

AN ABSTRACT OF THE THESIS OF

Sharron E. LaFollette for the degree of Doctor of Philosophy in Pharmacy presented on December 21, 1988. Title: Modification of the Acute Effects of Ethanol or Acetaldehyde on Drug Metabolism by Ascorbic Acid, Thiamine, or Cysteine In Vivo.

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Abstract approved: _____

The inhibition of mixed-function oxidase (MFO) activity by ethanol or acetaldehyde was examined in vivo by monitoring changes in salivary half-life of antipyrine or aminopyrine in adult male Sprague-Dawley rats. Ethanol (1-6 g/kg, po) or acetaldehyde (4-8 mmol/kg, ip) was administered 20 minutes prior to antipyrine (100 mg/kg, ip) or aminopyrine (80 mg/kg, ip). Ethanol increased the half-life of antipyrine in a dose-dependent (0-96%) manner. The effect of acetaldehyde was minimal (23%) and not dose-dependent. Neither compound affected the half-life of aminopyrine.

L-ascorbic acid (2 mmol/kg), thiamine-HCl (0.24 mmol/kg), or L-cysteine (2 mmol/kg) was given ip 90 minutes prior to ethanol (2 g/kg, po) or acetaldehyde (6 mmol/kg, ip) which was followed in 20 minutes by antipyrine. Ascorbic acid or cysteine pretreatments effectively prevented the ethanol-increased antipyrine half-life. Other pretreatments had only a weak affect on ethanol- or acetaldehyde- increased antipyrine half-life. Cysteine did not affect acetaldehyde-increased antipyrine half-life. The data suggested a

possible direct effect of ethanol on the MFO metabolism of antipyrine.

Low concentrations of either ethanol (10 mM) or acetaldehyde (1.4 mM) did not inhibit the N-demethylation of either aminopyrine to monomethyl-4-aminoantipyrine or antipyrine to norantipyrine (NOR) in isolated rat liver microsomes. The hydroxylation of antipyrine to 3-hydroxymethylantipyrine (3 HMA) was increased by 57% and 19% respectively. Ethanol (50 mM) did not inhibit the metabolism of antipyrine as measured by the oxidation of NADPH to NADP^+ , but did result in a 20% decrease in the formation of NOR and a 24% decrease in the formation of 3 HMA. The inability of in vitro assays to quantify the 4-hydroxyantipyrine metabolite of antipyrine may explain the lack of effect by low concentrations of ethanol or acetaldehyde on in vitro antipyrine metabolism.

Modification of the Acute Effects of
Ethanol or Acetaldehyde on Drug Metabolism by
Ascorbic Acid, Thiamine, or Cysteine In vivo

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Doctor of Philosophy

Completed December 21, 1988

Commencement June 1989

APPROVED:

Redacted for privacy

Professor of Pharmacy in charge of major

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Date thesis is presented December 21, 1988

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ACKNOWLEDGEMENTS

I would like to thank my husband, Bill Martz, for his patience and support through five years of separation while I was in Oregon and he in Illinois. He provided the advise and strength of experience as well as encouragement during all of my graduate education.

To Sandy and Olvar Bergland I extend the warm hand of friendship. Their hospitality, understanding and friendship was much needed and deeply appreciated.

I would also like to thank the members of my committee, Dr. Fink, Dr. Weber, Dr. Hendricks, Dr. Constantine, and Dr. Curtis for all their help during my stay at Oregon State University.

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MODIFICATION OF THE ACUTE EFFECTS OF ETHANOL OR ACETALDEHYDE ON DRUG METABOLISM BY ASCORBIC ACID, THIAMINE, OR CYSTEINE IN VIVO.

General Introduction

Ethanol use and alcohol-associated illness are major causes of premature mortality in the United States (CDC, 1985). Reduction of morbidity and mortality associated with the misuse of alcohol is a major target for the 1990 objectives of the US Department of Health and Human Services (USDHHS, 1980). The physiological effects of ethanol ingestion vary considerably with dose, duration of administration, duration of withdrawal, and parameters measured. The stress of ethanol consumption is also influenced by many contributing factors which include nutritional status, changes in hormones, neurohormones, and changes in electrolyte balance (Seixas and Eggleston, 1976).

Alcohol is known to affect the metabolism of endogenous substances such as fatty acids, carbohydrates, amino acids, steroids, bile acids, biogenic amines and porphyrins (Gastineau et al., 1979). It is also generally recognized that ethanol influences the metabolism of foreign compounds and drugs (Rubin et al., 1970a). Some of the effects of ethanol ingestion may be due to the direct action of ethanol or its metabolites while other effects may result from the modification of metabolic pathways by ethanol as a consequence of its metabolism.

Upon absorption, ethanol distributes itself throughout total body water. There is no storage of ethanol in the body and only 2-10% of the alcohol ingested is excreted by the lungs and kidneys. Therefore most of the alcohol consumed undergoes metabolism (Gastineau et al., 1979).

The metabolism (Figure 4.1) of alcohol occurs predominately in the liver. Approximately 80% of the ethanol ingested is metabolized by alcohol dehydrogenase (ADH) in the cytosol (Thurman et al., 1972). The oxidation of ethanol to acetaldehyde is coupled with the conversion of nicotinamide adenine dinucleotide (NAD^+) to its reduced form (NADH). The resulting cellular disturbance of the redox potential is responsible for a number of secondary reactions. These include an increase in the lactate/pyruvate ratio favoring hyperlactacidemia, increased lipogenesis, preferential utilization of ethanol instead of fatty acids as a main energy fuel for the mitochondria, and as a result, decreased fatty acid oxidation (Lieber, 1979 and Isselbacher, 1977).

Two minor pathways have been proposed to account for the remaining 10-18% of ethanol metabolism. Catalase can metabolize ethanol provided that hydrogen peroxide is present (Keilin and Hartree, 1945). The contribution of catalase toward the metabolism of ethanol is generally believed to be minor due to the normally limited supply of hydrogen peroxide in the cell.

The second minor pathway, located on the smooth endoplasmic reticulum (SER), is a cytochrome P450 containing system and has been labeled the microsomal ethanol-oxidizing system (MEOS). The requirements of this system for NADPH, molecular oxygen, physiological

pH optimum, membrane lipoproteins, and its partial inhibition by carbon monoxide resemble those criteria commonly found among mixed-function oxidase (MFO) (Lieber and DeCarli, 1970). Catalase- and alcohol dehydrogenase-free reconstituted systems containing cytochrome P450 suggest that MEOS is responsible for the major part of the non-ADH ethanol metabolism (Miwa et al., 1978). Induction of MEOS also accounts for the increased rate of ethanol metabolism in the chronic alcoholic (Ohnishi and Lieber, 1977).

Many endogenous chemicals, drugs, and other xenobiotics undergo biotransformation by cytochrome P450 containing MFO systems. Ethanol is known to affect the metabolism of many of these compounds. In vitro, ethanol inhibits the MFO metabolism of many of these compounds (Moldowan, 1981; Kennedy, 1979; Rubin et al., 1970a; Pirola, 1978; and Grundin, 1975). The high ethanol concentrations required to inhibit some of the in vitro chemical metabolism suggests nonspecific alterations in membrane fluidity or protein denaturation. The significance of this inhibition in vivo is questionable.

In vivo, fewer compounds have been examined, yet ethanol does affect the pharmacokinetics and metabolism of many of those examined (Lane et al., 1985 and Rubin et al., 1970b). Initial hypothesis for ethanol-inhibited MFO drug metabolism suggest competitive inhibition of MFO metabolic pathways (Rubin et al., 1970a), cellular redox changes inhibited mitochondrial-cytosol transfer of MFO required cofactors (Ontko, 1974), and the ethanol metabolite, acetaldehyde, binding to cellular proteins (Lieber et al., 1980).

Acetaldehyde, the primary metabolite of ethanol, is in many ways more potent and toxic than alcohol itself (Windholz, 1983 and Holtzman

and Schneider, 1974). Administration of acetaldehyde has been reported to affect hepatic carbohydrate metabolism (Prasanna and Ramakrishnan, 1984), alter biogenic amine metabolism (Weiner, 1981), decrease protein synthesis (Rothschild et al., 1980) and export (Lieber et al., 1980), depress mitochondrial functions (Cederbaum and Rubin, 1977, and Cederbaum et al., 1975), and cause fatty liver (Truitt et al., 1966). Acetaldehyde may also contribute to the effect of ethanol on drug metabolism. As the product of ethanol metabolism via MEOS, acetaldehyde concentrations may reach toxic subcellular concentrations. At high concentration, acetaldehyde has been shown to inhibit in vivo p-nitroanisoie O-demethylation (Dicker and Cederbaum, 1982).

Proposed mechanisms for the inhibition of drug metabolism by ethanol have relied almost entirely on data derived from subcellular methods. These studies ignore the possible physiological significance in the whole animal. The purpose of the research in this thesis is to compare the in vivo and in vitro inhibition of drug metabolism by ethanol. Emphasis was placed on physiologically and biochemically significant concentrations of ethanol. The potential role of acetaldehyde was explored as well as dietary means of managing ethanol or acetaldehyde inhibited drug metabolism.

The research can be divided into three phases. The first phase (Chapter 1) examines the effect of ethanol or acetaldehyde on aminopyrine or antipyrine metabolism in vivo. The second phase (Chapter 2) examines whether dietary factors such as cysteine, thiamine, or ascorbic acid can block the inhibition by ethanol or acetaldehyde on antipyrine metabolism in vivo. The third phase

(Chapter 3) discusses attempts to quantify the in vitro inhibition of MFO activity by ethanol or acetaldehyde under physiologically significant concentrations.

The first set of experiments described within the context of this report was designed to study the effect of ethanol or acetaldehyde on drug metabolism in vivo. In order to evaluate the acute effect of ethanol or acetaldehyde on drug metabolism, the antipyretic drugs antipyrine and aminopyrine were selected. Both drugs are distributed in total body water with negligible binding to tissue or plasma proteins, metabolized almost completely in the liver with a low hepatic extraction ratio, and the parent compound has negligible renal elimination (Brodie and Axelrod, 1950a and 1950b).

Antipyrine elimination rate has been successfully used as a tool for evaluating the liver's ability to metabolize drugs in both man (Vesell and Passanati, 1973; Huffman et al., 1974; and Welch et al., 1975) and animals (Welch et al., 1967; Statland et al., 1973; Vesell et al., 1973; and Capel et al., 1979). Antipyrine was selected because of the availability of a noninvasive method to measure free drug concentration in the saliva (Wilson, V.L. et al., 1982 and Welch et al., 1975).

Aminopyrine was chosen because it has been a common substrate to examine MFO activity in many in vitro (LaDu et al., 1955 and Matsuyama et al., 1983) and in vivo systems (Vesell et al., 1975; Lockwood et al., 1980; and Houston et al., 1981). Likewise, the rate of aminopyrine metabolism has recently been examined as a possible clinical liver function test (Schneider et al., 1980 and Kotake et al., 1982). A noninvasive method similar to that for antipyrine was

developed to measure free aminopyrine in the saliva of rats.

The second set of experiments was designed to examine possible dietary factors for the management of ethanol or acetaldehyde inhibited drug metabolism.. Dietary protection against ethanol- or acetaldehyde-inhibited drug metabolism could suggest in vivo mechanisms for ethanol hepatotoxicity. A review of the literature shows that some dietary factors associated with alcohol-related malnutrition were being examined for their roles in the overall toxicity of ethanol or acetaldehyde. Of these compounds, cysteine, ascorbic acid, and thiamine have received the most attention.

Thiol compounds, such as cysteine, are known to increase the survival rate of animals given a lethal dose of ethanol (MacDonald et al., 1977), to reduce ethanol-induced sleeping time (Nagasawa et al., 1975), and to prevent ethanol-induced fatty liver (Torrielli et al., 1978). Cysteine also protects against acetaldehyde-induced mitochondrial damage (Cederbaum and Rubin, 1976), to increase survival rate of animals given a lethal dose of acetaldehyde (Sprince et al., 1975), and lowers ethanol-derived circulating blood acetaldehyde in rats (Acholonu, 1983).

Thiamine, Vitamin B1, is a vitamin known to be deficient in many alcoholics due to inadequate nutritional intake, decreased absorption, or impaired utilization (Leevy, 1982). High thiamine intake has been clearly associated with a reduction in voluntary ethanol drinking (Eriksson et al., 1980). In mice, thiamine is very effective in reducing the mortality from acetaldehyde (Moldowan and Acholonu, 1982) and can lower ethanol-derived blood ethanol and blood acetaldehyde levels in rats (Acholonu, 1983).

Vitamin C has been shown to overcome the adverse side effects of disulfiram-ethanol reaction caused by the buildup of acetaldehyde from the inhibition of acetaldehyde dehydrogenase (Weissman and Koe, 1969). Ascorbic acid is also effective in reducing the mortality induced by acetaldehyde (Moldowan and Acholonu, 1982) and can lower ethanol-derived blood ethanol and blood acetaldehyde levels in rats (Acholonu, 1983). Ascorbic acid has also been shown to influence overall drug metabolism activities (Sato and Zannoni, 1976).

The third set of experiments examined the effect of ethanol or acetaldehyde on the metabolism of antipyrine or aminopyrine by isolated rat liver microsomes. The attempt was to develop necessary analytical techniques for the HPLC quantification of the primary metabolites of in vitro antipyrine metabolism. Comparisons between in vivo and in vitro results at biochemically significant concentrations of ethanol or acetaldehyde could then lead to a better understanding of the mechanism by which ethanol selectively inhibits MFO drug metabolism.

An established analytical technique for quantification of antipyrine metabolites in vitro was not available. Development and refinement of such methodology was not productive. It was therefore necessary to adapt three sets of experiments to examine antipyrine in vitro metabolism.

A well recognized analytical method (Nash, 1953) was used to examine the N-demethylation of aminopyrine to monomethyl-4-aminoantipyrine through quantifying formaldehyde formed. This method was adapted to examine the N-demethylation of antipyrine to norantipyrine. Total in vitro antipyrine metabolism was screened

by monitoring the conversion of NADPH to NADP^+ (Stratland et al., 1973) as a measure of total MFO activity. Norantipyrine, 3-hydroxyantipyrine, and 3-carboxyantipyrine metabolites were successfully quantified through the development of a high pressure liquid chromatographic assay.

A fourth chapter is included as general discussion of the advantages and disadvantages of experimental methods. Potential mechanisms by which ethanol inhibits drug metabolism are reviewed based on data provided by the research as well as new information available in the literature since the research was completed.

CHAPTER 1: The Effect of Ethanol or Acetaldehyde on Antipyrine or Aminopyrine Salivary Half-Life in Rats.

Summary

The inhibition of mixed-function oxidase activity by ethanol and acetaldehyde was examined in vivo by monitoring changes in salivary half-life, apparent volume of distribution, and clearance of antipyrine and aminopyrine in adult male rats. Ethanol (1-6 g/kg, po) or acetaldehyde (4-8 mmol/kg, ip) was administered 20 minutes prior to antipyrine (100 mg/kg, ip) or aminopyrine (80 mg/kg, ip). Ethanol increased the half-life of antipyrine in a dose-dependent (0-96% increase) manner over the control value of 171 ± 5 minutes. Acetaldehyde inhibition was less dramatic (23% increase) and not dose-dependent. The half-life of aminopyrine remained statistically unchanged from control (68 ± 7 minutes) with either treatment.

Introduction

The metabolism of ethanol by the microsomal ethanol oxidizing system (MEOS) is believed responsible for alcohol's effect on the metabolism of endogenous compounds, drugs, and other xenobiotics. In vitro, ethanol inhibits the metabolism of many of these compounds (Sato et al., 1980 and Strubelt, 1980). The significance of this inhibition in vivo has yet to be fully established. Ethanol does affect the pharmacokinetics and metabolism of many of those compounds already examined (Dossing et al., 1984; Schuppet, 1969; Minnigh and Zemaitis, 1982; Sato et al., 1981; Chung and Brown, 1976; Sellers, 1979; and Sellers et al., 1979) yet there is little reported information on the possible role of acetaldehyde in this inhibition.

Explanations as to the mechanism of inhibition of drug metabolism by ethanol have relied heavily on subcellular methods. Much higher concentrations of ethanol are often required to inhibit microsomal (Dicker and Cederbaum, 1982) and intact hepatic cells (Moldowan, 1981) drug metabolism than are physiologically feasible or biochemically significant. This may indicate different mechanisms for inhibition at the subcellular or cellular level than in the whole animal.

Early proposed mechanisms concentrate on the direct interaction of ethanol with components of the mixed-function oxidase (MFO) system (Cinti et al., 1973) disregarding any action of acetaldehyde formed during the metabolism of ethanol. Whether acetaldehyde is a possible mediator for ethanol-induced inhibition of chemical metabolism has not been thoroughly investigated. Recent reports have

shown that acetaldehyde in vitro can inhibit the metabolism of some chemicals (Dicker and Cederbaum, 1982). Few in vivo experiments have investigated the effects of acetaldehyde on MFO activity. The purpose of this study is therefore to investigate the effect of ethanol or acetaldehyde on in vivo drug metabolism.

