect. A) this rate was increased about two-fold after treating rats with either pyrazole or 4-methylpyrazole. A dose response curve for pyrazole inhibition of microsomal ethanol oxidation is shown in Fig. 23A.

Figure 23

Dose Response of Inhibition of Microsomal Ethanol Oxidation by the In-Vitro Addition of Pyrazole, 4-Methylpyrazole or DMSO

Inhibition of microsomal ethanol oxidation by the *in vitro* addition of varying concentrations of (A) pyrazole, (B) 4-methylpyrazole and (C) DMSO. The oxidation of 55 mM ethanol by microsomes from rats treated with either saline, pyrazole (200 mg/kg body weight/day for 2 days) or 4-methylpyrazole (200 mg/kg body weight/day for 3 days) was assayed as described in "Materials and Methods" in the presence of the indicated concentrations of pyrazole, 4-methylpyrazole or DMSO. Results are from 2-4 experiments.


A.
B.
C.
• 4MP
○ Pyr
▲ Saline
nmol/min/mg microsomal protein
[Pyrazole] mM
[4-Methylpyrazole] mM
[DMSO] mM

Microsomes from rats treated with pyrazole appear to be especially sensitive to inhibition by added pyrazole as there was an initial rapid decrease in activity such that most of the increase in ethanol oxidation was blocked by low concentrations of the inhibitor. Microsomes isolated from rats treated with 4-methylpyrazole also appear to be very sensitive to inhibition by added pyrazole as there was an initial rapid decrease in ethanol oxidation at low concentrations of pyrazole, followed by a less pronounced decrease at higher concentrations of pyrazole. The latter, which suggests resistant to inhibition of ethanol oxidation, resembles the pattern observed with microsomes from saline treated animals at all concentrations of pyrazole (Fig. 23A). One possible explanation for these results could be the presence of at least two populations of cytochrome P-450 isozymes; one that is very sensitive to inhibition by pyrazole, and one that is not especially sensitive to inhibition by pyrazole.

Figure 23B shows similar types of experiments with 4-methylpyrazole added as the in vitro inhibitor of microsomal ethanol oxidation. In all three preparations, but especially in those from rats treated with pyrazole or 4-methylpyrazole, there was an initial rapid inhibition of microsomal ethanol oxidation, followed by a less sensitive phase. The increase in ethanol oxidation produced by prior treatment of rats with pyrazole of 4-methylpyrazole was completely lost in the presence of low levels of 4-methyl-

pyrazole, analogous to results with pyrazole as the *in vitro* inhibitor.

For comparative purposes, the effects of dimethylsulfoxide on microsomal ethanol oxidation were also studied. It has been shown that DMSO interacted with microsomes from ethanol-treated animals, but not microsomes from control animals, to produce a modified type II spectral change (146). In microsomes from pyrazole- or 4-methylpyrazole-treated rats, but not microsomes from saline controls, DMSO produced a modified type II spectral change (Sect. B). DMSO was shown to be a very effective inhibitor of microsomal ethanol oxidation in liver microsomes isolated from imidazole-treated rabbits as compared to other inducers (148). In rabbits, imidazole and ethanol appear to induce the same cytochrome P-450 (149). The above results suggest that DMSO may have a special interaction with the cytochrome P-450 induced by alcohol or alcohol-like inducers (imidazoles, pyrazoles) and could, therefore, be a more effective inhibitor of cytochrome P-450-catalyzed reaction in these induced preparations.

Experiments were carried out employing DMSO added as the *in vitro* inhibitor of ethanol oxidation. Figure 23C shows that much higher concentrations of DMSO, as compared to pyrazole or 4-methylpyrazole, were required to inhibit microsomal ethanol oxidation. In contrast to results with

pyrazole or 4-methylpyrazole, the inhibition by DMSO was the same in all three preparations, i,e., prior treatment with pyrazole or 4-methylpyrazole did not produce a population of cytochrome P-450 isozymes with ethanol oxidizing activity that was especially sensitive to inhibition by DMSO. Indeed, at all concentrations of DMSO tested, the rate of ethanol oxidation by microsomes from rats treated with either pyrazole or 4-methylpyrazole remained higher than the rates by microsomes from saline controls.

C-2 Kinetics of Inhibition of Microsomal Ethanol Oxidation

To evaluate the kinetics of inhibition by pyrazole, 4-methylpyrazole and DMSO, the effects of these agents on the oxidation of varying concentrations of ethanol was studied in microsomes isolated from rats treated with saline, pyrazole or 4-methylpyrazole. In all experiments, DMSO was employed at a concentration of 50 mM. However, in view of the increased effectiveness of pyrazole and 4-methylpyrazole as inhibitors of microsomal ethanol oxidation, lower concentrations of pyrazole (0.25 and 0.5 mM) and 4-methylpyrazole (0.15 and 0.50 mM) were utilized in experiments with microsomes isolated from rats treated with these agents, than with microsomes from saline controls (1 and 3 mM of either pyrazole or 4-methyl-

Table XI
The Effect of the In-Vitro Addition of Pyrazole, 4-Methylpyrazole or DMSO on the Microsomal Oxidation of Varying Concentrations of Ethanol

Addition	Rate of Oxidation of Ethanol (nmol/min/mg protein) Concentration of Ethanol (mM)			
	11	27.5	55	110
SALINE MICROSOMES				
none	3.69±0.11	5.29±1.08	7.24±0.63	8.96±0.42
1 mM pyrazole	2.07±0.37	3.30±0.29	4.61±6.03	6.03±0.77
3 mM pyrazole	1.37±0.12	2.02±0.28	3.73±0.82	5.82±0.80
1 mM 4-MP	1.54±0.17	2.68±0.35	3.80±0.54	5.26±0.53
3 mM 4-MP	1.06±0.14	2.12±0.24	3.34±0.84	4.83±0.84
50 mM DMSO	1.56±0.43	2.82±0.37	3.87±0.59	5.30±0.35
PYRAZOLE MICROSOMES				
none	6.16±0.70	8.46±0.16	11.47±0.31	13.41±0.64
0.25 mM pyrazole	3.51±0.43	5.12±0.24	7.83±0.25	8.86±0.33
0.50 mM pyrazole	2.88±0.08	4.06±0.21	5.84±0.29	6.90±0.31
0.15 mM 4-MP	1.73±0.26	2.82±0.24	3.97±0.42	4.97±0.72
0.50 mM 4-MP	1.11±0.24	2.08±0.27	3.62±0.40	4.54±0.38
50 mM DMSO	2.44±0.45	4.98±0.39	7.10±0.95	8.37±0.72
4-METHYLPYRAZOLE MICROSOMES				
none	8.36±1.13	10.37±0.63	13.84±0.38	18.21±0.92
0.25 mM pyrazole	5.64±0.36	8.98±0.86	11.14±0.64	15.00±1.38
0.50 mM pyrazole	4.55±0.29	6.68±0.62	9.34±1.32	12.61±0.73
0.15 mM 4-MP	3.41±0.53	4.45±0.85	6.60±1.26	8.00±1.13
0.50 mM 4-MP	2.40±0.43	4.24±0.45	6.17±0.93	8.28±1.27
50 mM DMSO	4.57±0.17	7.41±0.73	10.08±0.88	13.44±1.00

The oxidation of ethanol was assayed as described in "Material and Methods". Experiments were carried out in the presence of the indicated concentrations of pyrazole, 4-methylpyrazole (4-MP) and DMSO added to microsomes isolated from rats treated with saline, pyrazole (200 mg/kg body weight/day for 2 days) or 4-methylpyrazole (200 mg/kg body weight/day for 3 days). The concentration of ethanol was varied from 11 to 27.5 to 55 to 110 mM. Results are from 3 experiments.

pyrazole). Results are summarized in Table XI. The oxidation of ethanol increased as the concentration of ethanol was increased over the range 11 to 110 mM in all three microsomal preparations (saline, pyrazole- and 4-methylpyrazole-treated). In the absence of any inhibitors, the rate of microsomal ethanol oxidation was higher in the microsomes from pyrazole- or 4-methyl-pyrazole-treated than microsomes from saline controls at all four concentrations of ethanol employed (Table XI - no additions). The presences of pyrazole or 4-methylpyrazole or DMSO caused an inhibition of microsomal ethanol oxidation at all ethanol concentrations studied in all three preparations. Pyrazole and 4-methylpyrazole, but not DMSO, were more effective inhibitors of ethanol oxidation in microsomes isolated from rats treated with pyrazole or 4-methylpyrazole than from saline controls (Table XI, note different concentrations of pyrazole and 4-methylpyrazole utilized).

To determine the kinetics of inhibition, the data shown in Table XI was plotted as Lineweaver-Burk plots. The reciprocal plots in the absence or presence of inhibitors were linear over the ethanol concentration range of 11 to 110 mM. The correlation coefficients ranged from 0.94-1.00 with a mean of 0.98. The kinetic values are summarized in Table XII. The kinetics of inhibition by pyrazole and 4-methylpyrazole were complex, as the inhibitors increased

Km values for ethanol while lowering the Vmax values in all three preparations. The kinetics of inhibition by DMSO although mixed, appeared to have a large competitive component (Table XII). Since all three inhibitors appeared to show mixed-type of inhibition, Ki values were estimated according to the equation (150):

$$Ki = \frac{\left[\frac{[I]}{a}\right]\left[\frac{Km^*}{Km}\right] - 1}{1 - \left[\frac{Km^*}{Km}\right]}$$

Km* - to the apparent Km in the presence of inhibitor. The values for "a" were estimated from the Lineweaver-Burk plots by graphically solving for the abscissa coordinate of the point of intersection of the control rate and the rate in the presence of the inhibitor; this coordinate value is equal to -1/aKm (150). The Ki values are summarized in the last column of Table XII. With microsomes from saline-treated rats, pyrazole had a Ki value of about 1.1 mM; this value was decreased about three fold in microsomes from either pyrazole- or 4-methylpyrazole treated rats. The Ki values for 4-methylpyrazole were about 0.7, 0.03 and 0.1 in microsomes from saline, pyrazole- and 4-methyl-pyrazole treated rats, respectively (Table XII). Thus, 4-methylpyrazole was a better inhibitor than pyrazole was, and was especially effective after in vivo treatment