Many attempts have been made to correlate the changes in drug pharmacokinetics and in serum enzymes with alcoholic liver disease states, chemical-induced hepatotoxicity, and general liver health. Measurements of serum glutamic-oxalacetic transaminase, alkaline phosphatase, and lactate dehydrogenase activities, or prothrombin clotting time and bilirubin content do not correlate well with the ability of the liver to metabolize drugs (Devgun et al., 1985; Andreasen et al., 1974; and Kotake et al., 1982). Instead, new in vivo methods which quantify antipyrine or aminopyrine metabolism have been developed. These offer more specific and sensitive measurements for evaluating the relationship between the clinical condition of the liver and its ability to metabolize drugs (Capel et al., 1979 and Haustein and Schenker, 1985).

In vivo MFO activity can be effectively studied using antipyrine or aminopyrine as test substrates (Figures 1.1 and 1.2). Both are analgesic-antipyretics which distribute evenly in total body water, have minimal binding to tissue protein, and are completely metabolized and excreted in the urine as a conjugated metabolite (Brodie and Axelrod, 1950a and 1950b). Therefore, the rate of disappearance of antipyrine or aminopyrine from plasma is a measure of the ability of the liver to metabolize the drug. In addition both can be conveniently studied through monitoring salivary disappearance of the

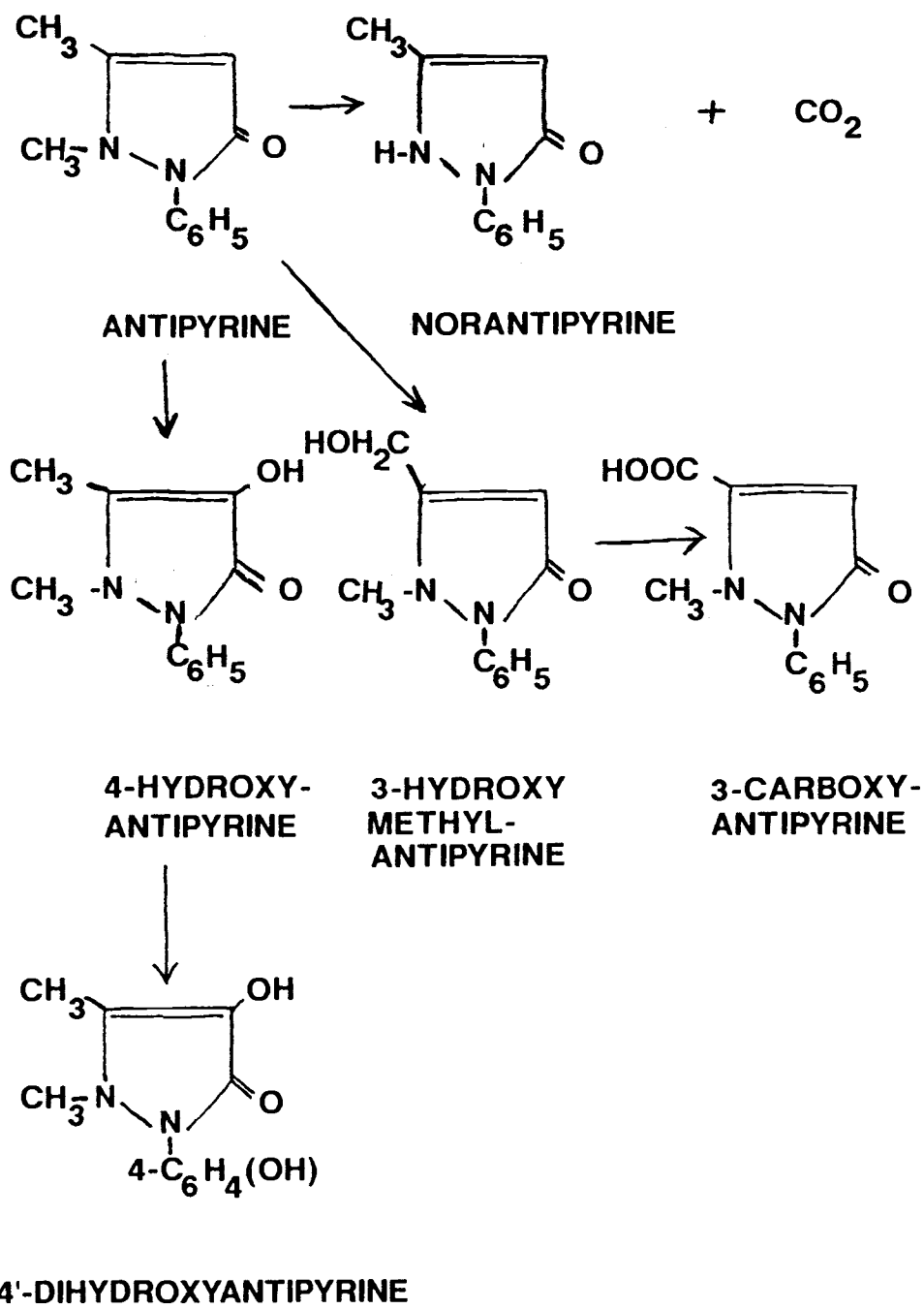
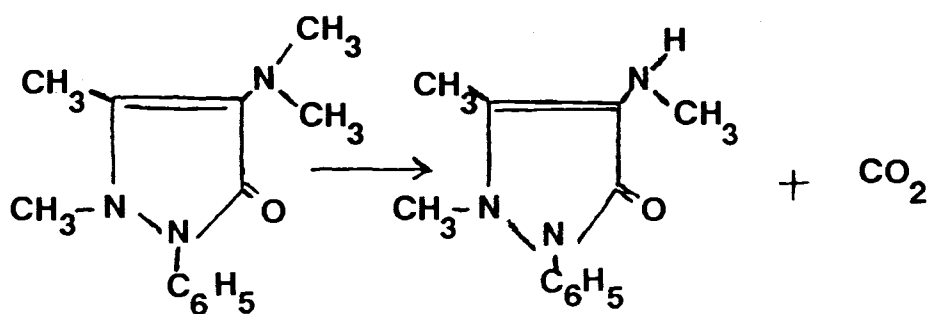
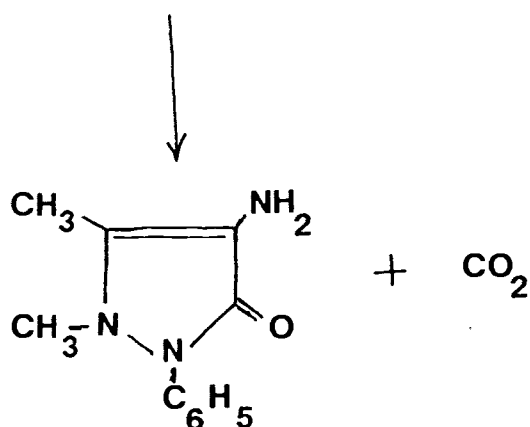


FIGURE 1.1: Antipyrine and Proposed Metabolites (Adapted from Inaba et al., 1980).



AMINOPYRINE

MONOMETHYL-
4-AMINOANTIPYRINE

4-AMINOANTIPYRINE

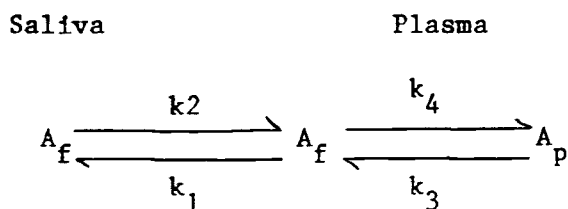
FIGURE 1.2: Aminopyrine and Proposed Metabolites (Adapted from Gillette *et al.*, 1957).

drug which avoids the problems of repeated plasma sampling (Wilson, V.L. et al., 1982 and Welch et al., 1975).

Antipyrine has gained acceptance as an indicator of many drug metabolizing activities. Vesell and associates, in 1973, were the first to utilize antipyrine plasma half-life as a measure of the liver microsomal drug metabolizing enzyme system. It was found that individual animals show a high degree of reproducibility of antipyrine half-life when animals were retested. Welch et al., in 1975, have shown that saliva elimination studies can accurately substitute for plasma elimination studies.

Capel and associates, in 1979, have compared the sensitivity of antipyrine metabolism versus hepatic enzyme markers (alpha-fetoprotein, gamma-glutamyltransferase, alkaline phosphatase, ornithine carbamoyltransferase, aspartate aminotransferase, and glutamate dehydrogenase). Antipyrine half-life values were the most sensitive indicator of hepatotoxicity with all pretreatments of cytotoxic drugs and carbon tetrachloride. They also suggest that antipyrine might provide an estimate of the relative impairment resulting from different treatments. Antipyrine elimination has also been used as a measure of predicting the heterocyclic hydroxylating capacity of the liver (Vesell, 1975).

A noninvasive quantitative assay has recently been developed (Wilson, V.L. et al., 1982) for the in vivo study of hepatic mixed-function oxidase activity in rats. This method involves monitoring salivary levels of antipyrine via high pressure liquid chromatography. Analyzing for drug content in saliva represents free drug which enters the saliva by passive diffusion:



Where A_f is free drug, A_p is plasma protein bound drug, and k_x is the rate constant for the process. For antipyrine the rate constant $k_1 = k_2$ between free antipyrine in the saliva and plasma. The rate constant for $k_3 \gg k_4$ and results in less than 10% of antipyrine being plasma protein bound (Brodie and Axelrod, 1950a). Since rates of plasma elimination parallel rates of saliva elimination (Vesell et al., 1975) analyzing for drug concentration in the saliva is convenient and economical, and allows for individual animal profiles rather than the pooling of samples from several animals necessary with plasma sampling. Pharmacokinetically, antipyrine is distributed in total body water with low percent plasma protein binding and the terminal linear portion of the decay curve follows a one compartment model (Brodie and Axelrod, 1950a).

Aminopyrine is structurally similar to antipyrine with the addition of a dimethylamino group at position 4 (Figure 1.2). Aminopyrine undergoes N-demethylation to its primary metabolite, monomethyl-4-aminoantipyrine (Brodie and Axelrod, 1950b). Aminopyrine is often one drug of choice to examine the effect of chemicals on in vitro MFO activity (Bast and Noordhoek, 1981 and Back et al., 1983). The aminopyrine breath test has been suggested as a clinical test to examine in vivo MFO activity (Schneider et al., 1980 and Kotake et al., 1982). Recently, the aminopyrine breath test was shown to

correlate with other laboratory liver function tests (Haustein and Schenker, 1985). Measuring salivary aminopyrine elimination can also be a means to examine in vivo MFO activity. Though 15% plasma protein bound, its elimination rate from saliva is indicative of its elimination rate from plasma (Vesell et al., 1975).

Materials and Methods

Male, Sprague-Dawley rats (200-300 g) were fed ad libitum a commercial diet (Appendix One: Oregon State University Rodent Feed) and given free access to tap water. They were housed in cages in groups of five at 22 degrees Centigrade. Animals were maintained on a 12 hour (7am-7pm) light/dark cycle. All animals were deprived of food for 12-15 hours prior to treatment.

Animals received antipyrine (100 mg/kg, ip) or aminopyrine (dose optimized using 50 mg/kg, 80 mg/kg, or 100 mg/kg, ip). Saliva samples were collected by using a 50 μ l capillary tube placed between the lower lip and gum (technique independent of intoxicated state of rat). These samples were analyzed for antipyrine or aminopyrine. Salivation was stimulated with pilocarpine (0.5 mg/0.1 ml saline, sc) as needed. This procedure does not change the antipyrine or aminopyrine concentration in the saliva (Welch et al., 1975).

Control animals received only antipyrine or aminopyrine (80 mg/kg, ip). Treated animals received graded doses of ethanol (30% v/v, po) or acetaldehyde (50% w/v, ip) 20 minutes prior to receiving the antipyrine or aminopyrine.

Saliva samples were analyzed within 2 hours of collection with a Waters Associates High Pressure Liquid Chromatograph (HPLC) fitted with a 30 cm reverse phase μ Bondapak (C_{18}) column, a 7 cm (C_{18}) guard column and Waters Model U6K Injector. The elution from the column (elute) was monitored as changes of absorbance read with a Waters Absorbance Detector Model 440 at 254 nm using absorbance units full

scale of 0.02-0.005.

Saliva samples containing antipyrine obtained optimal peak separation with a 30% acetonitrile in 5 mM phosphate buffer mobile phase, pH 6.7, at a flow rate of 2 ml/minute. The saliva of rats does not show background interference for antipyrine. One μ l of saliva sample containing antipyrine and one μ l aminopyrine (2 μ g/ml, external standard) were injected simultaneously on column. Retention times for antipyrine and aminopyrine were 2.62 minutes and 3.58 minutes respectively. The peak height ratio method was used to quantitatively analyze for antipyrine with a lower detection limit for antipyrine of 1.0 ng/ml.

Saliva samples containing aminopyrine and unidentified metabolites obtained optimal peak separation with a 35% methanol in 5 mM phosphate buffer mobile phase, pH 6.7, at a flow rate of 1.5 ml/minute. The saliva of rats does not show background interference for aminopyrine. One-18 μ ls of saliva sample were injected on column. Retention time of aminopyrine was 7.5 minutes. Two major metabolite (Figure 1.2) peaks were also present in the saliva. Monomethyl-4-aminoantipyrine eluted at 6 minutes (Bast and Noordhoek, 1981) and a second unidentified metabolite eluted at 4.0 minutes. Neither metabolite interfered with the quantitation of aminopyrine by this method. Two μ ls of blank saliva obtained prior to administration of aminopyrine was injected along with 2 μ l antipyrine (1 μ g/ml, external standard). Antipyrine's retention time was 4.8 minutes. Peak height ratio was used to quantitatively analyze for aminopyrine with a lower detection limit of 12 ng/ml.

Salivary antipyrine or aminopyrine was plotted as the log

concentration of the drug verses time. Linear regression was used to obtain the salivary half-life via $t_{1/2} = .693/k_e$, where k_e is the slope of elimination phase. The concentration at zero time (C_0) was estimated by extrapolation of the slope back to the ordinate. The volume of distribution (V_d) was calculated as approximate $V_d = D/C_0$, where D = dose. The clearance (Q) was calculated as $Q = V_d \times k_e$. The mean values were used to compare results by One-way Analysis of Variance coupled with a Least Squares Difference test. All values are reported as \pm standard error of the mean.

Antipyrine was purchased from Aldrich Company. Aminopyrine and pilocarpine were purchased from Sigma Chemical Company. Acetaldehyde was obtained from Mallinkrodt and was freshly distilled prior to each use. HPLC grade acetonitrile and methanol were purchased from JT Baker Chemical Company. All other reagents were purchased from Sigma Chemical Company.

Results

The optimum dose of antipyrine for rats had been previously determined (Welch et al., 1975) to be 100 mg/kg body weight. The saliva does not show background interference nor are any of the metabolites of antipyrine detectable at this dose. Control half-life, clearance, and volume of distribution were calculated as shown in Table 1.1. The range of the half-life values for antipyrine in control rats was from 120-180 minutes.

The control data represents a relatively large number of experimental animals. Each experimental day, control rats were run along with treatment groups. Data between daily control groups were compared and showed no difference over the lifetime of this research. Control animals were therefore pooled.

Ethanol treatment was via po administration. A dose-response curve for the inhibitory effect of ethanol on antipyrine is shown in Figure 1.3. The rectilinear plot was drawn by regression analysis. The results show that increasing the dosage of ethanol caused a linear increase (0-96%) in antipyrine half-life. No statistically significant change was seen in the clearance or volume of distribution of antipyrine (Table 1.1).

Acetaldehyde was given intraperitoneally at doses ranging from 4-8 mmol/kg rat. Only at a dose of 6 mmol/kg body weight of acetaldehyde was there a change from control half-life of antipyrine (23% increase) which was statistically significant. No change was observed in either clearance or volume of distribution at this dose.

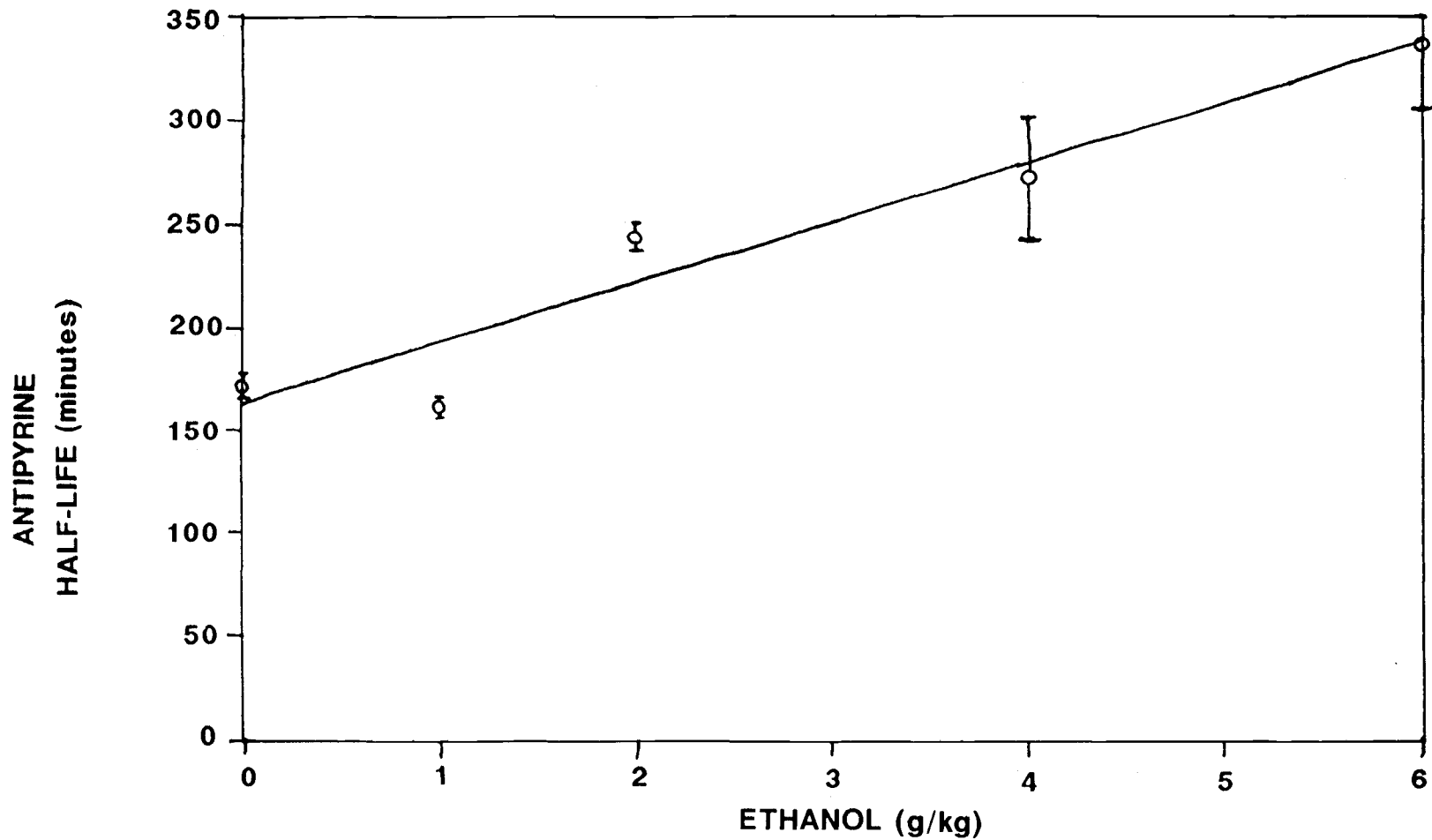


FIGURE 1.3: Dose Response Curve for the Inhibitory Effect of Ethanol on Antipyrine Salivary Half-life. Refer to Table 1.1 for number of rats used at each dose and standard error.

TABLE 1.1 Salivary Antipyrine Elimination in Control, Ethanol, and Acetaldehyde Treated Rats^a.

treatment (/kg rat)	no. rats	antipyrine ^b		
		$t_{\frac{1}{2}}$ (min)	V_d (L/kg)	Q (ml/kg/min)
control	29	171 \pm 5	.98 \pm .08	4.06 \pm .33
ethanol				
1 g (22 mmol)	5	161 \pm 4	.98 \pm .04	4.22 \pm .16
2 g (43 mmol)	4	^c 243 \pm 14	.94 \pm .15	2.78 \pm .58
4 g (87 mmol)	6	^c 273 \pm 28	1.18 \pm .17	3.05 \pm .38
6 g (130 mmol)	11	^c 335 \pm 44	1.38 \pm .30	2.77 \pm .44
acetaldehyde				
4 mmol (.176 g)	9	184 \pm 13	.74 \pm .05	2.97 \pm .29
5 mmol (.220 g)	5	202 \pm 21	1.09 \pm .05	3.88 \pm .34
5.5 mmol (.242 g)	5	174 \pm 14	1.17 \pm .05	4.80 \pm .50
6 mmol (.264 g)	14	^c 211 \pm 13	1.02 \pm .05	3.52 \pm .26
8 mmol (.352 g)	4	213 \pm 42	1.40 \pm .21	4.70 \pm .58

a. Ethanol (po) or acetaldehyde (ip) was administered 20 minutes prior to antipyrine (100 mg/kg, ip) to adult male (200–300 g) Sprague-Dawley rats. Salivary antipyrine half-life ($t_{\frac{1}{2}}$), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.

b. Results are $\bar{X} \pm$ SE.

c. Significantly different ($p < .05$, LSD) from control.

Higher doses of acetaldehyde resulted in mortality, severe physiological reaction, or inhibition of salivation making the use of higher doses unfeasible for this study.

To determine the optimum dose of aminopyrine, doses ranging from 50-100 mg/kg body weight were chosen. The salivary half-life was independent of dose over this range (Table 1.2). The dose of 50 mg/kg was found to be unsatisfactory due to the difficulty in quantifying drug at the end of the elimination curve. Only two animals of six initially dosed had measurable peak heights of aminopyrine after less than 2 hours sampling time. At 100 mg/kg, aminopyrine displayed two elimination phases making calculations of half-life more difficult than at the dose of 80 mg/kg which displayed only one elimination phase. The dose of 80 mg/kg was therefore selected as the optimum dose for this study. This dose was also used to study the effect of ethanol or acetaldehyde on aminopyrine metabolism (Table 1.3).

Neither the 2 g/kg body weight or the 6 g/kg body weight po dose of ethanol showed a statistical difference when compared with the control value of 68 ± 7 minutes. Treatment with acetaldehyde used 6 mmol/kg body weight. Acetaldehyde had no statistical effect on the half-life of aminopyrine at the administered doses. Both acetaldehyde and ethanol decreased the volume of distribution of aminopyrine by approximately 50% of the control value of $3.56 \pm .67$ L/kg. Both also decreased the clearance by about 50% from that of the control value of 38 ± 10 ml/kg/minute.

TABLE 1.2 Aminopyrine Salivary Elimination Profile^a.

^c dose	no. rats	^b t _{1/2} (min)	^b V _d (L/kg)	^b Q(ml/kg/min)
50 mg/kg	2	49 ± 1	2.54 ± .37	36 ± 6
80 mg/kg	4	68 ± 7	3.56 ± .67	38 ± 10
100 mg/kg	4	60 ± 6	2.96 ± .22	36 ± 3

a. Comparison of dose-dependency of ip dose of aminopyrine to adult male (200-300 g) Sprague-Dawley rats. Saliva aminopyrine half-life (t_{1/2}), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.

b. Results are $\bar{X} \pm \text{SE}$.

c. Not statistically different.

TABLE 1.3 Salivary Aminopyrine Elimination in Control, Ethanol, and Acetaldehyde Treated Rats^a.

treatment (/kg rat)	no. rats	aminopyrine ^b		
		$t_{\frac{1}{2}}$ (min)	V_d (L/kg)	Q (ml/kg/min)
control	4	68 \pm 7	3.56 \pm .67	38 \pm 10
ethanol				
2 g (43 mmol)	4	53 \pm 4	^c 1.57 \pm .06	^c 21 \pm 2
6 g (130 mmol)	11	72 \pm 9	^c 1.87 \pm .26	^c 20 \pm 3
acetaldehyde				
6 mmol (.264 g)	5	65 \pm 2	^c 1.70 \pm .10	^c 18 \pm 1

a. Ethanol (po) or acetaldehyde (ip) was administered 20 minutes prior to aminopyrine (80 mg/kg, ip) to adult male (200–300 g) Sprague-Dawley rats. Saliva aminopyrine half-life ($t_{\frac{1}{2}}$), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.

b. Results are $\bar{X} \pm$ SE.

c. Significantly different ($p < .05$, LSD) from control.

Discussion

The elimination of antipyrine from saliva has recently gained acceptance as a useful index for estimating the relative capacity of the liver to metabolize drugs (Welch et al., 1975 and Vesell et al., 1975). Since antipyrine elimination is totally dependent on its metabolism by the liver, the salivary half-life of antipyrine is a useful index of drug metabolism in animals (Statland et al., 1973; Vesell et al., 1973; and Welch et al., 1967). Moreover, antipyrine does not significantly bind to plasma protein (Soberman et al., 1949). Since saliva contains only the free form of the drug (Vesell et al., 1975) interpretation of changes in antipyrine salivary half-life are not clouded by alterations in plasma protein distribution, turnover, or binding.

Ethanol significantly increased (30%) the half-life of antipyrine at doses as low as 2 g/kg body weight in these experiments. Ethanol also increased antipyrine half-life in a dose-dependent manner. This agrees with the significant decrease in rate of elimination of metabolites of antipyrine in urine (Schuppel, 1969) and the inhibition of hexobarbital metabolism (Chung and Brown, 1976) after acute administration of ethanol.

The dosage range of ethanol (1-6 g/kg) for these experiments was selected based on research of others (Rubin et al., 1970b, Chung and Brown, 1976, and Sato et al., 1981). Others have established that in this dose range, blood ethanol levels will range from approximately 0.07% to 0.42 % (Majchrowicz, 1981) in male Sprague-Dawley rats. The

spectrum of intoxication ranged from no apparent influence at 1 g/kg of ethanol to severe impairment displayed as inability to right themselves or complete lack of response to external stimuli at 6 g/kg of ethanol. Degrees of intoxication in humans (Table 1.4) are very comparable through this range of blood ethanol levels (Casarett and Doull, 1986).

Acetaldehyde was more potent, but not as effective as ethanol in increasing the half-life of antipyrine . Only 6 mmol/kg (.264 g/kg) of acetaldehyde significantly prolonged the half-life of antipyrine. When 8 mmol/kg dose of acetaldehyde was used there was no statistical difference in the half-life from that of controls due to a large standard error. At 8 mmol/kg acetaldehyde most animals showed severe symptoms of acetaldehyde stress. A high rate of mortality limited the number of animals which could be studied. The inability to consistently stimulate salivation in surviving rats made accurate timing of samples and collection difficult. Saliva samples from these rats tended to be cloudy. Despite centrifugation of the samples, HPLC analyses had high background noise which increased the possibility of error in peak height measurements. These factors added to normal interindividual variations of drug metabolism may have contributed to the large standard error at this dose. It should be noted that all animals died at 10 mmol/kg acetaldehyde.

Although acetaldehyde has greater reactivity and lipid solubility than ethanol (Walsh, 1971), the physiological significance of these concentrations of acetaldehyde toward ethanol inhibited drug metabolism is questionable (Truitt and Walsh, 1971 and Korten et al., 1975). In Sprague-Dawley rats, the acetaldehyde concentration

TABLE 1.4: Symptoms of intoxication associated with increasing blood ethanol levels (BEL) in humans (adapted from Casarett and Doull, 1986 and Barry, 1979). Dosage and estimated blood ethanol level (Majchrowicz, 1981) for Sprague-Dawley rats used in these experiments.

Sprague-Dawley Rats		Humans	
Ethanol dose	BEL	Range BEL	Symptom
		0.01-0.05%	No influence
1 g/kg	0.07%	0.05-0.1%	Loss of dark adaptation & stereoscopic vision
2 g/kg	0.14%	0.1-0.15%	Euphoria, loss of inhibi- tions, prolonged rxn time
		0.15-0.2%	Moderate to severe poisoning, rxn time greatly prolonged, slight disturbances in equilibrium & coordination
4 g/kg	0.28%	0.2-0.35%	Severe degree poisoning, disturbance in equilibrium & coordination, retardation of thought processes & clouding of consciousness
6 g/kg	0.42%	0.35-.4%	deep possible fatal coma

resulting from ethanol (2-6 g/kg) metabolism in vivo is between 3-15 $\mu\text{g/g}$ in the liver and 1-8 $\mu\text{g/ml}$ in the blood (Watanabe et al., 1986). These acetaldehyde blood and tissue levels compared with data in Wistar rats (Espinet and Argiles, 1984). Higher levels (1-400 $\mu\text{g/ml}$) which may have been cited in literature have been recently attributed to artifacts of the analytical techniques (Ericksson, 1983).

Acholonu (1983) reported blood acetaldehyde levels in Sprague-Dawley rats of 39.6 $\mu\text{g/ml}$ after a 6 mmol/kg ip dose of acetaldehyde. This blood acetaldehyde level is approximate four times greater than that resulting from ethanol (6 g/kg) metabolism in the Watanabe study. Despite higher blood acetaldehyde levels generated by exogenous acetaldehyde, Watanabe went on to show that 2.5 $\mu\text{g/g}$ peak liver concentration limits the hepatocellular availability of acetaldehyde.

One possible mechanism by which ethanol inhibits hepatic drug metabolism is through the binding of acetaldehyde to the MFO. To do this acetaldehyde must be present at this subcellular level. It appears that 6 mmol/kg exogenous acetaldehyde may be necessary to arrive at a hepatocellular acetaldehyde concentration comparable to that generated by hepatocellular ethanol metabolism.

Antipyrine is a capacity limited, binding insensitive drug (Sellers, 1979) whose rate of hepatocellular metabolism should be independent of any changes in blood flow caused by ethanol or acetaldehyde. Since apparent volume of distribution and clearance remain unchanged by either ethanol or acetaldehyde, it is likely that their effect is directly on the metabolism of antipyrine.

Antipyrine is metabolized through the hepatic mixed-function

oxidase system (Brodie and Axelrod, 1950a). Ethanol is also metabolized through this system, though referred to as the microsomal ethanol oxidizing system (Lieber and DeCarli, 1970). Although ethanol inhibits antipyrine metabolism, the lack of effect of antipyrine on ethanol metabolism (Andreasen et al., 1974) suggests that antipyrine and ethanol may be metabolized by different metabolic pathways in the hepatic microsomes. Ethanol also did not affect the binding spectrum of antipyrine in vitro (data not shown). Chapters 2-4 examine further possible mechanisms for ethanol inhibition of antipyrine metabolism in vivo.

This thesis research shows that the ability of ethanol to inhibit the metabolism of drugs which are substrates of the MFO system is not limited by its ability to effect the half-life of the drug. Ethanol in this study did not significantly alter the half-life of aminopyrine. Ethanol did significantly decrease both the apparent volume of distribution (56%) and the clearance (47%) of salivary aminopyrine. This is in agreement with the finding of the aminopyrine breath test following a single dose of ethanol (Hanew et al., 1984). Acetaldehyde also did not inhibit the half-life of salivary aminopyrine, yet did significantly decrease both the apparent volume of distribution (52%) and the clearance (52%) of aminopyrine in the saliva.

Aminopyrine is approximately 15% plasma protein bound in control rats (Vesell et al., 1975). The volume of distribution for aminopyrine would therefore decrease with inhibition of plasma protein binding and result in a faster clearance of aminopyrine from the saliva. Inhibition of plasma protein binding alone would be

responsible for a 15% decrease volume of distribution and clearance.

Others have suggested that the trend toward a two compartment model of aminopyrine, plus binding changes, affect the extrapolation to the y-axis for the apparent volume of distribution (Sultatos et al., 1988). These problems would also affect clearance which relies on the volume of distribution for its calculation.

CHAPTER 2: Effect of L-Ascorbic Acid, Thiamine-HCl, or L-Cysteine on the Inhibition of Antipyrine Salivary Half-Life by Ethanol or Acetaldehyde.

Summary

The inhibition by ethanol and acetaldehyde of mixed-function oxidase activity has been demonstrated in vivo by monitoring changes in salivary half-life ($t_{1/2}$) of antipyrine. The ability of L-ascorbic acid (Vit C), thiamine-HCl (Vit B₁), or L-cysteine (Cys) to prevent ethanol or acetaldehyde increased $t_{1/2}$ of antipyrine was studied in adult (200-300 g) male rats. Ethanol (2 g/kg, po) or acetaldehyde (6 mmol/kg, ip) was administered 20 minutes prior to antipyrine (100 mg/kg, ip). At dosages of 2 mmol/kg Vit C or Cys given 90 minutes prior to ethanol, the $t_{1/2}$ of antipyrine remained unchanged from control (171 ± 5 minutes) compared with 243 ± 12 minutes in the presence of ethanol alone. When 0.24 mmol/kg Vit B₁ was given prior to ethanol the $t_{1/2}$ of antipyrine was not significantly changed from ethanol alone. When Vit C or Vit B₁ was given prior to acetaldehyde, the $t_{1/2}$ of antipyrine remained unchanged from control yet not statistically different when compared with 211 ± 13 minutes in the presence of acetaldehyde alone. Cys pretreatment had no effect on the acetaldehyde-increased antipyrine $t_{1/2}$. Using the criteria that pretreatment was effective if pretreatment groups were statistically unchanged from the control group, Vit C or Cys pretreatment prevented ethanol-increased antipyrine $t_{1/2}$ and Vit C or Vit B₁ pretreatment prevented acetaldehyde-increased antipyrine $t_{1/2}$.