Table XII
Kinetic Constants for the Inhibition of Microsomal Ethanol Oxidation by Pyrazole, 4-Methylpyrazole and DMSO

Treatment	Addition	Kinetic Value Km (mM)	Vmax (nmol/min/mg)	Ki (mM)
SALINE				
	none	18	9.6	-
	1 mM pyrazole	26	6.8	0.97
	3 mM pyrazole	40	6.2	1.22
	1 mM 4-MP	33	6.2	0.56
	3 mM 4-MP	66	7.4	0.80
	50 mM DMSO	36	6.6	27.1
PYRAZOLE				
	none	15	14.3	-
	0.25 mM pyrazole	21	10.1	0.25
	0.50 mM pyrazole	26	7.5	0.40
	0.15 mM 4-MP	27	5.9	0.04
	0.50 mM 4-MP	58	6.9	0.02
	50 mM DMSO	46	12.8	20.7
4-METHYLPYRAZOLE				
	none	12	17.3	-
	0.25 mM pyrazole	21	16.3	0.36
	0.50 mM pyrazole	28	13.2	0.39
	0.15 mM 4-MP	16	8.2	0.08
	0.50 mM 4-MP	37	10.4	0.12
	50 mM DMSO	26	15.2	34.5

The kinetic constants were derived from linear regressions of Lineweaver-Burk plots of the data shown in Table XI.

with pyrazole or 4-methylpyrazole. By contrast, the Ki values for DMSO appeared to be similar with all three microsomal preparations (Table XII).

D. Comparison with Other Inducers

The in vitro interactions of pyrazole and 4-methylpyrazole with hepatic microsomes isolated from either saline-, pyrazole- or 4-methylpyrazole-treated rats has been characterized above. The results appear to indicate that pyrazole and 4-methylpyrazole can interact with microsomes as well as inhibit microsomal ethanol oxidation. Furthermore, these interactions are increased after pyrazole or 4-methlypyrazole treatment, e.g. Ks of 4-methylpyrazole were lowered and the inhibitory effectiveness of both compounds were increased after pyrazole or 4-methylpyrazole treatment. The current experiments were carried out to evaluate the in vitro interactions of pyrazole and 4-methylpyrazole with microsomes isolated from rats induced with other drugs, such as phenobarbital, 3-methylcholanthrene and ethanol, and to compare the effects of the latter treatments to the effects produced by pyrazole and 4-methylpyrazole treatment.

D-1 The Effects of Phenobarbital and 3-Methylcholanthrene Treatment on the Kinetics of Binding of Pyrazole and 4-Methylpyrazole to Microsomes.

Rats were treated with phenobarbital, corn oil and 3-methylcholanthrene and the effects on the content of cytochrome P-450 was determined. Whereas corn oil had no effect on the total content of cytochrome P-450, phenobarbital and 3-methylcholanthrene resulted in a two- to three-fold increase in the total content of cytochrome P-450. The content of cytochrome P-450 were 0.57 ± 0.02, 1.53 ± 0.15 and 1.63 ± 0.12 nmol/mg microsomal protein for corn oil-, phenobarbital, and 3-methylcholanthrene-treated, respectively. Furthermore, treatment with 3-methylcholanthrene resulted in a shift of the carbon monoxide difference spectrum from 450 nm to 447 nm, indicative of the cytochrome P-448 that is induced by 3-methylcholanthrene.

Pyrazole and 4-methylpyrazole bound to microsomes from corn oil-, phenobarbital- and 3-methylcholanthrene-treated rats, resulting in a type II spectra similar to that described for microsomes from saline-, pyrazole- or 4-methylpyrazole-treated rats. Varying concentrations of pyrazole or 4-methylpyrazole were added to microsomes from phenobarbital-, corn oil-, or 3-methylcholanthrene-treated rats and the magnitude of the binding spectrum, as reflected by the change in absorbance (peak-trough), was

determined. With all three preparations of microsomes, the magnitude of the spectral change increased as the concentration for pyrazole or 4-methylpyrazole was increased over the range of 0.33 mM to 1.67 mM. A Hanes-Wolfe plot of these data was linear (correlation coefficients ranged from 0.984-1.000 with a mean of 0.996±0.001) and the kinetic constants were calculated from linear regression analyses. Spectral dissociation constants (Ks) and maximal spectral changes (Vs) were calculated from these plots and are summarized in Table XIII. The Ks values for pyrazole and 4-methylpyrazole binding to microsomes from corn oil treated rats were nearly the same, 0.387 mM and 0.337 mm, respectively and are almost identical with the Ks values from saline treated microsomes (compare Tables X and XIII). Analogous to results obtained with saline microsomes, the Vs values per mg microsomal protein or per nmol cytochrome P-450 from microsomes isolated from corn oil-treated rats were two-fold higher with 4-methylpyrazole as a substrate compared to pyrazole. Treatment with 3-methylcholanthrene or phenobarbital caused an increase in the Ks values for pyrazole. Phenobarbital treatment had no effect on the Ks for 4-methylpyrazole, whereas, 3-methylcholanthrene treatment caused a lowering of the Ks value for 4-methylpyrazole binding. The Vs values per mg microsomal protein were increased two to three-fold for both substrates by the phenobarbital and 3-methyl-

Table XIII

The Effect of Phenobarbital and 3-Methylcholanthrene Treatment on the Binding Constants for the Binding of Pyrazole and 4-Methylpyrazole to Microsomes

	Treatment		
parameter	Phenobarbital	Corn Oil	3-Methylcolanthrene
PYRAZOLE BINDING			
Ks (mM)	1.139	0.387±0.076	0.516±0.079
Vs (per mg protein)	0.0185	0.0077±0.0004	0.0240±0.0026
Vs (per nmol P-450)	0.0122	0.0136±0.0007	0.0148±0.0014
4-METHYLPYRAZOLE BINDING			
Ks (mM)	0.319	0.337±0.083	0.168±0.017
Vs (per mg protein)	0.0323	0.0151±0.0003	0.0383±0.0052
Vs (per nmol P-450)	0.0211	0.0266±0.0006	0.0236±0.0030

Varying concentrations of either pyrazole or 4-methylpyrazole (0.33-1.67 mM) were added to microsomes and the change in absorbance (peak at 429-426 nm - trough at 392-390 nm) was determined. Hanes-Wolfe plots were constructed from the spectral changes associated with the binding of either pyrazole or 4-methylpyrazole and the kinetic parameters were calculated by linear regression. The content of cytochrome P-450 was 1.53, 0.57, and 1.63 nmol/mg microsomal protein for the phenobarbital, corn oil and 3-methylcholanthrene treated, respectively. Results are from 2 (phenobarbital) to 4 (corn oil and 3-methylcholanthrene) experiments.

cholanthrene treatments, however, no such changes were observed when the Vs values were expressed per nmol cytochrome P-450 (Table XIII).

D-1.1 The Effects of Chronic Ethanol Consumption on the Kinetics of Pyrazole and 4-Methylpyrazole Binding to Microsomes.

Similar experiments were performed with rats that were chronically fed ethanol and their pair-fed controls. The Ks value for pyrazole binding in pair-fed control rats was more than two-fold higher than observed with other control rats (compare Tables X and XIII to XIV), 0.897 mM, whereas the Vs values were nearly the same as the other controls whether results are expressed per mg microsomal protein or per nmol cytochrome P-450 (Table XIV). The Ks value for 4-methylpyrazole binding, 0.269 mM, was lower when compared to the Ks for pyrazole in the pair-fed controls (Table XIV); however, when the results are compared to the other controls (Table X, saline; Table XIII, corn oil) there was little or no difference. Analogous to results obtained with both saline and corn oil control microsomes, the Vs values per mg microsomal protein or per nmol cytochrome P-450 were nearly two-fold higher with 4-methylpyrazole as a substrate compared to pyrazole (Table XIV). Chronic ethanol consumption resulted in a lowering of the Ks values for both pyrazole and 4-methylpyrazole, as well as a

Table XIV
The Effect of Chronic Ethanol Consumption on the Binding Constants for the Binding of Pyrazole and 4-Methylpyrazole to Microsomes

parameter	Pair-Fed	Alcohol-Fed
PYRAZOLE BINDING		
Ks (mM)	0.897 ± 0.117	0.149 ± 0.031
Vs (per mg protein)	0.0108 ± 0.0003	0.0259 ± 0.0047
Vs (per nmol P-450)	0.0165 ± 0.0022	0.0189 ± 0.0034
4-METHYLPYRAZOLE BINDING		
Ks (mM)	0.269 ± 0.032	0.147 ± 0.019
Vs (per mg protein)	0.0175 ± 0.0005	0.0382 ± 0.0053
Vs (per nmol P-450)	0.0265 ± 0.0039	0.0279 ± 0.0035

Varying concentrations of either pyrazole or 4-methylpyrazole (0.33-1.67 mM) were added to microsomes and the change in absorbance (peak at 429-426 nm - trough at 392-390 nm) was determined. Hanes-Wolfe plots were constructed from the spectral changes associated with the binding of either pyrazole or 4-methylpyrazole and the parameters were calculated by linear regression analyses. The content of cytochrome P-450 was 0.679 ± 0.099 and 1.371 ± 0.094 nmol/mg microsomal protein for the pair-fed and alcohol-fed, respectively. Results are from 3 experiments.

greater than two-fold increase in the Vs for both substrates when the results are expressed per mg microsomal protein (Table XIV). When the maximal binding values were expressed per nmol cytochrome P-450 these changes were not observed. Hence, ethanol treatment, but not phenobarbital or 3-methylcholanthrene treatment, increased the affinity for both pyrazole and 4-methylpyrazole

D-2 The Effects of Phenobarbital and 3-Methylcholanthrene Treatment on the Inhibition of Microsomal Ethanol Oxidation by Pyrazole and 4-Methylpyrazole

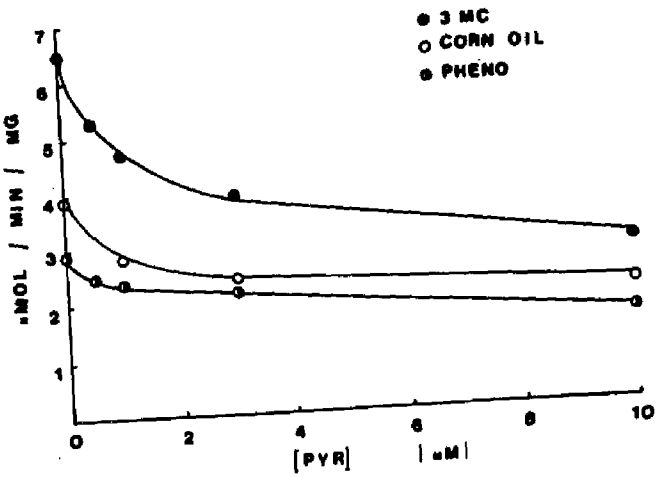
At a substrate concentration of 55 mM, ethanol was oxidized at a rate of 2.9, 3.9 and 6.5 nmol per min per mg microsomal protein in microsomes isolated from phenobarbital-, corn oil-, and 3-methylcholanthrene-treated rats, respectively (Fig. 24). These rates are lower than those found with microsomes isolated from pyrazole- or 4-methylpyrazole-treated rats. Indeed, these rates correspond to values of 1.9, 6.8, and 4.0 nmol per min per nmol cytochrome P-450, respectively; hence, phenobarbital and 3-methylcholanthrene actually lower the turnover number for microsomal oxidation of ethanol. A dose response curve for pyrazole inhibition of microsomal ethanol oxidation is shown in Figure 24A. In contrast to microsomes isolated from pyrazole- or 4-methylpyrazole-treated rats, neither of the three preparations were especially sensitive to

Figure 24

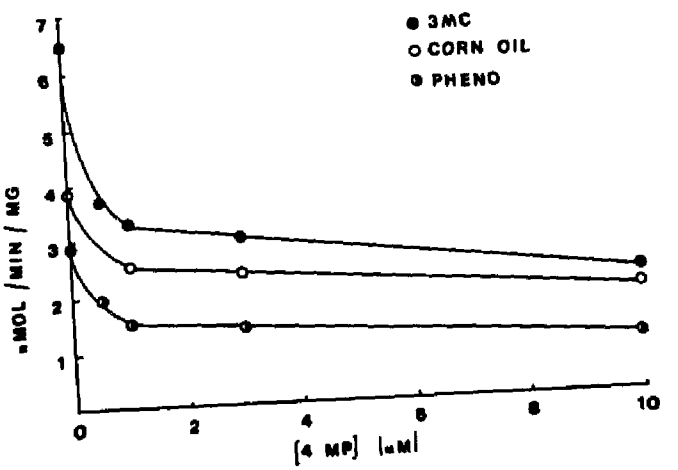
The Effect of Phenobarbital and 3-Methylcholanthrene Treatment on the Dose Response of Inhibition of Microsomal Ethanol Oxidation by the In-Vitro Addition of Pyrazole and 4-Methylpyrazole

Inhibition of microsomal ethanol oxidation by the *in vitro* addition of varying concentrations of (A) pyrazole and (B) 4-methylpyrazole. The oxidation of 55 mM ethanol by microsomes from rats treated with either phenobarbital, corn oil or 3-methylcholanthrene was assayed as described in "Materials and Methods" in the presence of the indicated concentrations of pyrazole or 4-methylpyrazole. Results are from 3 experiments.

A


● 3 MC
○ CORN OIL
● PHENO
nMOL / MIN / MG
[PYR] (uM)

B


● 3MC
○ CORN OIL
● PHENO
nMOL /MIN / MG
[4 MP] (uM)

inhibition by pyrazole, as there was only slight decreases in ethanol oxidation at all concentrations of the inhibitor (Fig. 24A). In this regard, their pattern of inhibition resembled the pattern that was observed with microsomes from saline treated rats (compare with Fig. 23A)

Figure 24B shows similar types of experiments with 4-methylpyrazole added as the in vitro inhibitor of microsomal ethanol oxidation. In all three preparations, methylpyrazole was a more effective inhibitor of ethanol oxidation than pyrazole. All three preparations showed an initial decrease in microsomal ethanol oxidation at low concentrations of 4-methylpyrazole followed by a phase that was less sensitive to inhibition (Fig. 24B). Similar to results with pyrazole as an inhibitor, neither of the three preparations appeared to be uniquely sensitive to inhibition by 4-methylpyrazole

D-2.1 The Effects of Chronic Ethanol Consumption on the Inhibition of Microsomal Ethanol Oxidation by Pyrazole and 4-Methylpyrazole

For comparative purposes, the effects of chronic ethanol consumption on the inhibition of microsomal ethanol oxidation by the in vitro addition of pyrazole or 4-methyl-pyrazole was also studied. Microsomes isolated from rats chronically fed ethanol oxidized ethanol at rats four-fold higher than dextrose controls (Table XV, no inhibitor

Table XV

The Effect of Chronic Ethanol Consumption on the Dose Response of Inhibition of Microsomal Ethanol Oxidation by the In Vitro Addition of Pyrazole or 4-Methylpyrazole

Addition	Alcohol-Fed		Pair-Fed	
	rate	% Inhibition	rate	% inhibition
PYRAZOLE				
none	13.1	-	2.7	-
0.25 mM	8.9	-32	-	-
0.50 mM	7.3	-44	2.8	+4
1.00 mM	6.8	-48	2.7	-0
3.00 mM	N.D.	N.D.	1.9	-30
4-METHYLPYRAZOLE				
0.25 mM	5.6	-57	2.3	-15
0.50 mM	5.2	-60	2.2	-19
1.00 mM	4.1	-68	2.1	-22
3.00 mM	N.D.	N.D.	2.05	-24

Inhibition of microsomal ethanol oxidation by the *in vitro* addition of either pyrazole or 4-methylpyrazole. The oxidation of 55 mM ethanol by microsomes from either ethanol-fed or their pair fed controls was assayed as described in "Materials and Methods" in the presence of the indicated concentration of either pyrazole or 4-methyl-pyrazole. Results are from 2-3 experiments.

rates). Chronic ethanol consumption also resulted in a two-fold increase in the content of cytochrome P-450 (see Table XIV, legend)

Microsomes isolated from rats chronically fed ethanol appeared to be particularly sensitive to inhibition by either pyrazole or 4-methylpyrazole: the addition of low concentration of either pyrazole or 4-methylpyrazole resulted in a rapid decrease in ethanol oxidation (Table XV). By contrast, microsomes from the pair-fed controls were very resistant to inhibition by either pyrazole or 4-methylpyrazole. Nevertheless, in both preparations 4-methylpyrazole was a better *in vitro* inhibitor of microsomal ethanol oxidation than pyrazole, as has been found for other preparations evaluated in this thesis. Thus, analogous to results with pyrazole and 4-methylpyrazole, treatment with ethanol results in a microsomal preparation whose MEOS activity is very sensitive to inhibition by added pyrazole or 4-methylpyrazole.

E. Metabolism of Pyrazole by Microsomes

Compounds which interact (bind), induce and inhibit cytochrome P-450 function, are often metabolized by cytochrome P-450 as well, e.g., phenobarbital and ethanol. The major product of pyrazole metabolism is found in the urine after *in vivo* administration of pyrazole 4-hydroxy-pyrazole (see introduction): this suggest the possibility

that pyrazole may be metabolized via hydroxylation. Since hydroxylation represents a primary mode of action of cytochrome P-450, it was of interest to see if pyrazole is metabolized via cytochrome P-450 and if pyrazole treatment would result in induction of its own metabolism.

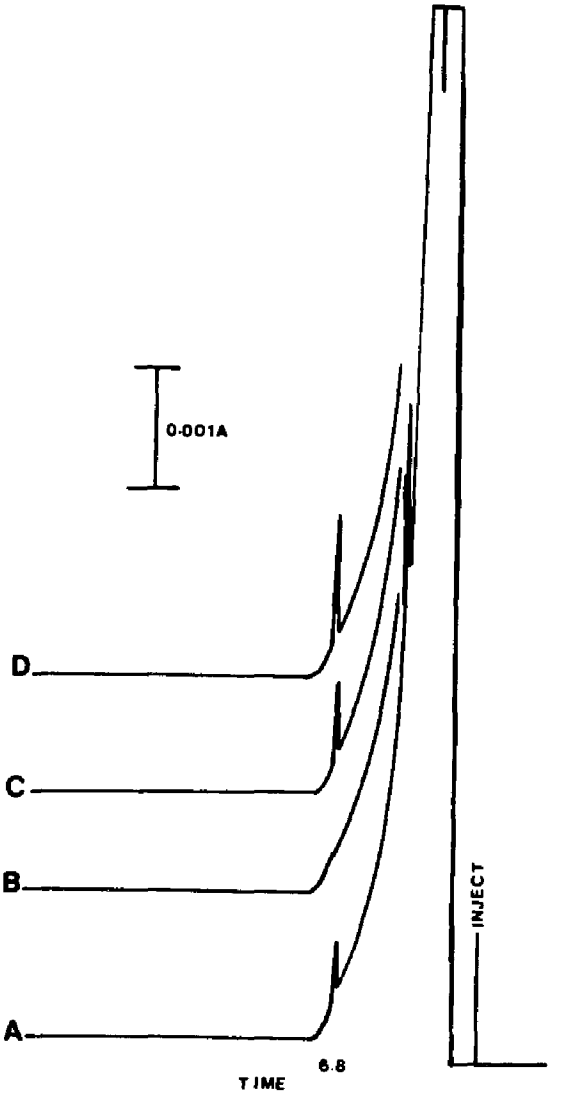
E-1 Pyrazole is Metabolized by Microsomes

Since preliminary in vivo results showed that pyrazole is metabolized to 4-hydroxypyrazole; therefore, a high pressure liquid chromatograghy (HPLC) procedure was devised to separate 4-hydroxypyrazole from the other components of a microsomal drug reaction system. Under extremely mild conditions, i.e., 100% H_2O, 4-hydroxypyrazole poorly interacted with C_{18} type columns, and eluted near the void volume of the column. Since pyrazole is positivity charge at pH values of less than 12, it was considered that the use of a negatively charged paired ion (paired-ion chromatography) would aid in the separation by chromatography. Using 5 mM octanesulfonic acid under acidic conditions (1% acetic acid pH 3.5), 4-hyrodrxy-pyrazole eluted at 4-10 minutes, depending upon the concentration of acetonitrile used. Figure 25A shows a HPLC profile of a microsomal drug reaction mixture that was allowed to run for 10 minutes without substrate (pyrazole). The reaction was terminated with PCA, followed by the addition of pyrazole to a final concentration of 1.0 mM.