INTRODUCTION

Ethanol can inhibit the in vivo metabolism of many mixed-function oxidase (MFO) substrates (Minnigh and Zemaitis, 1982; Rubin et al., 1970b; Strubelt, 1980; Chung and Brown, 1976; and Dossing et al., 1984) with antipyrine being just one example (Chapter 1). This influence of ethanol on mixed-function oxidase activity is most likely a result of ethanol oxidation by the microsomal ethanol oxidizing system (MEOS). In order to better understand the mechanism of ethanol inhibited MFO activity, researchers have examined the effect of ethanol on drug metabolism in perfused liver, isolated hepatocytes, and isolated and reconstituted hepatic microsomes. Poor drug selection and too high concentrations of ethanol make comparisons back to in vivo conditions difficult. Few reports address the contribution of acetaldehyde toward inhibition of MFO activity.

The oxidative metabolism of ethanol by MEOS may inhibit the metabolism of various drugs by MFO system in several ways. At the top of the list would be the interference by ethanol of drug binding to the cytochrome P450. Ethanol could either compete for the binding site (Rubin et al., 1970a), strip the binding site of endogenous cofactors necessary for drug binding (Cinti et al., 1973), or effect the chemical and structural property of the enzyme so as to interfere with drug binding (Taraschi and Rubin, 1985). Second on the list would be the interference of drug binding by acetaldehyde. Acetaldehyde may bind with the MFO (Nomura and Lieber, 1981) and either prevent drug binding or inhibit the activity of the MFO toward

the drug through its binding as has been demonstrated by acetaldehyde enzyme inhibition in the brain (Collins, 1988).

The purpose of this research is to select dietary factors which have demonstrated their ability to inhibit the degree of ethanol intoxication, affect quantity of ethanol consumed, or block the adverse effects of acetaldehyde. The dietary factors will then be studied for their ability to prevent ethanol or acetaldehyde inhibition of antipyrine metabolism in vivo and thereby inferring possible mechanisms by which ethanol inhibits MFO activity.

A review of the literature revealed that L-ascorbic acid, L-cysteine, or thiamine-HCl showed the best potential for interfering with the adverse effects caused by acetaldehyde or ethanol. This made them the best candidates to investigate a dietary means of interfering with ethanol-inhibited antipyrine metabolism.

The evidence for the value of L-ascorbic acid as a possible protectant is abundant. Ascorbic acid has been shown to reduce acetaldehyde-induced mortality in mice (Moldowan and Acholonu, 1982), loss of righting reflex (O'Neill and Rahwan, 1976), and reduce acetaldehyde blood levels (Sprince et al., 1980). Leukocyte ascorbic acid levels and the rate of ethanol clearance from the blood have been directly correlated (Krasner et al., 1974). Ascorbic acid also decreases blood ethanol concentration (Yunice et al., 1984) and ethanol-derived acetaldehyde (Acholonu, 1983). Yunice et al., 1984 also demonstrated a reduction of fatty infiltration of the liver in alcohol fed animals receiving high dietary ascorbic acid.

Thiamine's selection for use in these experiments originates from Williams' 1950 genotrophic theory on alcohol consumption. He

believed a genetically-determined exceptionally-high requirement for thiamine was involved in the genesis of human alcoholism. Eriksson et al., in 1980, showed that thiamine-deficient rats showed a significant tendency to increase ethanol drinking. Rats on a diet excessive in thiamine (20 mg/kg) drank 1/5 as much ethanol as rats given a diet considered sufficient in thiamine (4 mg/kg).

Cysteine was selected because it has been shown to activate alcohol dehydrogenase through enhanced binding of ethanol to the enzyme (Harris, 1964 and Theorell, 1967). Cysteine pretreatment increases the rate of survival from a 24 hour-LD90 (18 mmol/kg) dose of acetaldehyde in rats (Sprince et al., 1979) and mice (MacDonald et al., 1977). Cysteine also protects against the inhibition of mitochondrial functions by acetaldehyde (Cederbaum and Rubin, 1976).

The specific objective of this part of the thesis was to investigate, in vivo, the ability of L-ascorbic acid, L-cysteine, or thiamine-HCl to prevent ethanol- or acetaldehyde-inhibited antipyrine metabolism.

Materials and Methods

Male, Sprague-Dawley rats (200-300 g) were divided into control, ethanol-alone, acetaldehyde-alone, dietary factor-alone, dietary factor plus ethanol, and dietary factor plus acetaldehyde. All animals were deprived of food for 12-15 hours prior to treatment.

All animals received antipyrine (100 mg/kg, ip). Saliva samples were collected by using a 50 μ l capillary tube placed between the lower lip and gum. These samples were analyzed for antipyrine. Salivation was stimulated with pilocarpine (0.5 mg/0.1 ml saline, sc) as needed. This procedure does not change the antipyrine concentration (Welch *et al.*, 1975) in the saliva.

Control animals received only antipyrine. Treated animals received 2 g/kg ethanol (30% v/v, po) or 6 mmol/kg acetaldehyde (50% w/v, ip) 20 minutes prior to receiving the antipyrine. Those receiving L-ascorbic acid (2 mmol/kg, ip), thiamine-HCl (0.24 mmol/kg, ip), or L-cysteine (2 mmol/kg, ip) pretreatment were dosed 90 minutes before receiving ethanol or acetaldehyde.

Saliva samples were analyzed within 2 hours of collection with a Waters Associates High Pressure Liquid Chromatograph fitted with a 30 cm reverse phase Bondapak (C₁₈) column and a 7 cm (C₁₈) guard column and Waters Model U6K Injector. The elution from the column (elute) was monitored as changes of absorbance read with a Waters Absorbance Detector Model 440 at 254 nm using absorbance units full scale of 0.02-0.005.

Saliva samples containing antipyrine obtained optimal peak

separation with a 30% acetonitrile in 5 mM phosphate buffer mobile phase, pH 6.7, at a flow rate of 2 ml/minute. The saliva of rats does not show background interference for antipyrine, nor are any of its metabolites detectable at this dose. One μ l of saliva sample and one μ l of aminopyrine (2 μ g/ml, external standard) were injected simultaneously on column. Retention times were 2.62 minutes and 3.58 minutes respectively. The peak height ratio method was used to quantitatively analyze for antipyrine with a lower detection limit for antipyrine of 1.0 ng/ml.

Salivary antipyrine was plotted as its log concentration versus time. Linear regression was used to obtain the salivary half-life via $t_{1/2} = .693/k_e$, where k_e is the slope of the elimination phase. The concentration at zero time (C_0) was estimated by extrapolation of the slope back to the ordinate. The volume of distribution (V_d) was calculated as approximately $V_d = D/C_0$, where D = dose. The clearance (Q) was calculated as $Q = V_d \times k_e$. The mean values were used to compare results by One-way Analysis of Variance coupled with a Least Squares Difference test. All values are reported as \pm standard error of the mean.

Antipyrine was purchased from Aldrich Company. Aminopyrine, pilocarpine nitrate, thiamine-HCl, and L-cysteine were purchased from Sigma Chemical Company. L-ascorbic acid was purchased from Baker Chemicals Company. Acetaldehyde was obtained from Mallinkrodt and was freshly distilled prior to each use. HPLC grade acetonitrile was purchased from JT Baker Chemical Company. All other reagents were purchased from Sigma Chemical Company.

Results

The optimum dose of antipyrine had been previously determined (Welch et al., 1975) to be 100 mg/kg body weight. Mean values of salivary antipyrine elimination parameters in control animals were calculated as 171 ± 5 minutes half-life, $0.98 \pm .08$ L/kg volume of distribution, and $4.06 \pm .33$ ml/minute/kg clearance (Table 2.1).

Both ethanol (2 g/kg, po) and acetaldehyde (6 mmol/kg, ip) doses were selected as the lowest dose which significantly increased the half-life of antipyrine over that of the control value, 243 ± 14 minutes and 211 ± 13 minutes respectively (Chapter one). No statistically significant change was calculated in either the clearance (Q) or volume of distribution (V_d) of antipyrine among treatment groups (Table 2.1, 2.2, 2.3).

Pretreatment with 2 mmol/kg L-ascorbic acid had no effect on the salivary half-life, volume of distribution, or clearance of antipyrine (Table 2.1). This agrees with work done by Blanchard et al., 1984. Wilson, J.T. et al., in 1976, also found that Vitamin C failed to effect the pharmacokinetic profile of antipyrine in man. Pretreatment with either thiamine-HCl (0.24 mmol/kg) or L-cysteine (2 mmol/kg) also had no statistically significant effect on the elimination of antipyrine (Table 2.2 and 2.3 respectively).

Pretreatment with ascorbic acid (2 mmol/kg) prevented the ethanol-increased antipyrine half-life. This group had an antipyrine half-life of 150 ± 14 minutes which is statistically the same as the control group (171 ± 5 minutes). This group also had a significantly

TABLE 2.1 Effect of L-Ascorbic Acid on Salivary Antipyrine Elimination in Control, Ethanol, and Acetaldehyde Treated Rats ^a.

treatment (/kg rat)	no. rats	antipyrine ^b		
		$t_{1/2}$ (min)	V_d (L/kg)	Q (ml/kg/min)
Control	29	171 \pm 5	.98 \pm .08	4.06 \pm .33
Ethanol				
2 g (43 mmol)	4	243 \pm 14 ^c	.94 \pm .15	2.78 \pm .58
6 mmol Acet.	14	211 \pm 13 ^c	1.02 \pm .05	3.52 \pm .26
2 mmol L-AA	10	203 \pm 19	.97 \pm .09	3.57 \pm .49
+ 2 g EtOH	7	150 \pm 14 ^d	.73 \pm .06	3.43 \pm .28
+ 6 mmol Acet.	8	182 \pm 13	.78 \pm .02	3.09 \pm .23

a. L-ascorbic acid (L-AA, ip) was administered 90 minutes prior to treatment with ethanol (EtOH, po) or acetaldehyde (Acet., ip). Antipyrine (100 mg/kg, ip) followed 20 minutes after ethanol or acetaldehyde treatment. Salivary antipyrine half-life ($t_{1/2}$), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.

b. Results are $\bar{X} \pm$ SE.

c. Significantly different ($p < .05$, LSD) from control.

d. Significantly different ($p < .05$, LSD) from 2 g/kg ethanol alone.

TABLE 2.2 Effect of Thiamine-HCl on Salivary Antipyrine Elimination in Control, Ethanol, and Acetaldehyde Treated Rats^a.

treatment (/kg rat)	no. rats	antipyrine ^b		
		$t_{\frac{1}{2}}$ (min)	V_d (L/kg)	Q (ml/kg/min)
Control	29	171 \pm 5	.98 \pm .08	4.06 \pm .33
Ethanol				
2 g (43 mmol)	4	243 \pm 14 ^c	.94 \pm .15	2.78 \pm .58
6 mmol Acet.	14	211 \pm 13 ^c	1.02 \pm .05	3.52 \pm .26
0.24 mmol B ₁	5	160 \pm 10	1.03 \pm .13	4.52 \pm .68
+ 2 g EtOH	7	214 \pm 29 ^c	.81 \pm .07	2.84 \pm .42
+ 6 mmol Acet.	8	199 \pm 18	.89 \pm .06	3.21 \pm .28

- a. Thiamine-HCl (B₁, ip) was administered 90 minutes prior to treatment with ethanol (EtOH, po) or acetaldehyde (Acet., ip). Antipyrine (100 mg/kg, ip) followed 20 minutes after ethanol or acetaldehyde treatment. Salivary antipyrine half-life ($t_{\frac{1}{2}}$), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.
- b. Results are $\bar{X} \pm \text{SE}$.
- c. Significantly different ($p < .05$, LSD) from control.

TABLE 2.3 Effect of L-Cysteine on Salivary Antipyrine Elimination in Control, Ethanol, and Acetaldehyde Treated Rats^a.

treatment (/kg rat)	no. rats	antipyrine ^b		
		$t_{\frac{1}{2}}$ (min)	V_d (L/kg)	Q (ml/kg/min)
Control	29	171 \pm 5	.98 \pm .08	4.06 \pm .33
Ethanol				
2 g (43 mmol)	4	243 \pm 14 ^c	.94 \pm .15	2.78 \pm .58
6 mmol Acet.	14	211 \pm 13 ^c	1.02 \pm .05	3.52 \pm .26
2 mmol Cys	5	200 \pm 22	.76 \pm .01	2.78 \pm .33
+ 2 g EtOH	6	200 \pm 7 ^d	.78 \pm .03	2.70 \pm .07
+ 6 mmol Acet.	7	228 \pm 25 ^c	.90 \pm .03	2.79 \pm .23

- a. L-cysteine (Cys, ip) was administered 90 minutes prior to treatment with ethanol (EtOH, po) or acetaldehyde (Acet., ip). Antipyrine (100 mg/kg, ip) followed 20 minutes after ethanol or acetaldehyde treatment. Salivary antipyrine half-life ($t_{\frac{1}{2}}$), volume of distribution (V_d), and clearance (Q) were compared among treatment groups.
- b. Results are $\bar{X} \pm \text{SE}$.
- c. Significantly different ($p < .05$, LSD) from control.
- d. Significantly different ($p < .05$, LSD) from 2 g/kg ethanol alone.

faster half-life than the group which received ethanol alone 243 ± 14 minutes.

The group which received both ascorbic acid and acetaldehyde had an antipyrine half-life of 182 ± 13 minutes which is also similar to control values. This group was not statistically different from the group which received acetaldehyde alone (211 ± 13 minutes).

Thiamine-HCl (0.24 mmol/kg) pretreatment showed only a trend toward reducing the effect of ethanol on antipyrine half-life. Not statistically different from ethanol-alone, the thiamine pretreatment group (214 ± 29 minutes) falls between that of ethanol-alone (243 ± 14 minutes) and control (171 ± 5 minutes) groups.

The group which received both thiamine and acetaldehyde had an antipyrine half-life of 199 ± 18 minutes which is comparable to the control half-life. As was the case with ascorbic acid-acetaldehyde group the thiamine-acetaldehyde group was not statistically different from the acetaldehyde-alone group.

L-cysteine (2 mmol/kg) pretreatment prevented only ethanol-increased antipyrine half-life. A statistical difference was also apparent between ethanol-alone (243 ± 14 minutes) or cysteine pretreatment (200 ± 7 minutes) groups. Cysteine had no effect on acetaldehyde-increased antipyrine half-life.

Discussion

Dietary prevention of in vivo ethanol- or acetaldehyde-inhibited drug metabolism has the potential of clinical use for the management of ethanol toxicity and minimizing alcohol-induced hepatic disease states. In the case of ascorbic acid, cysteine, and thiamine they also suggest mechanisms by which ethanol inhibits MFO activity.

For the purpose of this discussion, all pretreatments with ascorbic acid, thiamine, or cysteine combined with either ethanol or acetaldehyde which had antipyrine half-life values statistically unchanged from controls are considered valuable for the management of ethanol or acetaldehyde inhibition of MFO activity. Therefore, ascorbic acid and cysteine were able to prevent ethanol-increased antipyrine half-life. Ascorbic acid and thiamine were able to interfere with acetaldehyde-increased antipyrine half-life.

Interindividual variations in response may explain the looseness in the data which interfered with tight statistic comparisons between treatment groups. This is best exemplified by the range of antipyrine half-life in control animals (120-180). A better study would be to use each rat as his own control. Antipyrine half-life has been shown to be reproducible in human males (Welch et al., 1975), but delays of 4-10 days between samples are needed to prevented antipyrine induction of MFO activity which results in antipyrine-decreased antipyrine half-life (Vesell, 1979).

Both ethanol and acetaldehyde significantly increase the salivary half-life of antipyrine. Compared with ethanol, acetaldehyde was a

more potent, but not as effective, inhibitor of antipyrine metabolism. Administration of acetaldehyde has also been reported as more potent (rat LD50_{acetaldehyde} = 1.93 g/kg versus rat LD50_{ethanol} = 10 g/kg) and toxic (degree physiologic impairment) than ethanol itself (Windholz, 1983 and Holtzmann and Schneider, 1974).

a) L-ascorbic acid as a possible protectant against acetaldehyde or ethanol hepatotoxicity (Figure 2.1).