Figure 25

HPLC Profile of the Separation of 4-Hydroxypyrazole from the Components of a Microsomal Drug Oxidizing System.

4-hydroxypyrazole was separated from the components of a microsomal drug oxidizing system as described in "Materials and Methods". A) Reaction system was allowed to run for 10 minutes without substrate (pyrazole), terminated, pyrazole was added to a final concentration of 1 mM, and 10 ul of the prepared supernatant was co-injected with 0.5 ul of 0.1 mM 4-hydroxypyrazole and the absorbance was monitored at 254 nm. B) 10 ul injection of the prepared supernatant from a "zero-time" control: reaction system was allowed to run for 10 minutes without substrate (pyrazole), terminated, pyrazole was added to a final concentration of 1 mM. C) 10 ul injection of the prepared supernatant from a 10 minute reaction using saline microsomes. D) 10 ul injection of the prepared supernatant from a 10 minute reaction using saline microsomes, co-injected with 0.5 ul of 0.1 mM 4-hydroxypyrazole.


0·001A
D
C
B
A
INJECT
6.8
TIME

10 ul of supernatant, which was prepared as described in "Material and Methods" was co-injected with 0.5 ul of 0.1 mM 4-hydroxypyrazole. Under conditions employing 10% acetonitrile, 1% glacial acetic acid and 5 mM octanesulfonic acid, sodium salt, 4-hydroxypyrazole had a retention time of 6.8 minutes. Figure 25B and C show a typical profile for a "zero"-time control and a sample after a 10 minute reaction, respectively. These results show that pyrazole is metabolized to a product which co-elutes with standard 4-hydroxypyrazole (Fig. 25D). Of importance is the lack of peak splitting of the metabolite plus standard 4-hydroxypyrazole profile when both are mixed together, indicating identical co-elution. This was observed under a variety of other solvent conditions.

E-2 The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Metabolism of Pyrazole

The effect of pyrazole or 4-methylpyrazole treatment on the oxidation of pyrazole was determined and the results are summarized in Table XVI. Since the product co-eluted with 4-hydroxypyrazole under several conditions, the product formation was compared to standard curves of 4-hydroxypyrazole. In the saline controls, pyrazole was oxidized at a rate of 0.143 nmol of product per minute per mg of microsomal protein. Pyrazole treatment resulted in 2.5-fold increase in pyrazole metabolism and 4-methyl-

Table XVI

The Effect of Pyrazole and 4-Methylpyrazole Treatment on the Microsomal Oxidation of Pyrazole

	Treatment		
	Saline	Pyrazole	4-Methylpyrazole
A)	0.14 ± 0.03	0.38 ± 0.05	0.63 ± 0.09
B)	0.24 ± 0.06	0.71 ± 0.11	0.40 ± 0.08

Rats were treated with either saline or with 200 mg/kg body weight/day of either pyrazole or 4-methylpyrazole for 2 or 3 days, respectively. Rats were fasted overnight prior to sacrificing. The oxidation of pyrazole was assayed as described in "Material and Methods". Experiment A refer to nmol product per minute per mg microsomal protein. Experiment B refers to nmol product per minute per nmol cytochrome P-450: the results in experiment A were divided by the respective content of cytochrome P-450 for each experiment. The results are from 3-4 experiments.

pyrazole treatment resulted in a greater than 3.5-fold increase in activity when results are expressed per mg microsomal protein (Table XVI, exp.A).

The above data was recalculated by dividing by the content of cytochrome P-450 in order to express the results as turnover numbers (nmol/min/nmol cytochrome P-450). Table XVI shows that pyrazole treatment resulted in nearly three-fold increase in the turnover number for the oxidation of pyrazole, whereas, 4-methylpyrazole treatment resulted in about a two-fold increase in the turnover number for pyrazole metabolism (Table XVI, exp.B).

E-3 Characterization of Pyrazole Metabolism by Microsomes

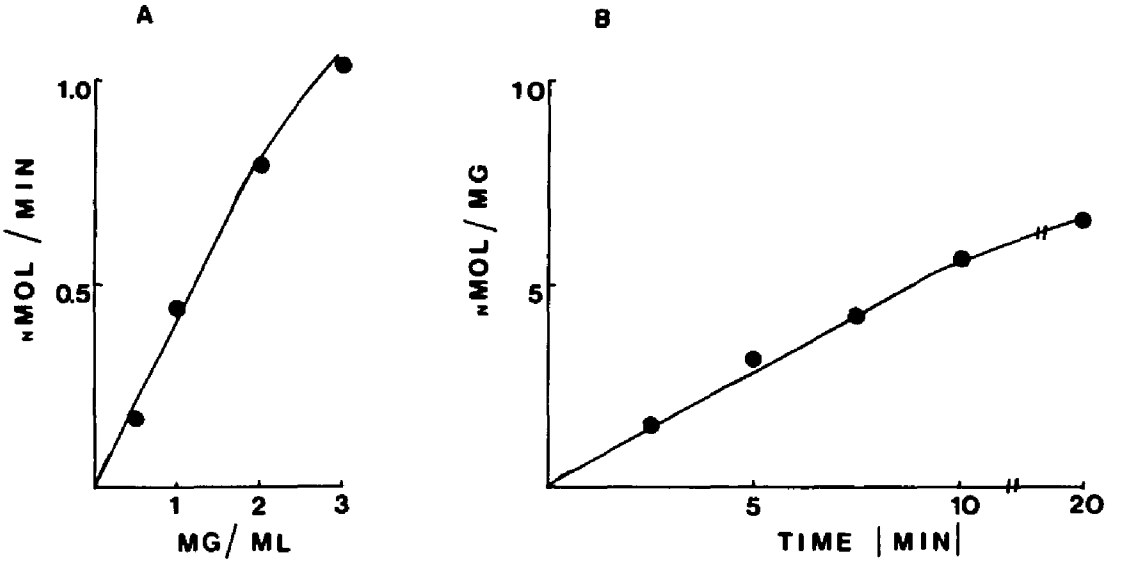
Since uninduced microsomes metabolized pyrazole at a rate that was near the limit of detection, characterization of microsomal oxidation of pyrazole was carried out in microsomes isolated from pyrazole treated rats. Figure 26A shows that pyrazole metabolism was linear with respect to protein up to 2 mg microsomal protein per ml of reaction mixture. Figure 26B shows that pyrazole metabolism was linear for at least 10 minutes. Analogous results were found with microsomes from methylpyrazole treated rats.

Cytochrome P-450 reactions are usually NADPH-dependent and are sensitive to inhibition by carbon monoxide as well as by substrates which would compete for cytochrome P-450. Therefore experiments were carried out to evaluate if NADH

Figure 26

Time course and protein curve of the oxidation of pyrazole by liver microsomes from pyrazole treated rats.

Microsomes were isolated from rats treated with pyrazole (200 mg/kg body weight/day for 2 days). The oxidation of pyrazole (final concentration, 1 mM) was assayed as described in material and methods. Reactions were either carried out of 10 minutes varying the concentration of protein (A), or varying lengths of time at constant protein (approximately 2mg/ml). Results are the mean of 2 experiments.


A
1.0
0.5
NMOL / MIN
1
2
3
MG/ ML
B
10
5
NMOL / MG
5
10
20
TIME | MIN|

could support pyrazole metabolism and if carbon monoxide or competitive substrates would inhibit pyrazole metabolism.

Results in Table XVII show that NADH supported pyrazole metabolism; however, the rate was about 25% that of the NADPH-dependent rate. It has previously been shown that the combination of NADH and NADPH is often synergistic towards the oxidation of certain drugs (151). The combination of NADH and NADPH was found to metabolize pyrazole at a rate 20% greater than the sum to the two individual rates (Table XVII). The *in vitro* addition of aniline resulted in inhibition of pyrazole metabolism; furthermore, pyrazole metabolism was sensitive to inhibition by carbon monoxide compared to nitrogen controls. The *in vivo* administration of ethanol simultaneously with pyrazole was shown to result in an inhibition of pyrazole metabolism *in vivo* (135). Results in Table XVII show that ethanol inhibited pyrazole metabolism in a dose dependent manner. It is of interest that a concentration as low as 1 mM produced inhibition of pyrazole metabolism by microsomes.

E-3.1 Kinetics of Microsomal Pyrazole Metabolism

Figure 27 shows plots of the rate of microsomal pyrazole oxidation as a function of the concentration of pyrazole over the range of 0.1 to 2.0 mM. Typical saturation kinetics were observed for microsomes isolated

Table XVII
The Effect of NADH and Cytochrome P-450 Inhibitors on the Microsomal Oxidation of Pyrazole

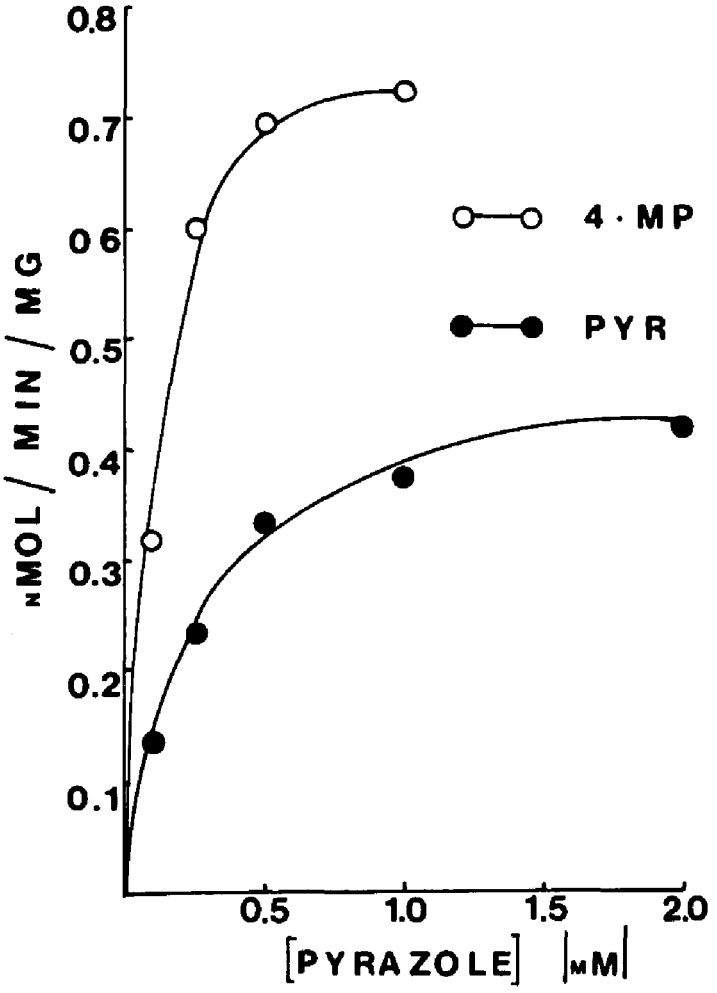
	Additions	Rate (nmol product/ min/mg microsomal protein
A)	NADPH (CONTROL)	0.396
	NADH (-NADPH)	0.099
	NADH + NADPH	0.594
	Aniline (0.5 mM)	0.198
	10 ml Carbon Monoxide (33%)	0.240
	10 ml Nitrogen (33%)	0.420
	20 ml Carbon Monoxide (66%)	0.150
	20 ml Nitrogen (66%)	0.410
B)	Control	0.312 ± 0.026
	1.0 mM Ethanol	0.215 ± 0.039
	5.0 mM Ethanol	0.175 ± 0.025
	10 mM Ethanol	0.109 ± 0.035
	50 mM Ethanol	0.078 ± 0.029

The oxidation of pyrazole by microsomes isolated from pyrazole treated rats was assayed as described in "Materials and Methods". Unless otherwise indicated, all reactions contained a NADPH generating system. NADH was added to a final concentration of 1.0 mM. In experiment A, the final concentration of pyrazole was 1.0 mM and in experiment B, 0.5 mM. Results are from 2 (exp. A) to 3 (exp. B) experiments.

Figure 27

The oxidation of pyrazole by rat hepatic microsomes as a function of the concentration of pyrazole.

Microsomes were isolated from rats either treated with pyrazole or 4-methylpyrazole (200mg/kg body weight/day) for 2 or three days, respectively. Reaction were carried out for 10 minutes, varying the concentration of pyrazole, and were assayed as described in "Materials and Methods". Results are from 3 experiments.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
nMOL / MIN / MG
4 · MP
PYR
0.5
1.0
1.5
2.0
[PYRAZOLE] |mM|

from either pyrazole- of 4-methylpyrazole treated rats. The relative kinetic constants for pyrazole metabolism were determined by linear regression analysis of Hanes-Wolfe plots of the data shown in Figure 27. Hanes-Wolfe plots were linear, with correlation coefficients ranging from, 0.98 to 1.00 with a mean of 0.99 ± 0.003. Microsomes from pyrazole and 4-methylpyrazole treated rats had Km values for pyrazole of 0.285 ± 0.048 and 0.136 ± 0.063 mM, respectively, and Vmax values of 0.478 ± 0.08 and 0.733 ± 0.116 nmol product/mg microsomal protein, respectively.

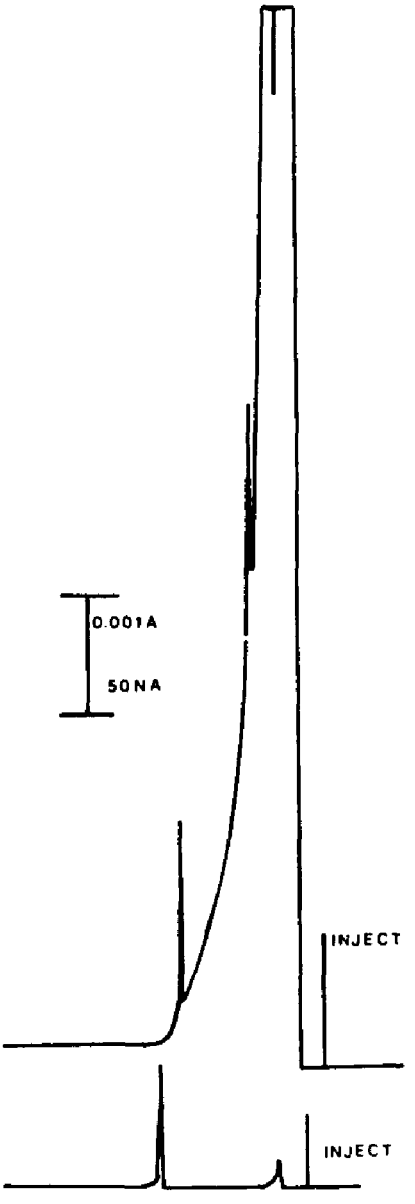
E-4 Electrochemical Detection of 4-Hydroxypyrazole

The data presented in this thesis have shown that pyrazole is metabolized by microsomes in a concentration-, protein-, and a time-dependent manner. The product of pyrazole metabolism, as assayed by this HPLC technique, coelutes with 4-hydroxypyrazole under several conditions and it was reasoned that this product was indeed 4-hydroxypyrazole. However, it was felt that additional evidence was needed to substantiate this hypothesis. Since the absorbance of 4-hydroxypyrazole profoundly drops above 254 nm, and the acetic acid in the buffer interferes at lower wavelengths, typical ratios of absorbance at two wavelengths could not be used. Therefore, an electrochemical detector was used. Figure 28 shows the simultaneous U.V. and electrochemical detection of the

Figure 28

HPLC profile of the microsomal oxidation of pyrazole using U.V. and electrochemical detection simultaneously.

The microsomal oxidation of pyrazole was carried out as described in "Material and Methods". 10 ul of sample (from a reaction system using microsomes isolated from a pyrazole treated rat) was injected and was simultaneously monitored with a U.V. detector (upper trace) and an electrochemical detector (lower trace) with an applied voltage of 0.9 volts.


0.001A
50NA
INJECT
INJECT

metabolite of microsomal pyrazole metabolism. By varying the applied voltage, one can use electrochemical detection to show that both the metabolite and the sample have the same electrochemical characteristics and therefore are the same compound. Figure 29 shows a plot of n-amps (peak height) as a function of applied voltage of a "zero-time" control spiked with 4-hydroxypyrazole and a sample from a 10 minute time point. Figure 29 clearly shows that both the standard and the product of microsomal pyrazole oxidation have the same electrochemical characteristics. Recent studies have shown that use of electrochemical detection plots such as these are very sensitive indices for verification by HPLC of the identity of metabolite to standard (152).

E-5 The effect of Classical Cytochrome P-450 inducers on the Metabolism of Pyrazole by Microsomes

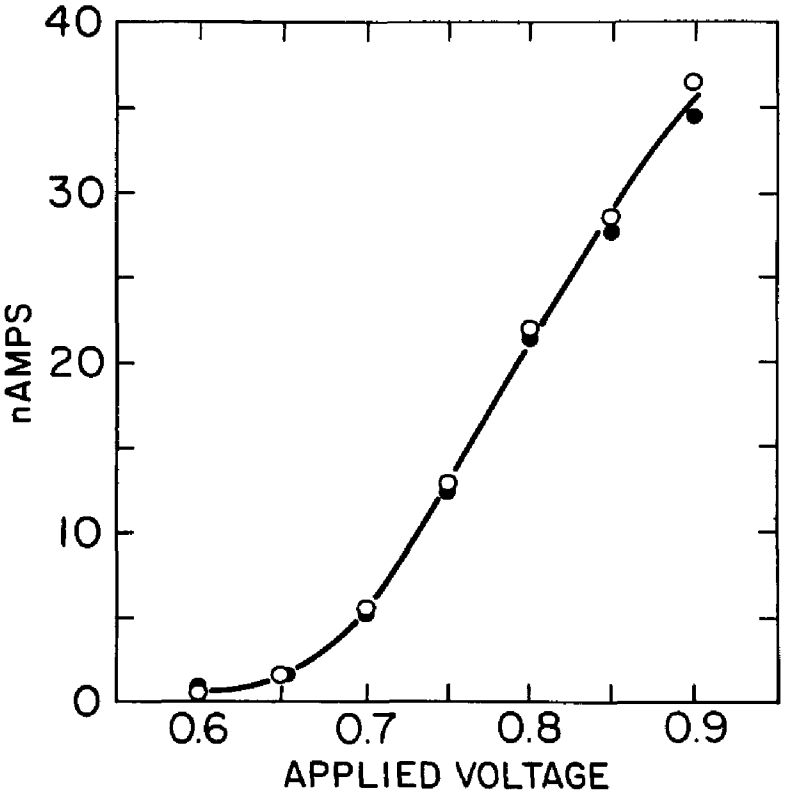
The above results have shown that pyrazole is metabolized by microsomes in a cytochrome P-450 dependent manner, and that microsomes isolated from rats that were treated with pyrazole or 4-methylpyrazole showed two- to three-fold higher rates of metabolism. It was of interest to see if other inducers of cytochrome P-450 would induce pyrazole metabolism.

Rats were treated with saline, phenobarbital, corn oil,

Figure 29

The Electrochemical Characteristics of 4-Hydroxypyrazole and the Product of Microsomal Pyrazole Oxidation.

A plot of the electrochemical detection of a "zero-time" spiked with 4-hydroxypyrazole (O-O) and the product of pyrazole metabolism (●-●), as a function of the applied voltage.