Ascorbic acid deficiency is known to influence MFO drug metabolism. and Sato, 1975, have reported that Vitamin C deficient guinea pigs have a reduced capacity to catabolize xenobiotics. In guinea pigs, increasing the ascorbic acid intake resulted in a dose-related increase in drug metabolizing capacity (Peterson et al., 1983). There is a limit beyond which increasing ascorbic acid intake is without further effect (Sutton et al., 1982). This concentration of ascorbic acid (25 mg/100 g liver) is the rate of ascorbic acid synthesis in species such as the mouse and rat (Sato and Zannoni, 1974). This is consistent with data from these experiment which showed that 2 mmol/kg L-ascorbic acid pretreatment (203 \pm 19 minutes) had no significant effect of antipyrine half-life as compared with control (171 \pm 5 minutes).

Pretreatment with 2 mmol/kg L-ascorbic acid prevented both ethanol- and acetaldehyde-inhibited mixed-function oxidase activity toward antipyrine. Ascorbic acid may be affording protection either directly by binding with acetaldehyde or indirectly by its action as a strong reducing agent. Ascorbic acid may undergo nonenzymatic condensation with acetaldehyde as it does with formaldehyde (Sprince

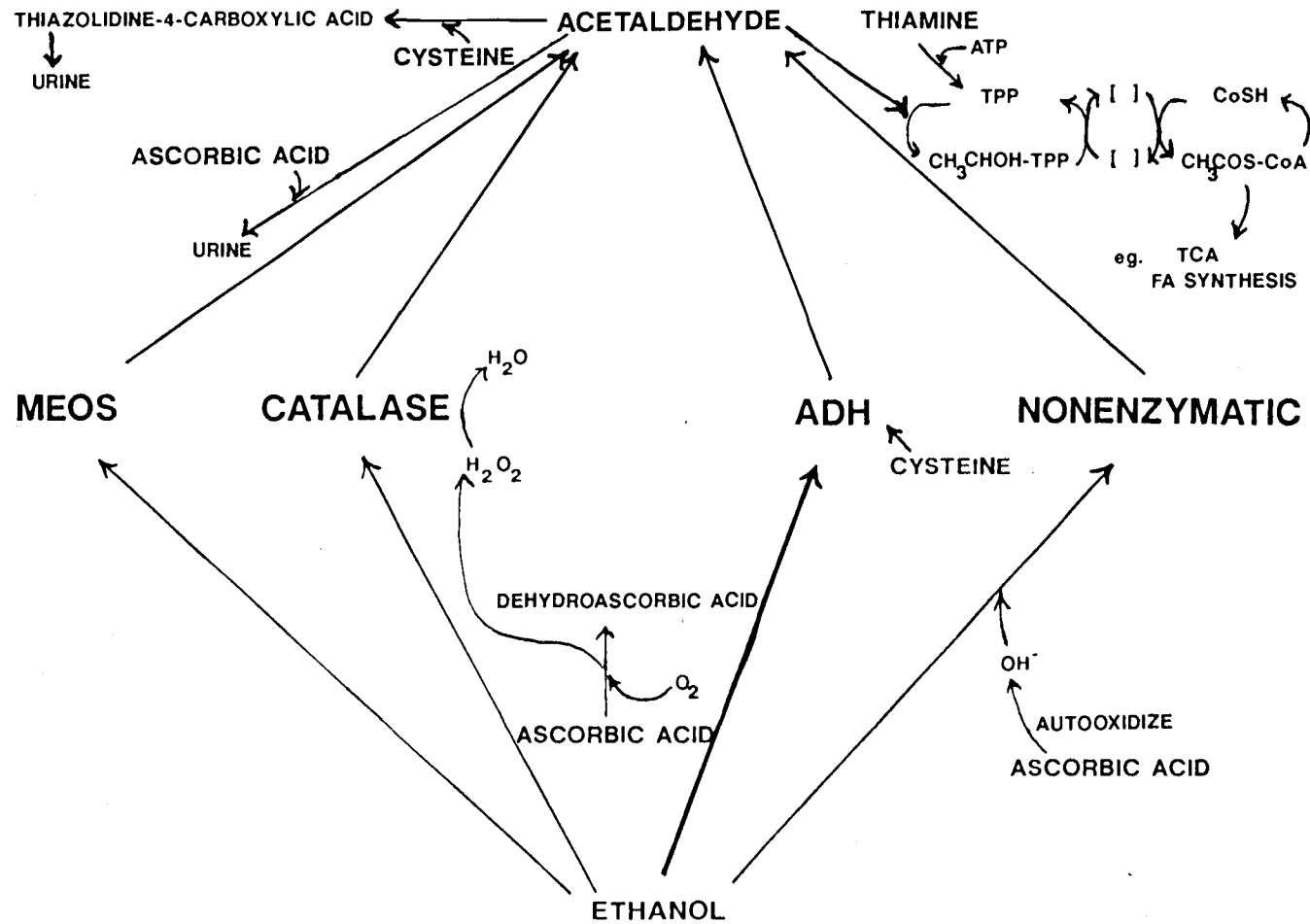


FIGURE 2.1: Proposed Mechanisms for the Effect of Ascorbic Acid, Thiamine, or Cysteine on the Inhibition by Ethanol or Acetaldehyde on the Half-life of Antipyrine.

et al., 1979). The resulting product formed may no longer be able to bind with cellular protein. Ascorbic acid may thereby block the inhibitory effect of acetaldehyde on mixed-function oxidase activity toward antipyrine.

Ascorbic acid condensation with acetaldehyde may also relieve the metabolite's back inhibition of alcohol dehydrogenase activity (Ryle et al., 1985 and 1987). Since the metabolism of ethanol can proceed unchecked by metabolite formation, the cellular ethanol concentration is effectively reduced. Direct inhibition of ethanol on drug binding with cytochrome P450 would therefore be decreased.

Ascorbic acid may also prevent ethanol-inhibited antipyrine metabolism by directly facilitating the metabolism of ethanol. Ascorbic acid reacts with oxygen nonenzymatically to form dehydroascorbic acid and hydrogen peroxide (Giblin, et al., 1984). The hydrogen peroxide then could facilitate the metabolism of ethanol by increasing catalase-hydrogen peroxide-dependent ethanol metabolism. This may explain why pretreatment with ascorbic acid an hour prior to ethanol (ip) decreased blood ethanol concentration (Yunice and Lindeman, 1977) since this decrease could not be accounted for by either decreased absorption of ethanol or increases in the volume of distribution of ethanol.

Since ascorbic acid is a strong antioxidant, it may autooxidize , generating hydroxyl radicals (Cohen, 1977) to be scavenged by ethanol for utilization in nonenzymatic oxidation to acetaldehyde (Feierman et al., 1985). Studies have indicated that the greater the generation of hydroxyl radicals in microsomal assays, the greater the rate of ethanol metabolism (Cederbaum et al., 1979 and Krikum et al.,

1984).

It appears that ascorbic acid is an effective protectant against ethanol-inhibited drug metabolism (antipyrine). This protection is likely afforded by decreasing the amount of ethanol at the enzyme (MFO) by increasing the rate at which ethanol is metabolized. This means less ethanol is present to bind with the enzyme to prevent MFO activity. Ascorbic acid also protects by removal of acetaldehyde, thereby preventing the acetaldehyde-inhibited drug metabolism.

b) Thiamine-HCl as a possible protectant against acetaldehyde or ethanol hepatotoxicity.

Pretreatment with thiamine-HCl seems to have reduced acetaldehyde-increased antipyrine salivary half-life. Although thiamine showed a trend toward lowering the half-life from that of ethanol treatment alone, the data was not statistically significant and thiamine failed to return the half-life to that of the control values.

Thiamine's capacity to reduce acetaldehyde-inhibited antipyrine metabolism could be related to thiamine's capacity to decrease acetaldehyde blood levels. Thiamine-HCl (0.24 mmol/kg) has been shown to reduce blood levels of acetaldehyde (Acholonu, 1983). Thiamine may inactivate acetaldehyde through the formation of thiamine pyrophosphate (TPP) which in turn complexes with acetaldehyde to eventually form acetyl Coenzyme A (Sprince et al., 1980).

The thiamine pretreatment data showed a trend toward reducing the ethanol-inhibited antipyrine metabolism, but was not statistically significant. Others have shown that the concentration of thiamine in

the diet affects ethanol concentrations in blood, liver, and hearts of rats given a po dose of 2 g/kg ethanol (Abe et al., 1979). When comparison was made between thiamine deficient and sufficient groups, the blood ethanol concentrations were higher in the deficient groups than the corresponding sufficient groups. Acholonu, 1983, showed that 0.24 mmol/kg thiamine was able to reduce blood levels of ethanol as well as acetaldehyde. Thiamine may also do this by trapping acetaldehyde as acetyl Coenzyme A and thereby release acetaldehyde inhibition of ethanol metabolism causing an increase in the rate by which ethanol is metabolized.

c) L-cysteine as a possible protectant against acetaldehyde and ethanol hepatotoxicity.

The response to pretreatment with L-cysteine was different for ethanol and acetaldehyde treated rats. Only the ethanol-inhibited mixed-function oxidase activity toward antipyrine was decreased. L-cysteine pretreatment did not decrease the acetaldehyde-inhibited antipyrine metabolism.

Cysteine has been shown to delay the absorption of ethanol when administered by the same route but not when cysteine was given ip and ethanol po (Nagasawa et al., 1977 and Beauge et al., 1976). Therefore delay in absorption of ethanol can not be a factor in the decrease of ethanol-increased antipyrine salivary half-life by cysteine in this experiment.

Though cysteine does not affect the absorption of ethanol it still may be interfering with the metabolism of ethanol. Cysteine in

vivo accelerates the clearance of ethanol from both the blood and the liver (Hirayama et al., 1983). By removing ethanol, it may be removing the direct effect of ethanol on MFO drug metabolism.

Cysteine may be influencing the metabolism of ethanol by either direct interaction with alcohol dehydrogenase or indirectly by the sequestering of acetaldehyde. Cysteine has been shown to activate alcohol dehydrogenase through enhancing the binding of ethanol to the enzyme (Harris, 1964 and Theorell, 1967). More likely is that cysteine forms an adduct with acetaldehyde (Cederbaum and Rubin, 1976), thus removing the acetaldehyde generated in ethanol metabolism. Because the equilibrium constant favors the reduction of acetaldehyde rather than the oxidation of ethanol, the cysteine-acetaldehyde complex shifts the enzymatic equilibrium resulting in accelerated ethanol oxidation (Hirayama et al., 1983).

Cysteine can also decrease the peak blood acetaldehyde level generated by the metabolism of ethanol (Acholonu, 1983). Because cysteine can react with acetaldehyde it is tempting to speculate that acetaldehyde therefore binds with cysteine which protects against the binding of acetaldehyde with microsomal protein responsible for drug metabolism. 5mM L-cysteine has been shown to decrease metabolically derived acetaldehyde protein binding in liver homogenates by 50% (Donohue et al., 1983).

Although cysteine can react with acetaldehyde, these experiments failed to show an effect by cysteine on acetaldehyde-inhibited antipyrine metabolism. One possible explanation is that the 6 mmol/kg acetaldehyde dose exceeded the capacity of 2 mmol/kg cysteine to protect against acetaldehyde toxicity. Increasing the dose of

cysteine was not possible due to increased mortality. Another explanation may be related to cysteine's rapid catabolism and/or the readily dissociation in vivo of the thiazolidine-4-carboxylic acid formed by its condensation with acetaldehyde (Nagasawa et al., 1987).

IN CONCLUSION, it appears that ascorbic acid and cysteine provided the most effective protection against ethanol inhibition of antipyrine metabolism in vivo. The data provided through pretreatment with either ascorbic acid, thiamine, or cysteine strongly suggests that the protection of ethanol-inhibited antipyrine metabolism appears to be through interference with the direct effect of ethanol on antipyrine metabolism. This either by increasing the metabolism of ethanol directly and/or by removing acetaldehyde's inhibition of alcohol dehydrogenase.

CHAPTER 3: The Effect of Ethanol or Acetaldehyde on Antipyrine or Aminopyrine Metabolism by Isolated Rat Liver Microsomes.

Summary

The influence of ethanol or acetaldehyde on the oxidation of antipyrine or aminopyrine by isolated rat liver microsomes was examined. Low concentrations of either ethanol (10 mM) or acetaldehyde (1.4 mM) did not inhibit the in vitro metabolism of either aminopyrine or antipyrine as seen in these sets of experiments. In fact, an increase in 3-hydroxymethylantipyrine formation was observed at these concentrations. Higher concentrations of ethanol (50 mM) did not inhibit the metabolism of antipyrine as measured by the oxidation of NADPH to NADP^+ by the isolated microsomes. Of the metabolites of antipyrine studied, norantipyrine and 3-hydroxymethylantipyrine formation were inhibited at 50 mM concentration of ethanol. Acetaldehyde (7.2 mM) inhibited only the hydroxylation of antipyrine to 3-hydroxymethylantipyrine.

Introduction

There is extensive clinical value in monitoring antipyrine or aminopyrine salivary half-life as a tool for assessing liver damage and the effect of exogenous substances on drug metabolism. Monitoring antipyrine salivary half-life is also a handy tool for assessing means of managing the effect of ethanol or acetaldehyde on drug metabolism. By managing or preventing the toxic effect of ethanol or acetaldehyde on drug metabolism, the mechanism by which ethanol influences drug metabolism can be explored.

Although the in vivo study of ethanol-influenced drug metabolism is necessary, inferred mechanisms of action should be also be studied in vitro. Studies in vivo may be confused by physiological ramifications of the administered compound as well as the cellular and subcellular inhibition of drug metabolism. Physiological changes which confuse mechanistic studies can included changes in blood flow, hormonal secretions, and neurological effects to name a few. Since physiological changes may overshadow the mechanism involved at the enzyme level, research of ethanol or acetaldehyde inhibition of drug metabolism should therefore be compared in vivo and in vitro.

The microsomal ethanol oxidizing system plays a significant role in the oxidation of ethanol at high cellular ethanol concentrations and appears to be responsible for the acceleration of the rate of ethanol elimination observed in the heavy drinker (Lieber and DeCarli; 1968, 1969, and 1970). Most lipophilic drugs are also metabolized by mixed-function oxidases of the hepatic smooth endoplasmic reticulum

(SER). Observations of kinetic interactions between ethanol and drugs have therefore been ascribed to induction and inhibition occurring at the mixed-function oxidases in the SER (Strubelt, 1980 and 1984; Mezey, 1976; Sellers and Holloway, 1978; Coon et al., 1984; and Hayashi et al., 1985).

An ever increasing number of studies are documenting the inhibition of drug metabolism by ethanol in vitro. Molar concentrations of ethanol can inhibit MFO activity toward both type I and type II compounds. Since a large concentration of ethanol is required, this nonspecific inhibition is probably acting through membrane perturbation or protein denaturation (Cohen and Mannering, 1973 and Cinti et al., 1973).

The effect of ethanol on xenobiotic biotransformation appears to be dose dependent. At lower, physiologically and biochemically significant, in vitro concentrations, the inhibitory effect of ethanol is selective. This suggests a more specific action of ethanol, in vitro, when concentrations are in the millimolar range than has been observed when ethanol is given at the higher, molar concentrations. With some systems (eg. aniline hydroxylase) the inhibition by ethanol is competitive in nature (Rubin et al., 1970a). Pentobarbital hydroxylase shows a mixed-type of inhibition by ethanol (Rubin et al., 1970a). P-hydroxylation of acetanilide is inhibited at low concentrations while N-demethylation of ethylmorphine, O-de-ethylation of acetophenetidin, and N-demethylation of p-chloro-N-methylaniline are not inhibited by ethanol in mmolar concentrations (Cohen and Mannering, 1973).

The evidence for the role of acetaldehyde in ethanol inhibited

drug metabolism lags behind that being compiled for ethanol. High concentrations of acetaldehyde inhibit p-nitroanisole O-demethylation in isolated hepatocytes. Physiological concentrations do not affect the metabolism of p-nitroanisole (Dicker and Cederbaum, 1982). The requirement of mmolar concentrations of acetaldehyde probably indicate a nonspecific inhibition of drug metabolism, as with high concentrations of ethanol.

The following set of experiments investigate the influence of ethanol or acetaldehyde on the oxidation of antipyrine or aminopyrine in isolated rat liver microsomes. Three assays were used to examine the effect of ethanol or acetaldehyde on antipyrine metabolism in vitro. The conversion of NADPH to NADP^+ was monitored as an indicator of total MFO activity toward antipyrine. N-demethylation of antipyrine to norantipyrine was studied through quantification of formaldehyde formed. Norantipyrine, 3-carboxyantipyrine, and 3-hydroxymethylantipyrine metabolites of antipyrine were successfully quantified via HPLC peak separation. 4-hydroxyantipyrine could not be successfully separated from antipyrine by this method. Aminopyrine N-demethylation was studied through quantification of formaldehyde formed.