40
30
20
10
0
nAMPS
0.6
0.7
0.8
0.9
APPLIED VOLTAGE

or 3-methylcholanthrene and the rate of microsomal pyrazole oxidation was determined. The rate of microsomal pyrazole metabolism for saline controls of rats not treated were identical. Results in Table XVIII show that pyrazole is not a good substrate for the phenobarbital induced cytochrome P-450, since these rates expressed per mg microsomal protein were 35% less than saline microsomes. Moreover, when rates are expressed per nmol cytochrome P-450, the rates with microsomes from phenobarbital treated animals were 70% lower than saline animals. 3-Methyl-cholanthrene treatment resulted in a 3.5-fold increase in microsomal pyrazole oxidation over rates found with corn oil controls. However, this increase was due to the increase in total cytochrome P-450 content; when the results are expressed per nmol cytochrome P-450, there were no differences between corn oil- and 3-methylcholanthrene-treated. Treatment of rats with corn oil resulted in a 50% increase in the rate of microsomal pyrazole oxidation as compared to saline controls (Table XVI) whether results are express per mg microsomal protein or per nmol cytochrome P-450. It is of interest that such results emphasize the need for appropriate controls, e.g., corn oil, saline or pair-fed.

Similar experiments were performed with microsomes isolated from rats chronically fed alcohol. Table XIX shows that pyrazole metabolism was linear for at least 10 minutes

Table XVIII
The Effect of Phenobarbital and 3-Methylcholanthrene Treatment on Microsomal Pyrazole Oxidation

	Treatment		
	Phenobarbital	Corn oil	3-Methylcolanthrene
A)	0.089 ± 0.014	0.215 ± 0.064	0.708 ± 0.191
B)	0.068 ± 0.004	0.371 ± 0.112	0.411 ± 0.078

Microsomes were isolated from either phenobarbital-, corn oil or 3-methylcholanthrene treated animals. The metabolism of pyrazole was assayed as describe in "Material and Methods" using a U.V. detector. Experiment A refers to nmol/min/ mg microsomal protein and experiment B refers to nmol/min/nmol cytochrome P-450. Results are from 3-4 experiments.

in microsomes from dextrose or alcohol treated animals. Chronic consumption of alcohol resulted in a four-fold increase in microsomal pyrazole oxidation over pair-fed contols. The oxidation of pyrazole was sensitive to inhibition by carbon monoxide and competitive substrates of cytochrome P-450 function.

Table XIX
The Effect of Chronic Ethanol Consumption on Microsomal Pyrazole Oxidation

	Addition	Treatment: Alcohol	Treatment: Dextrose
A)	2.5 minutes	1.91 ± 0.47	-
	5.0 minutes	4.06 ± 0.92	0.79 ± 0.13
	10 minutes	8.09 ± 0.87	2.19 ± 0.11
B)	carbon monoxide (33%)	5.22 ± 1.27	1.03 ± 0.22
	20 mM Ethanol	2.16	1.09 ± 0.34
	100 mM Ethanol	0.75 ± 0.29	0.39 ± 0.39
	50 mM DMSO	1.48 ± 0.28	0.71 ± 0.53
	10 mM Aniline	0.96 ± 0.36	0.23 ± 0.03

Microsomal pyrazole oxidation was assayed as describe in "Material and Methods" using U.V. detection. Experiment A refer to nmol/mg microsomal protein, and experiment B refers to nmol/mg microsomal protein/10 minutes. Results are from 3 experiments.

CHAPTER IV SUMMARY AND DISCUSSION

Pyrazole and especially 4-methylpyrazole are widely used to inhibit alcohol dehydrogenase and, thus, block metabolic effects which are dependent on the oxidation of ethanol. These agents are also useful in the treatment of methanol (112) and ethylene glycol (113,114) poisoning. Pyrazole has been shown to influence the mixed-function oxidase system dependent oxidation of several drugs (116,126,128,129), including ethanol (116,130). Results in this thesis show that 4-methylpyrazole also can affect the mixed-function oxidase system, but several of the effects of 4-methylpyrazole treatment are different than those found with pyrazole. One of the more striking differences between pyrazole and 4-methylpyrazole treatment is the effect on the content of cytochrome P-450. Whereas pyrazole treatment has no effect (quantitatively), 4-methylpyrazole treatment results in a 2-fold increase in the content of cytochrome P-450 (Table II). This increase in the content of cytochrome P-450 can be observed after a single dose of 100 mg/kg body weight of 4-methylpyrazole (Table III). Neither pyrazole nor 4-methylpyrazole treatment has any effect on the activity of NADPH-cytochrome P-450 reductase (Table II). Thus, pyrazole treatment had no quantitative effect on the two major enzymes which comprise the mixed function oxidase system, whereas

4-methylpyrazole treatment results in the induction of cytochrome P-450, without a corresponding increase in the activity of NADPH-cytochrome P-450 reductase.

Pyrazole treatment results in an increase in the oxidation of alcohol by microsomes, whether results are expressed as nmol of product/min/mg microsomal protein or per nmol of cytochrome P-450 (Fig. 2,3). These results suggest the possibility that the pyrazole treatment may result in qualitative changes in the cytochrome P-450 isozyme composition, e.g., pyrazole may induce an alcohol-preferring cytochrome P-450 isozyme. In accordance with this possibility is the increase in the binding spectrum with ethanol as the substrate (Fig. 15), and the presence of a binding spectrum with DMSO and 2-butanol as substrates (Fig. 14). Chronic consumption of ethanol by rats is known to increase the magnitude of the binding spectrum of microsomes with ethanol (147), and to result in a microsomal binding spectrum with DMSO (82,146) and 2-butanol (23). The turnover number for aminopyrine oxidation is not increased by pyrazole treatment (Fig. 4,5): aminopyrine is not an effective substrate for the alcohol-preferring cytochrome P-450 (103). A single treatment with 200 mg of pyrazole/kg body weight increases microsomal oxidation of alcohols by 50% to 100% (Fig. 2,3). Treatment for 2 days with as little as 50 mg of pyrazole/kg body weight can also increase microsomal oxidation of alcohols by 50 to 100%

(Fig. 5). These time course or dose-response effects on alcohol oxidation are similar to the effects on binding spectra with ethanol, DMSO, and 2-butanol as substrates (Fig. 16) and suggest an association between the binding studies and the oxidation of alcohols.

The microsomal oxidation of alcohols is also increased after 4-methylpyrazole treatment; however, the effect of the 4-methylpyrazole treatment is more complex than that of the pyrazole treatment. The increase in the oxidation of alcohol appears primarily to be due to the increase in the total content of cytochrome P-450 and to a lesser extent to the induction of an alcohol-preferring cytochrome P-450, since increases are observed when results are expressed per mg microsomal protein, but are less significant when expressed per nmol cytochrome P-450 (Fig. 2,3,6,7). Analogous to the results with ethanol oxidation, the magnitude of the microsomal ethanol-binding spectrum increases with the dosage of 4-methylpyrazole when data are express per mg microsomal protein (Fig. 17), but not when expressed per nmol cytochrome P-450 (Fig. 18). In line with the above, the results in Figures 2 and 3 suggest the possibility that 4-methylpyrazole may cause some induction of the microsomal oxidation of alcohols after a single treatment, but whereas a second treatment with pyrazole is especially effective in increasing the oxidation of alcohols by microsomes, a second or a third treatment with

4-methylpyrazole appears ineffective. Perhaps 4-methylpyrazole treatment may initially induce an alcohol-preferring cytochrome P-450; however, further treatment may result in the induction of other isozymes of cytochrome P-450 which are not especially effective in oxidizing alcohols. The concomitant induction of alcohol-nonpreferring isozymes may mask a significant contribution by an alcohol-preferring cytochrome P-450. Support for some induction by 4-methylpyrazole treatment of an alcohol-preferring cytochrome P-450 may be afforded by microsomal binding spectra found when DMSO or 2-butanol are used as substrates (Fig. 14B and C). On the other hand, there may be binding spectra between DMSO and 2-butanol and other isozymes of cytochrome P-450 induced by 4-methyl-pyrazole treatment. In this regard, whereas the DMSO-binding spectrum is identical when comparing microsomes from pyrazole- and 4-methylpyrazole-treated rats (Fig. 14A and B), the 2-butanol binding spectrum is different (Fig. 14C). The different responses of the DMSO and 2-butanol binding spectra to increasing concentrations of 4-methyl-pyrazole (Fig. 18) may reflect induction of more than one isozyme of cytochrome P-450. It was therefore considered that perhaps use of lower doses of 4-methylpyrazole or shorter periods of exposure might be useful in evaluating whether an alcohol-preferring cytochrome P-450 can be induced by 4-methylpyrazole without the concomitant induction of other isozymes which may occur upon longer

treatment with 4-methylpyrazole. Treating rats with a single exposure to varying concentration of 4-methylpyrazole results in a 50% increase in the microsomal oxidation of ethanol and 2-butanol, but not aminopyrine, at the lower concentrations of 4-methylpyrazole, e.g., 50 mg/kg body weight. However, further studies were required to characterize the cytochrome P-450 isozyme(s) induced by 4-methylpyrazole treatment. Therefore, studies of the substrate specificity of the isozyme(s) induced by 4-methylpyrazole as well as the effect that 4-methylpyrazole treatment has on SDS-gel electrophoretic profiles of microsomes, and comparison of these effects to the effects that pyrazole has on these parameters was conducted.

Pyrazole increases the content of a cytochrome P-450 isozyme with a molecular weight of about 52,000 (128, Fig. 8). Associated with this is an increase in the oxidation of certain drugs, e.g., aniline, p-nitroanisole and dimethylnitrosamine at low concentrations, but not others, e.g., aminopyrine, 7-ethoxycoumarin, and dimethylnitrosamine at high concentrations whether results were expressed per mg microsomal protein or per nmol cytochrome P-450 (Tables IV,V and VI). The microsomal oxidation of ethanol, 2-butanol is also increased and there is a stereochemical preference for the (+)-2-butanol isomer over the (-)-isomer (Fig. 13). These properties are identical

to those found after chronic ethanol treatment, which increases the metabolism of ethanol itself (65), aniline (82,153), p-nitroanisole (154) and low concentrations of dimethylnitrosamine (145) and demonstrates a stereochemical preference for (+)-2-butanol (127). Drugs such as aminopyrine or 7-ethoxycoumarin, whose oxidation is not increased by pyrazole or 4-methylpyrazole are good substrates for inducers such as phenobarbital and 3-methylcolanthrene but not ethanol. Other similarities between pyrazole and ethanol treatments described include binding spectra with DMSO and 2-butanol. Taken as a whole, these results strongly suggest that in rats, pyrazole and ethanol treatments may induce similar isozymes of cytochrome P-450, and that the former may serve as a convenient model for the later. Koop et al. (149) recently reported that antibodies against the ethanol inducible cytochrome P-450 isozyme 3a of rabbit liver cross reacted with liver microsomes isolated from rabbits treated with several inducers, including imidazole and pyrazole. In rabbits, imidazole treatment also appears to induce the same cytochrome P-450 that ethanol treatment induces (149). It is noteworthy, however, that in rats, imidazole treatment was recently shown not to induce a cytochrome P-450 isozyme with properties similar to the ethanol-inducible isozyme (155), suggesting that species differences between rats and rabbits with regard to

imidazole induction. Similarly, DMSO was shown to be a very effective inhibitor of ethanol oxidation in liver microsomes isolated from imidazole-treated rats as compared to other inducers (148) but not in microsomes isolated from pyrazole treated rats (Fig. 23C). Results in this thesis suggests that, unlike imidazole, pyrazole and ethanol appear to induce similar types of cytochrome P-450 isozymes in the rat.

The oxidation of all the drugs tested, as well as alcohols, are increased per microsomal protein after 4-methylpyrazole treatment. This increase is due primarily to the increase in total content of cytochrome P-450. Since the 4-methylpyrazole treatment did not alter (or slightly increased) the content of microsomal protein per gram liver, or the liver wet weight per kg body weight (Table I), the increase in drug metabolism per mg microsomal protein would also occur when expressed per gram liver or per kg body weight. Hence, this inductive effect of 4-methylpyrazole on hepatic drug metabolism should be kept in mind in view of the potential value of this agent in treating a variety of conditions associated with the ingestion of ethanol and other alcohols.

However, the oxidation of those drugs which are not good substrates for the ethanol-inducible cytochrome P-450 increased only 50%, e.g., 7-ethoxycoumarin, aminopyrine and dimethylnitrosamine at high concentration (high Km), whereas the oxidation of those drugs that are good

substrates for the ethanol inducible isozyme increased 200-400%, e.g., ethanol, 2-butanol, p-nitroanisole, dimethylnitrosamine at low concentration and aniline. SDS-gel electrophoresis shows that 4-methylpyrazole increase the content of at least 2 cytochrome P-450 isozymes, one which migrates to the same position as the pyrazole (and ethanol) inducible isozyme, and the second (which may actually be two isozymes) to a lower molecular weight position (Fig.8). The finding that one of the isozymes appears to be similar on gels to the pyrazole and ethanol inducible isozymes correlates with certain other similarities between these three treatments, e,g., binding spectra with DMSO and 2-butanol (Fig. 14), stereochemical preference for the (+)-2-butanol isomer (Fig.13, Table IX), increased sensitivity to inhibition by pyrazole or 4-methylpyrazole (Fig. 23, Table XV), greater affinity for 4-methylpyrazole (Table X) and a increased turnover numbers for certain drug (aniline, and p-nitroanisole) (Table V). However, no increase in the turn over number is found with ethanol, (+)-2-butanol, low concentration of dimethyl-nitrosamine or increase in affinity for pyrazole is found after 4-methylpyrazole treatment, in contrast to the pyrazole treatment. The finding that 4-methylpyrazole treatment increase the content of 2 or 3 isozymes of cytochrome P-450 can probably explain these divergent effects on turnover number and affinities. It is possible

that only the 52,000 molecular weight isozyme shows high activity in metabolizing alcohol and dimethylnitrosamine and high affinity for pyrazole whereas the 48,000-50,000 molecular weight isozyme shows poor activity of affinity with these substrates. Hence, an increase of an alcohol-preferring cytochrome P-450 is compensated by enrichment of an alcohol-nonpreferring cytochrome P-450 (s). On the other hand, both the 52,000 and 48,000-50,000 (to a lesser extent) molecular weight isozymes may show good activity with substrates such as aniline and p-nitroanisole as well as have high affinity for 4-methylpyrazole (hence, specific activity and affinity increased with these substrates, respectively), and both isozymes may show poor activity with certain other substrates, e.g., aminopyrine, 7-ethoxycoumarin, dimethylnitrosamine (hence, specific activity is decreased with these substrates). Further evaluation of these will require eventual purification of the 52,000 and 48,000-50,000 molecular weight isozymes.

Results in this thesis demonstrate that in vitro, pyrazole and 4-methylpyrazole can inhibit the microsomal oxidation of ethanol, and that the inhibitory effectiveness of these agents is increased in rats treated with pyrazole, 4-methylpyrazole or ethanol (Fig. 23, Table XV). Microsomal oxidation of ethanol is increased after treatment with ethanol, pyrazole and 4-methylpyrazole (65,82,127, Fig. 2,5,6,9,23 and Tables III, VII, XII, XV).

The increased sensitivity of microsomal ethanol oxidation to inhibition by pyrazole or 4-methylpyrazole after treatment with these agents (or ethanol) probably reflects the induction of an alcohol-preferring cytochrome P-450 isozyme by the pyrazole or 4-methylpyrazole treatment. Indeed, the increased rate of microsomal ethanol oxidation produced by the pyrazole, 4-methylpyrazole or ethanol treatment is especially sensitive to in vitro inhibition by either pyrazole or 4-methylpyrazole (Fig. 23, Table XV), suggesting that the cytochrome P-450 isozyme(s) responsible for the increase in microsomal ethanol oxidation reflect the isozyme(s) which are sensitive to inhibition by pyrazole and 4-methylpyrazole. By contrast, microsomes isolated from pair-fed, saline or corn oil controls, or 3-methylcholanthrene or phenobarbital treated rats did not display an increase in sensitivity to inhibition by pyrazole or 4-methylpyrazole (Fig. 24). These results show that not all inducible cytochrome P-450 isozymes are especially sensitive to inhibition by pyrazole or 4-methylpyrazole, further suggesting that those isozymes which have high activities towards ethanol oxidation are the isozymes which display high sensitivity to these agents.

Pyrazole and 4-methylpyrazole produce type II binding spectra with the microsomes and the magnitude of the spectral changes or affinity for these compounds (or both

magnitude and affinity) increased after treatment with pyrazole, 4-methylpyrazole or ethanol (Fig. 19,20 and Tables X, XIV). These increased interactions with pyrazole and 4-methylpyrazole with cytochrome P-450 after treatment with ethanol, pyrazole or 4-methylpyrazole may explain, in part, the increased inhibitory effectiveness towards microsomal ethanol oxidation. For example, the Ki for pyrazole in inhibiting microsomal oxidation of ethanol is lowered about three-fold after pyrazole or 4-methylpyrazole treatment (Table XII). Associated with this three-fold decrease in Ki is a two fold increase in values for the maximal spectral change (Vs) associated with pyrazole binding after pyrazole or 4-methylpyrazole treatment (Table X). The Ki for 4-methylpyrazole as an inhibitor of microsomal ethanol oxidation is lowered about ten-fold after pyrazole or 4-methylpyrazole treatment. Associated with this decrease in Ki is a decrease in Ks for 4-methyl-pyrazole in microsomes from pyrazole- and 4-methylpyrazole-treated rats, as well as an increase in the Vs (Table X). Analogous to this, is the increase in sensitivity to inhibition of microsomal ethanol oxidation by pyrazole and 4-methylpyrazole found in microsomes from rats chronically fed ethanol. Associated with this, is a decrease in the Ks of these compounds, as well as an increase in the Vs for these compounds (Table XIV). By contrast, ethanol oxidation by microsomes from phenobarbital or 3-methyl-

cholanthrene treated rats, was not especially sensitive to inhibition by pyrazole. Both of the latter microsomal preparations showed an increase in the Vs; however, microsomes isolated form phenobarbital and 3-methylcolanthrene treated rats showed a decrease in their affinity for pyrazole (Table XIII). Thus, the increased inhibitory effectiveness of pyrazole and 4-methylpyrazole after treatment with pyrazole, 4-methylpyrazole or ethanol appears to be associated with increased interaction with the cytochrome P-450 isozyme(s) induced by these compounds. Recently, the possibility that 10 mM pyrazole, in the presence of NADPH, may act as a suicide inhibitor of cytochrome P-450 was suggested (156). However, under the conditions of our experiments, the combination of pyrazole plus NADPH was not any more effective than NADPH alone in causing loss of cytochrome P-450 carbon monoxide binding spectrum (about a 10% loss of the carbon monoxide binding spectrum was found after a 5 minute incubation period with microsomes from pyrazole-treated rats). No cytochrome P-420 was observed despite this 10% loss of cytochrome P-450 binding spectrum. In other experiments neither 1 mM pyrazole nor 1 mM 4-methylpyrazole had any effect on the activity of the NADPH-cytochrome P-450 reductase.

The kinetics associated with 4-methylpyrazole inhibition of microsomal ethanol oxidation appeared somewhat unusual in that, as the concentration of 4-methylpyrazole is

raised (from 1 mM to 3 mM for saline controls or from 0.15 mM to 0.5 mM in microsomes from pyrazole- or 4-methylpyrazole-treated rats), there is an increase in the Km for ethanol, but Vmax does not decrease any further (Table XII). One interpretation for these results could reflect several populations of cytochrome P-450 isozymes in the microsomal preparations which 4-methylpyrazole interacts with, e.g., lower concentrations appear to interact with one population of cytochrome P-450 isozymes and inhibit ethanol metabolism via a mixed-type of inhibition, while a higher concentration may react with another population of cytochrome P-450 isozymes and inhibit ethanol metabolism via a competitive type mechanism. That 4-methylpyrazole may interact with several populations of cytochrome P-450 can be suggested from the increase in total cytochrome P-450 content observed after 4-methylpyrazole treatment, but not after pyrazole treatment (Table II). It should be noted that rates of ethanol oxidation or Vs values for pyrazole or 4-methylpyrazole are increased when results are expressed per mg microsomal protein, but not per nmol cytochrome P-450. This, again, could reflect varying population of cytochrome P-450, with some showing good activity or affinity for ethanol and 4-methylpyrazole, while others may show poor activity or affinity for these substrates. Further evaluation of these considerations will require studies with purified isozymes.