Materials and Methods

Male Sprague-Dawley rats weighing 200-300 g were fed OSU laboratory chow (Appendix One). The animals were killed by cervical dislocation.

MICROSOMAL PREPARATION. The following steps were performed at 4° Centigrade. Livers were removed and homogenized with a Rockwell drill press in 0.25 mM sucrose. The homogenate was then centrifuged using a Beckman Model J2-21 centrifuge fitted with a JA17-123 rotor at 11,000 x g for 15 minutes. The supernatant was decanted and recentrifuged for 15 minutes at 15,000 x g. The microsomes were pelleted from this supernatant using a Beckman Model L3-50 Ultracentrifuge equipped with a 42.1 rotor at 105,000 x g for one hour. The pellet was resuspended in 0.1M Tris/1.15% KCl buffer, pH 7.4 and resedimented at 105,000 x g for one hour. The final microsomal pellet was suspended in the Tris/KCl buffer, pH 7.4, in a final volume of 1 ml buffer per 1 g initial liver weight. Protein was determined by the method of Lowry et al., 1951. Spectral determination of cytochrome P450 protein was performed on the Aminco DW-2A UV Spectrophotometer. All microsomal suspensions were kept cold on ice and used within 2 hours of isolation.

ASSAY FOR AMINOPYRINE N-DEMETHYLATION. Aminopyrine metabolism was measured as formaldehyde formed from N-demethylation. The assay mixture contained a final concentration of 5 mM MgCl₂, 1 mM NADP⁺, 5 mM glucose-6-phosphate, 4.9 mM semicarbazide, 0.1 M Tris/1.15% KCl buffer, pH 7.4, 1.5 mg/ml microsomal protein and 2 IU/ml

glucose-6-phosphate dehydrogenase in a total volume of 3.0 mls. The reaction was started by the addition of aminopyrine (0.008-5.0 M final concentration). Triplicate flasks were capped and incubated 15 minutes at 37° C in a Forma Scientific CH/P Model 2564 shakerbath at 100 cycles/minute under air. Blanks were run as boiled blanks. The reaction was stopped by transfer to an ice bath and the addition of 1.0 ml of 8.9% ZnSO_4 , 1.5 ml saturated Ba(OH)_2 , and 0.5 ml saturated $\text{Na}_2\text{B}_4\text{O}_7$. Aminopyrine N-demethylase activity was expressed as the amount of formaldehyde formed per mg protein per minute (Nash, 1953). The reaction was linear to 20 minutes and 2.0 mg protein per ml.

ASSAY FOR TOTAL ANTIPYRINE METABOLISM: Total antipyrine metabolism was measured by monitoring the conversion of NADPH to NADP^+ . Assays were performed using matched cuvettes by the method of Stratland et al., 1973. The reference cuvette contained no antipyrine whereas the sample cuvette had a concentration of 16 mM antipyrine. The 645 μ ls of reaction mixture in each cuvette contained a final composition of 5 mM MgCl_2 , 0.1 M Tris/1.15% KCl buffer, pH 7.4, 4-6 mg protein/ml, and 100 μ M NADPH. The change in absorbance was recorded on an Aminoco DW-2A UV Vis Spectrophotometer for 10 minutes at 37° C at 365 nm. Activity was computed over 10 minutes and expressed as nmol NADPH oxidized per mg protein per minute.

ASSAY FOR ANTIPYRINE N-DEMETHYLATION: Antipyrine undergoes N-demethylation to norantipyrine. Quantification of norantipyrine was measured as formaldehyde formed per mg protein per minute by the Nash reaction (1953- see assay for aminopyrine N-demethylation). The reaction mixture was incubated with 24 mM antipyrine and was linear to 4.5 mg protein and 30 minutes.

ASSAY FOR METABOLITES OF ANTIPYRINE: Antipyrine metabolism to norantipyrine, 4-hydroxyantipyrine, 3-hydroxymethylantipyrine, 3-carboxyantipyrine, and 4,4'-dihydroxyantipyrine were measured in a total incubation volume of 1 ml containing the NADPH generating system previously described (see aminopyrine N-demethylase assay). Triplicate flasks containing 24 mM antipyrine and 3 mg/ml protein were capped and incubated 12.5 minutes at 37° C in the Forma Scientific CH/P Model 2564 shakerbath at 100 cycles/minute under air. The reaction was stopped and protein precipitated with 1 ml of saturated BaSO₄ solution. Samples were then centrifuged at 1000 x g. The supernatant was removed to which 5-10 mg sodium metabisulfite and 1.0 ml 1 M NaOH were added.

Unmetabolized antipyrine was removed by double extraction with 20 ml of chloroform. The aqueous layer was then adjusted to pH 7.0 with 1.0 ml 1 M phosphate buffer, pH 7.0. Metabolites were extracted sequentially with 10 mls ethyl acetate and 10 mls dichloromethane. The organic phases were blown to dryness sequentially in conical glass tubes (15 ml) in a stream of nitrogen at 60° C. Samples were reconstituted in 300 μ ls methanol.

Samples were analyzed with a Waters Associates High Pressure Liquid Chromatograph fitted with a 30 cm reverse phase μ Bondapak (C₁₈) column and a 7 cm (C₁₈) guard column. Absorbance was read by a Waters Absorbance Detector Model 440 at 254 nm using absorbance units full scale of 0.02-.005. Injections were made with a Waters WISP 710B automatic injector system. Optimal peak separation was obtained with a 12% methanol, 87% water, 1% acetic acid, and PIC B7 mobile phase at a flow rate of 2.2 ml/min. Retention times were 2.2 minutes for

3-carboxyantipyrine, 4.2 minutes for 3-hydroxymethylantipyrine, 6.6 minutes for 4,4'-dihydroxyantipyrine, 9.4 minutes for norantipyrine, 10.9 minutes for 4-hydroxyantipyrine, and 11.2 minutes for antipyrine. 4,4'-dihydroxyantipyrine was not produced in sufficient quantity for study. 4-hydroxyantipyrine was only separated from antipyrine when less than 0.5% of the initial antipyrine remained. Extraction procedures removed only 97% of the total antipyrine. Therefore, it was not possible to study 4-hydroxyantipyrine by this method.

ETHANOL AND ACETALDEHYDE INHIBITION: Triplicate samples with ethanol, 10 mM or 50 mM final concentration, or acetaldehyde, 1.4 mM or 7.2 mM final concentration, were compared with that of control values for each in vitro assay. Ethanol or acetaldehyde was added after flasks were capped to prevent evaporation during the course of incubation. All data was analyzed by the Student's t test.

PRODUCTION OF ACETALDEHYDE FROM ETHANOL: Ethanol is metabolized by the microsomal ethanol oxidizing system to acetaldehyde. Therefore, it is necessary to know the quantity of acetaldehyde generated in those flasks containing ethanol. Reactions were carried out in center-well flasks made with epoxy glued plastic caps inside stoppered 25 ml Erlenmeyer flasks. The center-well contained 0.6 ml of 15 mM semicarbizide-HCl in 180 mM potassium phosphate, pH 7.4. The reaction blank was the same as above minus drug. Reactions were terminated by the addition of trichloroacetic acid to a final concentration of 4.5% w/v. The sealed flasks were incubated 24 hours at room temperature. 0.2 ml aliquots of the center-well contents were diluted to 3 mls with water and the absorbance of the aldehyde-semicarbazone complex determined at 224 nm (Cederbaum and

Cohen, 1984). Standard Curves were prepared by adding known amounts of acetaldehyde to boiled blank controls. The assay was found to be linear to 900 nmoles of acetaldehyde.

MATERIALS: Antipyrine and aminopyrine were purchased from Sigma Chemical Company. Norantipyrine (Alfred Bader Library of Rare Chemicals) and 4-hydroxyantipyrine were purchased from Aldrich Company. 3-hydroxymethylantipyrine and 4,4'-dihydroxyantipyrine were a generous gift from Dr. D.D. Breimer, Sylvius Laboratories, Leiden, The Netherlands. 3-carboxyantipyrine was a generous gift from Dr. H. Bassmann, Institute of Pharmacology and Toxicology, Braunschweig, West Germany. Acetaldehyde was obtained from Mallinkrodt. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (E.C.1.1.1.4.9) and nicotine adenine dinucleotide phosphate were purchased from Cal Biochem Behring. 1-hexane sulfonic acid counter ion PIC B7 was purchased from Waters and Associates. All other chemicals were purchased from Sigma Chemical Company.

Results

AMINOPYRINE N-DEMETHYLATION: The colorimetric evaluation of formaldehyde formation (Nash, 1953) for studying aminopyrine N-demethylation in vitro yields comparable results to radiometric and high pressure liquid chromatographic assays (Bast and Noordhoek, 1981). The Nash reaction was therefore used to analyze the effect of ethanol or acetaldehyde on the in vitro metabolism of aminopyrine. The assay mixtures contained 1.5 mg microsomal protein/ml and 15 minutes incubation time. The microsomes were preincubated with 10 mM ethanol, 1.4 mM acetaldehyde, or buffer control for 10 minutes prior to the addition of aminopyrine. This allowed time for complete mixing and decreased the variation between samples in the same treatment group.

Controls were compared with that of either 10 mM ethanol or 1.4 mM acetaldehyde. Aminopyrine metabolism was not significantly inhibited by any of these treatments (Fig 3.1). Others have found that aminopyrine is only inhibited in vitro by high concentrations of ethanol (Rubin et al., 1970b).

ANTIPYRINE METABOLISM IN VITRO: A literature search failed to turn up a well established, accurate method for studying antipyrine metabolism in vitro. Therefore three assays were used to investigate the inhibition of ethanol or acetaldehyde on the metabolism of antipyrine in vitro. The oxidation of NADPH to NADP^+ was used as a monitor of total antipyrine metabolism and mixed-function oxidase activity. N-demethylation to norantipyrine was checked by quantifying

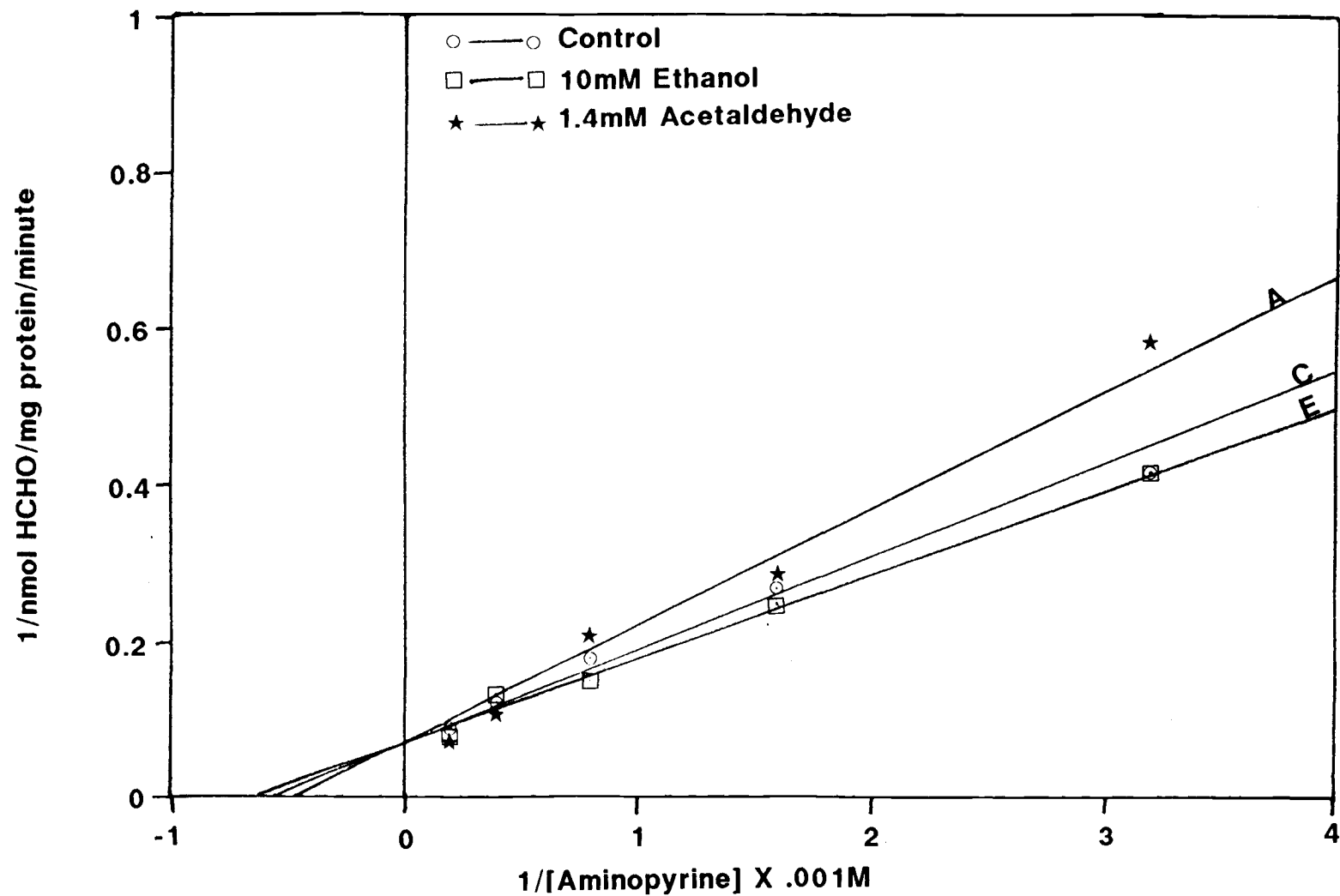


FIGURE 3.1: Effect of Ethanol or Acetaldehyde on the *In vitro* Metabolism of Aminopyrine. Treatment groups not statistically different from control as compared by student's t test.

formaldehyde formed. High pressure liquid chromatography was used in an attempt to quantify known metabolites of antipyrine metabolism.

Monitoring the disappearance of NADPH as a method to examine total antipyrine metabolism is greeted with criticism in the literature (McManus and Ilett, 1979). No stoichiometric relationship between NADPH disappearance and antipyrine metabolism has been consistently demonstrated. Despite this criticism, few alternative, reproducible methods have been developed to study the metabolism of antipyrine in vitro.

Isolated microsomes were incubated with 16 mM antipyrine. An average of $8.78 \pm .19$ nmol/mg protein/minute of NADPH disappeared in control cuvettes (Table 3.1). Neither ethanol (10 mM or 50 mM) or acetaldehyde (1.4 mM or 7.2 mM) had any effect on the disappearance of NADPH. These in vitro results were not consistent with in vivo inhibition of antipyrine metabolism documented in Chapter One. It is possible ethanol or acetaldehyde metabolism by the microsomes also caused NADPH to be oxidized as in other cytochrome P450 moderated reactions, thereby preventing detection of ethanol or acetaldehyde inhibition of antipyrine metabolism by this method.

Since the Nash reaction can also be used to monitor the N-demethylation of antipyrine to norantipyrine, the effect of ethanol or acetaldehyde on N-demethylation of antipyrine was studied using 3.0 mg protein/ml with an incubation time of 12.5 minutes (Figure 3.2). Samples were incubated 5 minutes with either ethanol or acetaldehyde prior to the addition of antipyrine. This allowed enough time for each sample to completely mix and decreased the variation between like-treated samples.

Table 3.1 Oxidation of NADPH to NADP⁺ as an indicator of total Antipyrine metabolism. Measured as nmol NADPH oxidized/mg protein/min.

Treatment	no. samples	range	mean \pm standard error
Control	7	8.03-9.60	8.78 \pm .19
Ethanol			
10 mM	6	8.92-9.88	9.34 \pm .16
50 mM	6	8.23-9.60	9.02 \pm .22
Acetaldehyde			
1.4 mM	5	8.57-8.74	8.92 \pm .11
7.2 mM	5	8.62-9.26	8.96 \pm .13

None of the experimental groups are statistically different from controls ($p > .05$).