4-Methylpyrazole is a better inhibitor of microsomal oxidation of ethanol than is pyrazole, especially after treatment with either pyrazole or 4-methylpyrazole. This could relate to increased hydrophobicity of the 4-methylpyrazole. 4-Methylpyrazole is a more potent inhibitor of alcohol dehydrogenase than pyrazole, and quantitative structure activity relationships have suggested that hydrophobic factors are most important in determining the potency of 4-substituted pyrazoles against alcohol dehydrogenase (157). It would be of interest to perform similar structure-activity relationships with regard to inhibition by pyrazole and pyrazole derivatives of microsomal oxidation of ethanol. In this context, pyrazole and 4-methylpyrazole are often utilized to assess the contribution of alcohol dehydrogenase towards the overall metabolism of ethanol. The assumption is usually made that these agents block only the ADH-dependent pathway of ethanol metabolism. However, pyrazole and 4-methylpyrazole also inhibit the microsomal pathway of ethanol oxidation. The increased sensitivity to inhibition by pyrazole or 4-methylpyrazole after treatment with these agents can be extended to an increased microsomal sensitivity to these agents after chronic ethanol consumption. Microsomes isolated from rats chronically fed the Lieber-DeCarli ethanol diet are indeed more sensitive to inhibition by pyrazole or 4-methylpyrazole than microsomes from pair-fed

controls (Table XV). There is considerable debate over the role of alcohol dehydrogenase and MEOS in contributing towards the metabolic tolerance after chronic ethanol consumption. Most studies in this area have employed pyrazole and 4-methylpyrazole to block ethanol metabolism in rats fed ethanol chronically and their pair-fed controls (66,76). In view of the increased sensitivity of microsomal oxidation of ethanol to inhibition by pyrazole or 4-methylpyrazole after chronic ethanol treatment, extreme caution would be required in the use of pyrazole or 4-methylpyrazole to assess the role of alcohol dehydrogenase-dependent and -independent (e.g., microsomal) pathways in contributing towards the metabolic tolerance associated with chronic ethanol consumption.

DMSO was shown to interact with microsomes or cytochrome P-450 form ethanol treated rats or rabbits to produce modified type II spectral change (82,146). This suggested the possibility of a special interaction between DMSO and the alcohol-inducible cytochrome P-450 isozyme. DMSO was a very effective inhibitor of ethanol oxidation by microsomes isolated from imidazole-treated rabbits (148), and this appears to reflect induction of the same cytochrome P-450 isozyme by imidazole and ethanol (149). However, in rats (unlike rabbits) imidazole treatment does not appear to induce an alcohol preferring isozyme of cytochrome P-450 (155). Since pyrazole and 4-methylpyrazole appear to be

"alcohol-like" inducers in rats, it was considered that analogous to results with imidazole, DMSO would be an especially effective inhibitor of microsomal ethanol oxidation after treatment with pyrazole or 4-methyl-pyrazole. This proved not to be the case as the Ki for inhibition by DMSO was similar in all three microsomal preparations (Table XII). Thus, although DMSO produces a binding spectrum with microsomes from pyrazole- or 4-methylpyrazole treated rats (Fig. 14) (but not with controls), the significance of this interaction is unclear since it does not result in enhanced inhibitory effectiveness by DMSO towards ethanol oxidation. There are several possibilities that could explain these results. One possibility is that although the alcohol-preferring cytochrome P-450 isozymes(s) gives a binding spectrum with DMSO, its affinity for DMSO is very low. Another possibility is that perhaps another isozyme(s) of cytochrome P-450 is induced along with the alcohol-preferring isozyme that binds DMSO; however, it does not have high activity with respect to ethanol metabolism. In any event, unlike pyrazole or 4-methylpyrazole, DMSO does not appear to be an especially effective inhibitor of the alcohol preferring cytochrome P-450 isozyme in rat liver microsomes.

Pyrazole can induce, interact (bind) and inhibit cytochrome P-450 dependent reactions. Analogous to other

compounds which can affect cytochrome P-450 functions as well as be metabolized by cytochrome P-450, e.g. ethanol and phenobarbital, pyrazole is also metabolized by microsomes in a cytochrome P-450 dependent manner. The reaction is dependent on NADPH, time and protein (microsomal) and is inhibited by substrates which compete for the active site of cytochrome P-450, e.g., ethanol and aniline. These reactions are inhibited by carbon monoxide, which competes with oxygen for one of the axial ligands of the heme in reduced cytochrome P-450 and thereby, inhibits the reaction which is dependent on oxygen (Table XVII).

An HPLC procedure to detect 4-hydroxypyrazole was developed during the course of these studies. Using paired-ion chromatograghy, the metabolite of pyrazole oxidation, 4-hydoxypyrazole, can be separated from the remaining microsomal reaction mixture components. Under the conditions described in this thesis, 4-hydroxy-pyrazole had a retention time of 6.8 minutes (Fig. 25). The product of microsomal pyrazole metabolism had an identical retention time and coeluted with the standard. Using electrochemical detection, both the standard and the product of microsomal pyrazole metabolism had the same electrochemical properties, and thus, are probably the same compound (Fig. 29). These results identify for the first time that liver microsomal cytochrome P-450 is responsible for the oxidation of pyrazole to 4-hydroxypyrazole, the

metabolite of pyrazole detected in the urine of rats treated *in vivo* with pyrazole.

Phenobarbital induced microsomes did not show enhanced rates of pyrazole metabolism, whereas, microsomes from 3-methylcholanthrene treated rats did show enhanced rates of pyrazole metabolism when results are expressed per mg microsomal protein (Table XVIII). However when the rates are expressed per nmol cytochrome P-450, the rates with microsomes from 3-methylcholanthrene treated rats were no different from those found with microsomes from rats treated with corn oil (controls). Microsomes from ethanol-, pyrazole- or 4-methylpyrazole-treated rats did display an increased rate of microsomal pyrazole oxidation whether results are expressed per mg microsomal protein or per nmol cytochrome P-450 (Tables XVI and XIX). Since these three treatments appear to result in the induction of an alcohol preferring cytochrome P-450, this suggest the possibility that pyrazole is a good substrate of the alcohol-inducible cytochrome P-450. Since pyrazole and 4-methylpyrazole have been suggested as effective agents to block the metabolic effects of ethanol and other alcohols, the increased oxidation of pyrazole by microsomes isolated from rats chronically treated with ethanol suggests that careful monitoring of the dosage of these agents, especially in alcoholics, is required in order to maintain effective levels. This analogous to the increased

oxidation of a variety of drugs observed after ethanol treatment.

Microsomes isolated from rats treated with pyrazole or 4-methylpyrazole metabolized pyrazole at rates 2.5 to 3.5 times greater than control animals. In vivo, pretreatment of rats with pyrazole was shown to accelerate the elimination of pyrazole from the blood (135). Microsomes from rats chronically fed ethanol also showed increased activity with pyrazole as the substrate (Table XIX). In should be noted that in vitro, the addition of ethanol inhibited pyrazole metabolism by microsomes (Table XVII). Analogous to this, the in vivo administration of ethanol simultaneously with pyrazole inhibited the metabolism of pyrazole to 4-methylpyrazole (135). Taken as a whole, these results suggest that cytochrome P-450 is probably responsible for the in vivo metabolism of pyrazole to 4-hydroxypyrazole and is the enzyme sensitive to ethanol inhibition of pyrazole metabolism and the induction of cytochrome P-450 is probably responsible for the enhanced in vivo metabolism of pyrazole after pyrazole treatment.

In summary, pyrazole and 4-methylpyrazole treatment can affect the hepatic microsomal mixed-function oxidase system in several ways. Pyrazole and 4-methylpyrazole treatment appear to result in the induction of an alcohol-preferring cytochrome P-450 as reflected by alcohol and drug oxidation data and binding spectra with several substrates. Pyrazole

treatment does not change the content of cytochrome P-450 or the activity of the cytochrome P-450 reductase. By contrast, treatment with 4-methylpyrazole increases the content of cytochrome P-450 about two-fold. Microsomes isolated from these treated rats exhibit several properties which are similar to microsomes isolated from rats chronically fed ethanol. This suggests the possibility that pyrazole- or 4-methylpyrazole-treatment may serve as good models to study the effects that ethanol has on the hepatic mixed-function oxidase system. Pyrazole and 4-methylpyrazole can inhibit microsomal oxidation of ethanol in vitro, and the effectiveness of these agents as inhibitors is increased in microsomes isolated from rats treated with pyrazole, 4-methylpyrazole or ethanol. Furthermore, pyrazole is metabolized by microsomes in a cytochrome P-450 dependent manner and its metabolism is increased by pyrazole-, 4-methylpyrazole or ethanol-treatment. In view of the above, extreme caution would be required in the use of pyrazole or 4-methylpyrazole to assess the role of alcohol dehydrogenase dependent and independent (e.g., microsomal) pathways in contributing towards overall metabolism of ethanol, especially in induced animals. Furthermore, pyrazole and 4-methyl-pyrazole can have similar drug interactions as ethanol, i.e., paradoxical effects with respect to metabolism, toxicity or teratogenicity of drugs and/or other foreign

substances. In the presence of pyrazole or 4-methyl-pyrazole, drug metabolism would be depressed because these agents would compete with drugs at the cytochrome P-450 level. However, in the absence of pyrazole or 4-methyl-pyrazole, but after 2 or 3 days or even a single treatment with these agents, drug metabolism (of at least certain drugs) would be increased because of the effect that these agents have on the cytochrome P-450 isozyme population. Consequently, caution should be used when these compounds are used clinically.

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