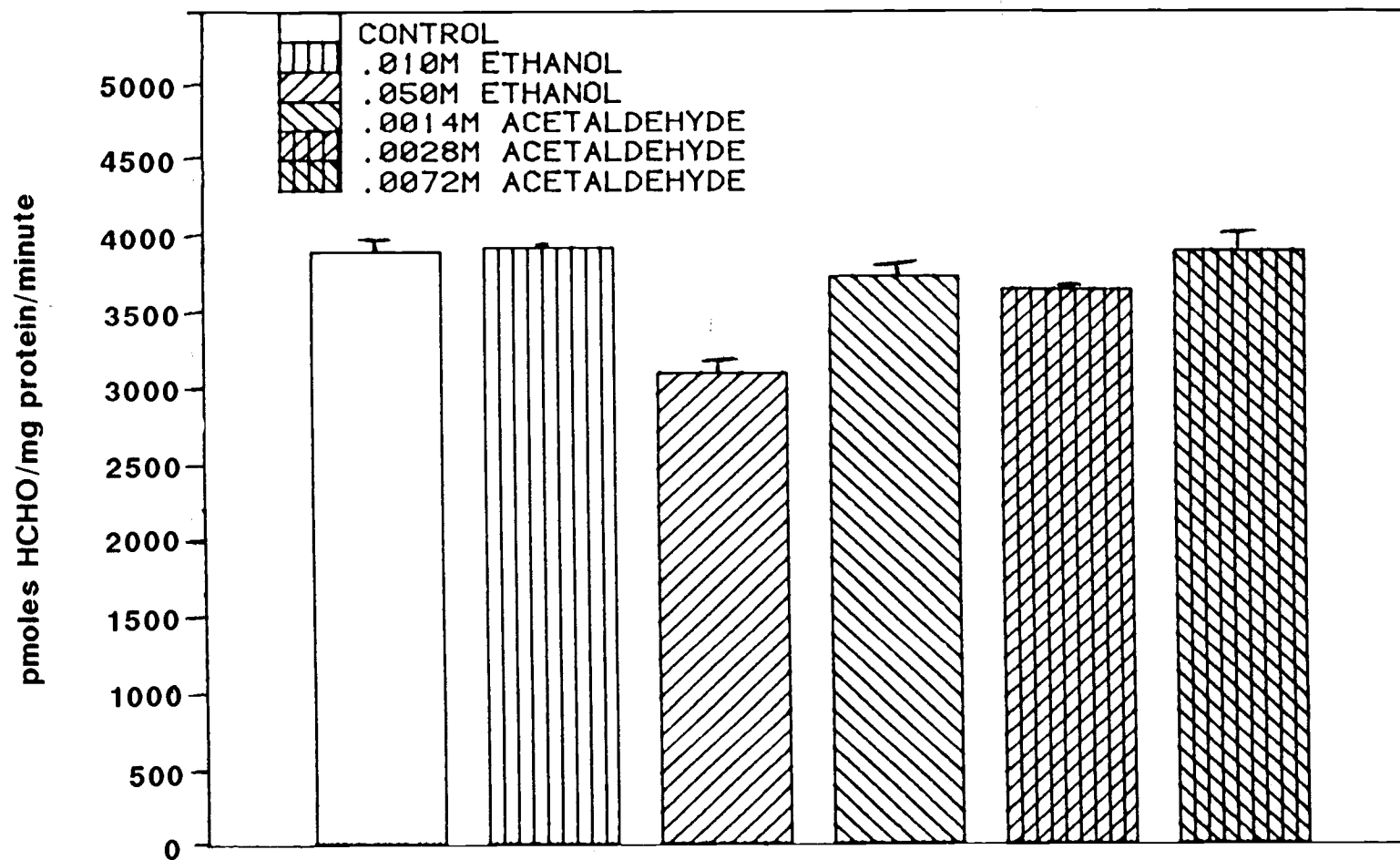


FIGURE 3.2: N-demethylation of Antipyrine In vitro. Control verses 5 minutes preincubation with ethanol or acetaldehyde. Values represent formaldehyde formed during incubation of 24 mM antipyrine with 3 mg protein for 12.5 minutes. Ethanol or acetaldehyde was added 5 minutes prior to the addition of antipyrine in order to completely mix the samples.

Control values (3787 ± 69 pmoles/mg protein/minute formaldehyde formed) were compared with values for those samples incubated with ethanol (10 mM or 50 mM) or acetaldehyde (1.4 mM, 2.8 mM, or 7.2 mM). Antipyrine N-demethylation in vitro was significantly inhibited (20%) only by 50 mM ethanol. Acetaldehyde was not able to inhibit the formation of formaldehyde from antipyrine.

Three of the known metabolites of antipyrine were in concentrations which could be quantitated by the above HPLC method. These metabolites could be detected in a linear fashion over the standard curve tested (Figure 3.3). Norantipyrine, 3-hydroxymethylantipyrine, and 3-carboxyantipyrine were each compared in control and ethanol or acetaldehyde incubated samples (Figure 3.4). No effect was seen on 3-carboxyantipyrine formation by any treatment.

In control microsomes norantipyrine was formed at a rate of 3790 pmoles/mg protein/minute. This value compares favorably with formaldehyde formed of 3787 ± 69 pmoles/mg protein/minute. When treatment was compared with control values, only 50 mM ethanol statistically inhibited norantipyrine formation with only 300 pmoles/mg protein/minute of norantipyrine measured.

The effect of 50 mM of ethanol on antipyrine N-demethylation was studied by monitoring both formaldehyde (3022 ± 80 pmoles/mg protein/minute) and norantipyrine (300 pmoles/mg protein/minute) formed. Unlike the control values, the values of ethanol-inhibited N-demethylation measured by the two methods do not agree. It is possible that ethanol metabolism to acetaldehyde accounts for the high values monitored through the use of the Nash reaction, although this could not be verified when known concentration of acetaldehyde were

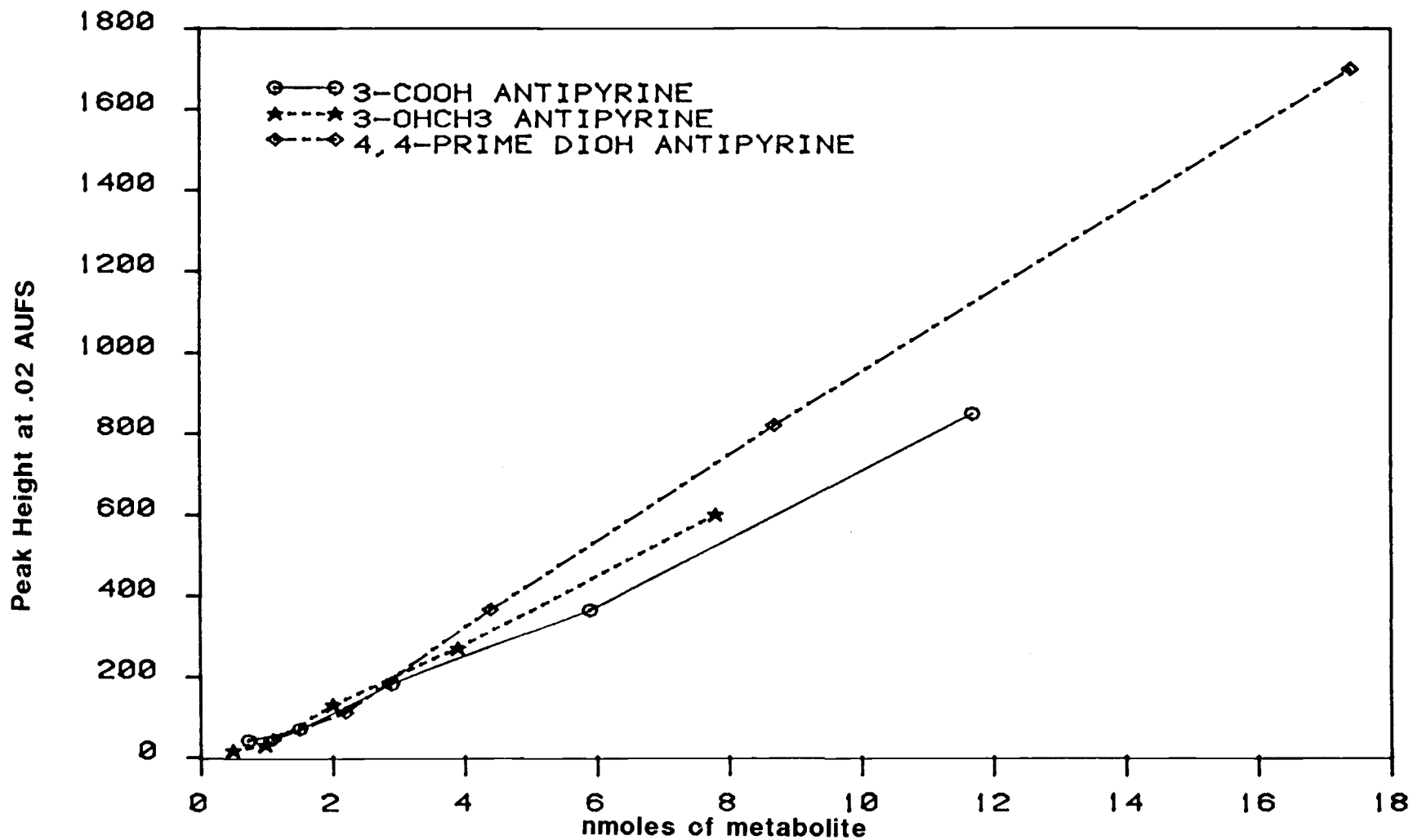


FIGURE 3.3: Standard Curves for 3-carboxyantipyrene (3-COOH), 3-hydroxymethylantipyrene (3-OHCH3), and 4,4'-dihydroxyantipyrene (4,4-prime DIOH).

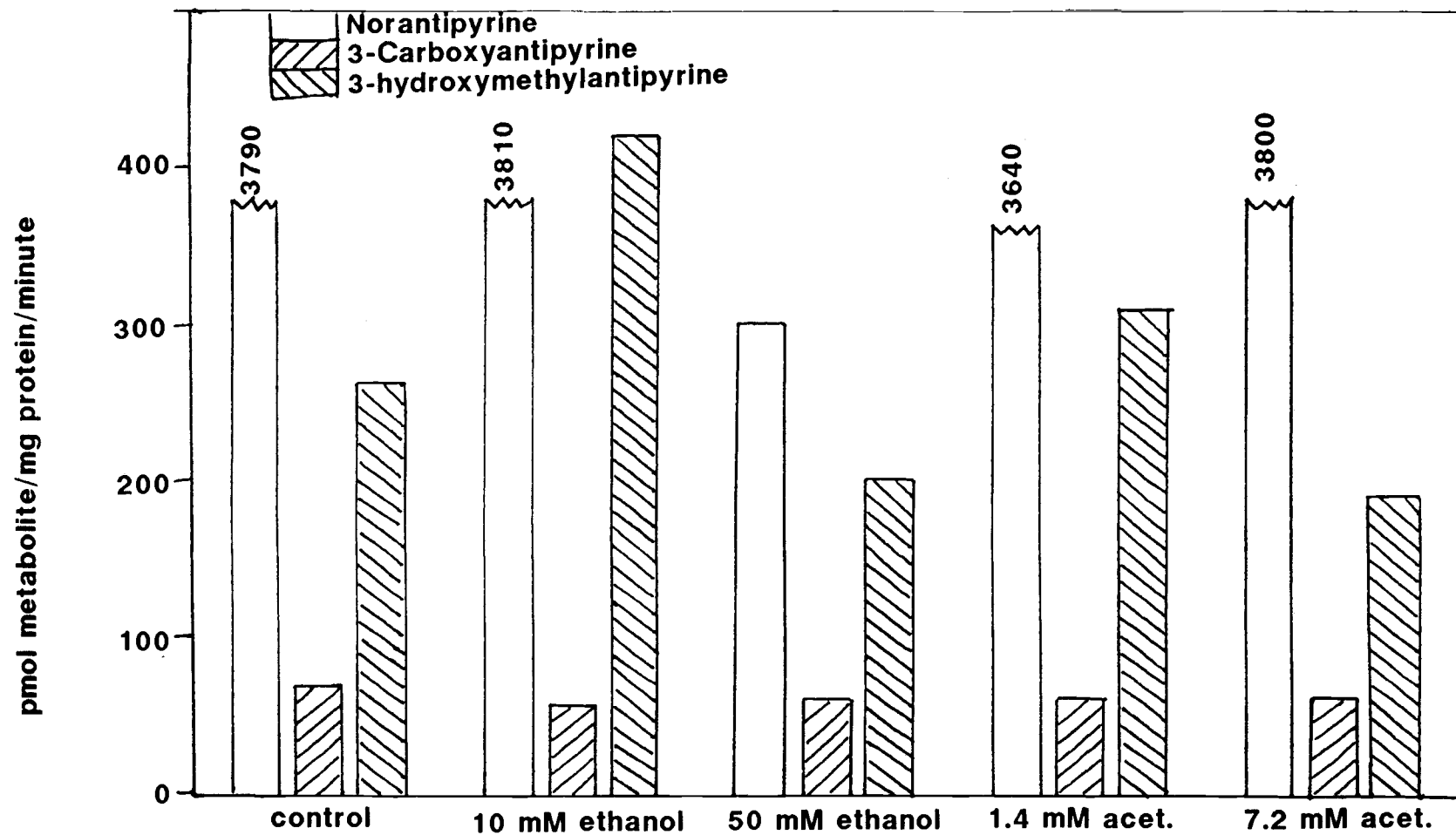


FIGURE 3.4: Effect of Acetaldehyde or Ethanol on the Formation of 3 Metabolites of Antipyrine In vitro.

analyzed using the Nash reaction.

A mixed response to the effect of ethanol or acetaldehyde on 3-hydroxymethylantipyrine formation was seen. Compared with control values (263 pmoles/mg protein/minute), 10 mM of ethanol increased (57%) the production of 3-hydroxymethylantipyrine to 420 pmoles/mg protein/minute while 50 mM ethanol decreased (24%) it to 200 pmoles/mg protein/minute. 1.4 mM acetaldehyde increased (14%) the production to 300 pmoles/mg protein/minute while 7.2 mM acetaldehyde decreased (28%) production to 190 pmoles/mg protein/minute.

The mixed response of 3-hydroxymethylantipyrine formation was not observed by the other metabolites of antipyrine. It is possible that at low concentrations of ethanol or acetaldehyde the enzyme responsible for hydroxylation of antipyrine at position three has a more favorable stereochemistry, thus accounting for greater production of 3-hydroxymethylantipyrine. At higher concentrations of ethanol or acetaldehyde enzyme may be inhibited.

Antipyrine was originally selected to study the effect of ethanol or acetaldehyde on hydroxylation, primarily to 4-hydroxyantipyrine. This metabolite could not be studied by the above method. If hydroxylation at the four and three positions is through the same pathway (enzyme), it is possible that low concentrations of ethanol or acetaldehyde block the metabolism of antipyrine to 4-hydroxyantipyrine in favor of 3-hydroxymethylantipyrine formation. Higher concentrations of ethanol or acetaldehyde would likely interfere with either metabolite formation.

ETHANOL METABOLISM IN VITRO: Because ethanol can be metabolized in vitro by microsomes, the amount of acetaldehyde generated was

measured. Both the 50 mM and 10 mM ethanol were tested for acetaldehyde generated over a period of 30 minutes (Figure 3.5). The highest levels of acetaldehyde generated were 210 ± 5 μ moles and 194 ± 26.9 μ moles respectively.

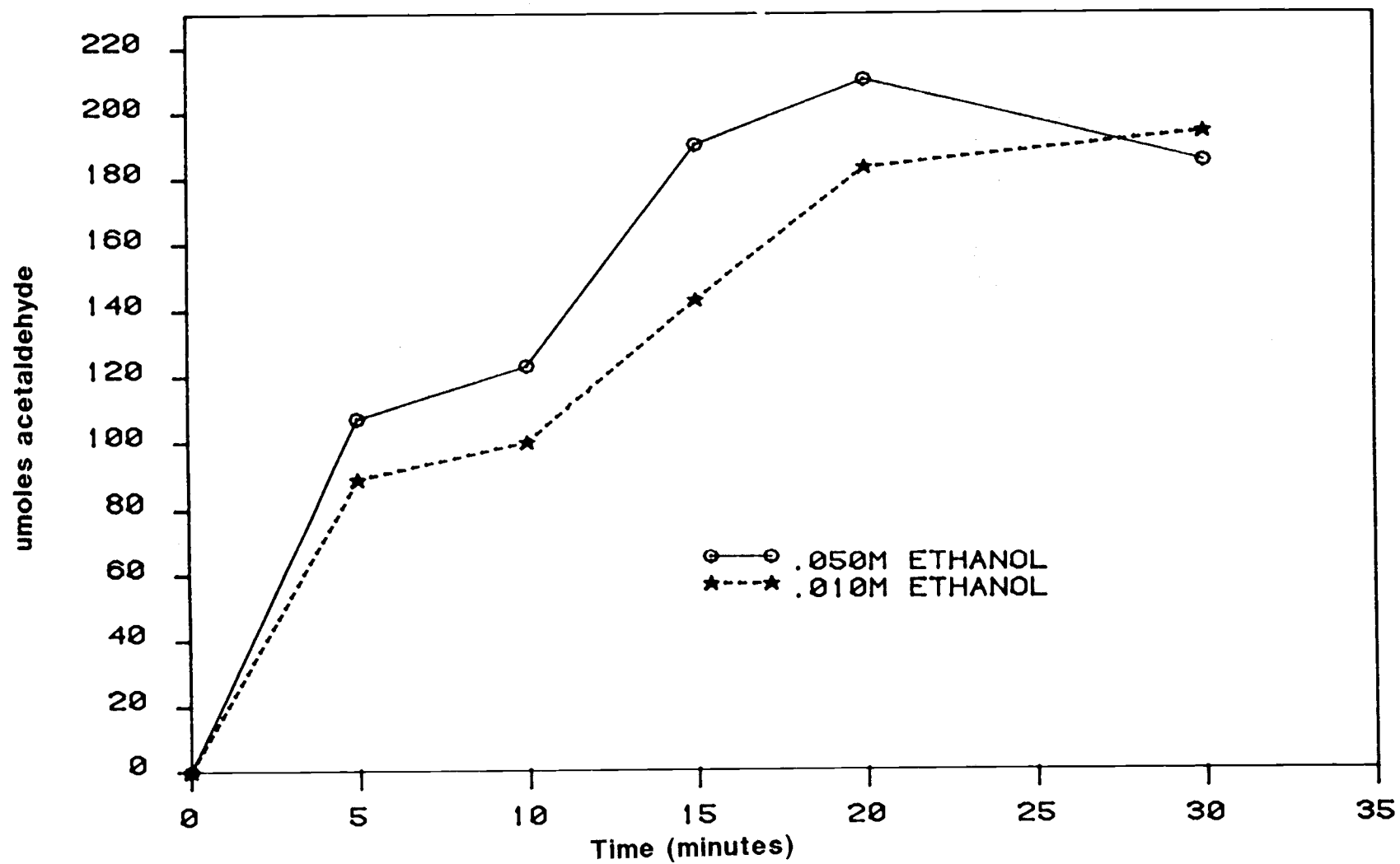


FIGURE 3.5: In vitro Formation of Acetaldehyde from 10 mM or 50 mM Ethanol.

Discussion

The inhibition of ethanol or acetaldehyde on drug metabolism in vitro is only significant when studied at physiologically significant concentrations. An intracellular concentration of ethanol (10mM from 2 g/kg) is physiologically attainable under conditions of ethanol intoxication. The concentration of circulating acetaldehyde (1.4 mM from 6 mmol/kg) may be physiologically attainable only after acetaldehyde administration, but not after ethanol administration even when the amount from ethanol plus cigarette smoking generated acetaldehyde is considered. The higher concentration of ethanol (50 mM from 6 g/kg) used is attainable without death in the Sprague-Dawley rat but rarely seen in humans (Pennington et al., 1978, Eriksson, 1983, and Espinet and Argiles, 1984). Acetaldehyde (7.2 mM from 40 mmol/kg) is above the 24 hour LD90 for Sprague-Dawley rats (Sprince, 1985).

Low concentrations of either ethanol (10 mM) or acetaldehyde (1.4 mM) were unable to inhibit the in vitro metabolism of either aminopyrine or antipyrine as described herein. In fact an increase in 3-hydroxymethylantipyrine formation was observed at these concentrations.

Neither 50 mM ethanol or 7.2 mM acetaldehyde inhibited the net metabolism of antipyrine. Of the metabolites of antipyrine studied, norantipyrine and 3-hydroxymethylantipyrine formation were inhibited by this concentration of ethanol. Acetaldehyde (7.2 mM) inhibited only 3-hydroxymethylantipyrine.

AMINOPYRINE N-DEMETHYLATION: The lack of effect of either ethanol or acetaldehyde on aminopyrine metabolism in vitro agrees with data in vivo following salivary aminopyrine half-life (Chapter 1). Studies of others have found that 100 mM ethanol is necessary to inhibit the metabolism of aminopyrine in vitro (Rubin et al., 1970b and Cinti et al., 1973).

Ranges of acetaldehyde (.010 mM-10 mM) previously tested by (Cinti et al., 1973) have also not inhibited the in vitro metabolism of aminopyrine. High concentrations of ethanol (50 mM) and acetaldehyde (>.400 mM) are difficult if not impossible to attain in vivo (Pennington et al., 1978, Eriksson, 1983, and Espinet and Argiles, 1984).

The inability of low concentrations of ethanol or acetaldehyde to affect in vitro metabolism of aminopyrine is not unexpected. This correlates well with studies of other type I drugs which are also not inhibited in vitro by low concentrations of ethanol (Rubin et al., 1970b, Reinke et al., 1983, and Cohen and Mannering, 1973).

One possible mechanism proposed for ethanol inhibited drug metabolism is a competition between the drug and ethanol for the substrate binding site of cytochrome P450. Ethanol has a modified type II binding spectra (Cohen and Mannering, 1973) and does not prevent the binding of type I substrates with cytochrome P450 (Rubin et al., 1971). Aminopyrine is a type I substrate whose binding spectra is not affected by the presence of ethanol or acetaldehyde (data not shown). Geometric details of the enzyme-substrate complex between cytochrome P450 and aminopyrine indicate a loose binding (Woldman et al., 1985). This binding may not be affected by

concentrations of ethanol or acetaldehyde at the level of the enzyme which are attainable in vivo.

ANTIPYRINE METABOLISM: Many type II drugs are inhibited by ethanol (Cohen and Mannering, 1973). It should be anticipated that antipyrine would be similarly affected, particularly hydroxylation of antipyrine. In vivo, both ethanol and acetaldehyde inhibited the salivary half-life of antipyrine suggesting some effect on MFO metabolism (Chapter One).

Low concentrations of ethanol or acetaldehyde did not inhibit the in vitro metabolism of antipyrine as measured by these analytical methods. The inability of the analytical methods to quantify 4-hydroxyantipyrine may be a significant factor in interpreting these results. This metabolite would be predicted to be most affected by ethanol or acetaldehyde since it results from cytochrome P448-like metabolism (Rhodes et al., 1984). 3-hydroxymethylantipyrine formation is a secondary hydroxylation pathway for antipyrine (Danhof et al., 1982). At low concentrations the ethanol or acetaldehyde effect on 3-hydroxylation would not significantly affect the net metabolism of antipyrine.

Low concentrations of ethanol or acetaldehyde had no effect on N-demethylation of antipyrine to norantipyrine. This was predicted since ethanol and acetaldehyde had no effect on the N-demethylation of aminopyrine at these concentrations.

The literature supports the idea that ethanol is metabolized by the mixed-function oxidase system (Teschke et al., 1977; Miwa et al., 1978; Ohnishi and Lieber, 1977; Koop et al., 1984; Coon et al., 1984; and Gellert et al., 1986). This suggests competition by ethanol for

binding sites on cytochrome P450. This binding appears to be a competition for type II drugs (Rubin et al., 1970a) as lower concentrations of ethanol do not interfere with type I drug binding or metabolism (Cinti et al., 1973). The reverse is also true. The metabolism of ethanol is inhibited by agents that produce a type II binding spectrum and is unaffected by those whose binding yields a type I spectrum (Rubin et al., 1971).

The data also may support a different explanation particularly since some type II drugs such as antipyrine do not interfere with ethanol metabolism in isolated microsomes (Andreasen et al., 1974) and ethanol or acetaldehyde did not affect the binding spectra of antipyrine (data not shown). Some have suggested that ethanol is not metabolized by binding with the cytochrome P450 but instead may be metabolized by hydroxyl radicals generated by the microsomes (Krikum et al., 1984). Hydroxyl radical scavengers (eg. mannitol) which inhibit ethanol metabolism support this idea (Cederbaum et al., 1979). These scavengers do not affect the metabolism of type I drugs such as aminopyrine. No studies are available on the effect of hydroxyl radical scavengers on antipyrine hydroxylation.

The importance of acetaldehyde in ethanol's inhibition of drug metabolism is not resolved in the literature. In Sprague-Dawley rats, the liver acetaldehyde concentration resulting from ethanol (2-6 g/kg) metabolism in vivo is between 3-5 μ g/g liver (Watanabe et al., 1986) yet 6 mmol/kg (.264 g/kg) acetaldehyde (Chapter 1) was necessary to inhibit the half-life of antipyrine. Watanabe's data showed that after exogenous acetaldehyde dosing, liver acetaldehyde levels are not elevated proportionally to acetaldehyde dose or rising blood

acetaldehyde levels but remain at a ceiling level of 2.5 μ g/g liver. Rapid extrahepatic metabolism of circulating acetaldehyde may be one explanation (Brien and Loomis, 1983).

Binding of acetaldehyde to cellular proteins is one potential mechanism of ethanol toxicity reported in the literature (Collins, 1988). In vitro, the binding to liver macromolecules of endogenously formed acetaldehyde from the metabolism of ethanol is consistently greater than an equivalent amount of exogenous acetaldehyde (Nomura and Lieber, 1981). This also might explain the inability of exogenous acetaldehyde (1.4 mM) in this research to significantly affect the in vitro metabolism of antipyrine.

CHAPTER 4: General Discussion

Research on the effect of ethanol on the body is a popular topic today. One body of Information being collected includes all facets of ethanol toxicity with a primary focus on its effect on the central and peripheral nervous systems. A second body of research has explored the metabolism of ethanol and the accompanying metabolic and physiologic changes throughout the body. Both types of studies have searched for a mechanism by which ethanol toxicity can be explained.

The research in this thesis has explored the effect of ethanol on drug metabolism via the mixed-function oxidase pathway. The literature alludes to much confusion regarding the elucidation of the in vivo mechanism by which ethanol affects drug metabolism due to the lack of an effective in vivo method by which to study ethanol's effect at the level of the mixed-function oxidases. In an attempt to find such an in vivo method, the research in this paper was developed along three lines.

1. Select mixed-function oxidase substrates whose metabolism was primarily via hepatic mixed-function oxidase. The drugs should ideally be affected only by changes at the level of mixed-function oxidase activity and not by physiological changes caused by ethanol. Methodology for the in vivo study of these drugs should best be studied without requiring pooling of animals to gather pharmacokinetic information.

2. Ethanol and its primary metabolite, acetaldehyde, should be screened for their role in the effect of ethanol on drug metabolism. Comparisons of in vivo to in vitro collected data on ethanol or acetaldehyde affected drug metabolism should be made with physiologically meaningful concentrations of ethanol or acetaldehyde for biochemical interpretation.
3. Select potential dietary constituents which might prevent the effect of ethanol on drug metabolism. Ideally these compounds should suggest a possible mechanism of action for the effect of ethanol on hepatic drug metabolism.

Mixed-Function Oxidase Substrates

There is extensive clinical value in having a noninvasive in vivo tool for assessing liver damage and the effect of exogenous substances on drug metabolism. The benefits include prediction of liver damage, monitoring effects of treatment, and matching patient with an appropriate and effective drug regime. Antipyrine and aminopyrine are two drugs whose clinical potential in these areas are presently being studied by researchers (Capel et al., 1979, and Haustein and Schenker, 1985).

Aminopyrine N-demethylation has been used extensively as an in vitro tool (Nash, 1953) to study drug metabolism and recently the aminopyrine breath test quantifying $^{14}\text{CO}_2$ biproduct of N-demethylation of labeled aminopyrine in vivo has been examined as a clinical liver function test (Haustein and Schenker, 1985). Antipyrine methodologies monitoring plasma and saliva antipyrine and those monitoring urine antipyrine and conjugated metabolites have gained recognition as tools for studying drug metabolism in vivo and have been examined in humans for use as clinical liver function tests (Capel et al., 1979). In vitro, study of antipyrine is limited due to a lack of an effective and reproducible analytical procedure.

Antipyrine and aminopyrine salivary half-life were effective tools in the study of ethanol inhibited drug metabolism. Despite significant findings (Chapters 1-3), studies of each drug presented less than ideal data.

Aminopyrine: Monitoring saliva for aminopyrine was selected

rather than the $^{14}\text{CO}_2$ breath test or plasma monitoring as the most convenient and economical means to monitor aminopyrine metabolism. Free drug in the saliva has been shown to be eliminated at the same rate as drug from the plasma which is 15% plasma protein bound (Vesell et al., 1975). Unfortunately, even this degree of plasma protein binding appears to affect extrapolations of apparent volume of distribution (Sultatos et al., 1980) and therefore affect calculations of clearance (Chapter 1). Meaningful data comparisons of treatment groups should therefore rely only on half-life comparisons.

Better studied would have been metabolites of aminopyrine in plasma or urine. To date, methodology for quantification of metabolites in plasma is unavailable and urine analysis for aminopyrine and its metabolites has received little attention. Currently, only N-demethylation of aminopyrine can be conveniently be examined.

Antipyrine: Monitoring for salivary antipyrine content was selected over plasma content due to the convenience of the analytical procedure. Data collected showed antipyrine salivary elimination followed first order kinetics. Data from plasma and urine also show antipyrine to display single compartment, first order kinetics (Huffman et al., 1974). Since saliva elimination can accurately substitute for monitoring plasma elimination (Welch et al., 1975), the saliva methodology was selected as the means to monitor individual rat antipyrine pharmacokinetics.

Unfortunately, monitoring the half-life of antipyrine in saliva is, at best, the net rate resulting from two hydroxylation reactions and one N-demethylation reaction, each proceeding at different rates

(Chapter 1: Figure 1.1). No analytical procedure currently exists to monitor these metabolites in plasma or saliva. Labeled antipyrine and the corresponding conjugated metabolites can be monitored in the urine (Kahn et al., 1981). This method is not practical in rats since conjugated metabolites of antipyrine and antipyrine follow flow-dependent renal clearance (Taylor and Blaschke, 1984). Even in humans complete urine collection, as well as accurate timing of urine collection, is difficult to ensure.

Since some of antipyrine's primary metabolites undergo secondary metabolism before conjugation, urinary profiles do not necessarily reflect activities of the liver enzymes (Boobis et al., 1981). Fortunately, comparisons can still be made between half-life of elimination and antipyrine metabolism. The half-life of elimination shows significant correlation with the appearance of conjugated 4-hydroxyantipyrine in the urine (Huffman et al., 1974) and not the other significant metabolites of antipyrine. Therefore changes in half-life of antipyrine may be a result primarily of changes in 4-hydroxylase activity.

In this study, half-life, apparent volume of distribution, and clearance for antipyrine were calculated. Literature shows that a linear relationship should exist between clearance and half-life for antipyrine (Sultatos et al., 1980). Yet significant changes in antipyrine half-life did not result in significant clearance changes. It has been suggested that this might be due to extrapolation to the y-axis for the volume of distribution rather than by examining the area under the curve as a means for calculating the volume of distribution (Sultatos et al., 1980). Problems of collecting

unlimited numbers of saliva samples made it impossible to accurately calculate area under the curve. Others have suggested that normalization for liver weight would solve discrepancies between half-life and clearance (Teunissen et al., 1984) for antipyrine.

The most difficult problem arising from the selection of antipyrine was the lack of an in vitro analytical method. Since it is believed that changes in 4-hydroxylation are related to changes in antipyrine half-life (Huffman et al., 1974), 4-hydroxyantipyrine analysis in vitro was necessary to confirm ethanol's inhibition of 4-hydroxylase activity. Further methodology development and monies would have been necessary to study this aspect.

Both antipyrine and aminopyrine methodologies suffer from lack of commercially available metabolite and labeled metabolite standards. Standards must be either synthesized and purified or solicited from others.

Ethanol or Acetaldehyde: In vivo verses In vitro

The liver is considered the main site of alcohol metabolism, whereas other organs such as kidney or lungs play only a very limited role in the elimination of alcohol. Hepatic enzyme systems capable of oxidizing ethanol in vitro include alcohol dehydrogenase, catalase, and the microsomal ethanol oxidizing system (Figure 4.1). The latter of these enzymes has characteristics common to other microsomal drug metabolizing enzymes (Teschke et al., 1977). Reconstituted microsomal systems (Ohnishi and Lieber, 1977) are capable of metabolizing not only ethanol but also other drugs commonly employed as substrates for the microsomal drug metabolizing enzyme system.

The centrilobular or Zone III of the hepatic acinus is the common site of drug metabolism as well as primary site of ethanol induced hepatotoxicity (Jauhonen et al., 1982). Acute studies of ethanol toxicity often focus on the metabolism of ethanol to examine the "imbalance" which might contribute to ethanol caused hepatotoxicity. Decreases in the NAD^+/NADH ratio, thereby altering the redox potential of the cell is often the suggested mechanism. Change in redox potential alone is not adequate to explain ethanol-inhibited drug metabolism. Ethanol inhibition of ethanol metabolism best exemplifies this (Ryle et al., 1985 and 1987). Ryle's data showed that treatment of rats with either Naloxone (2 mg/kg, ip) 1 hour after 2 g/kg, ip, ethanol or 3-palmitoyl-(+)-catechin (100 mg/kg, po) 1 hour before ethanol prevented ethanol induced redox state changes without affecting the ethanol elimination rate. Cysteine and malotilate which

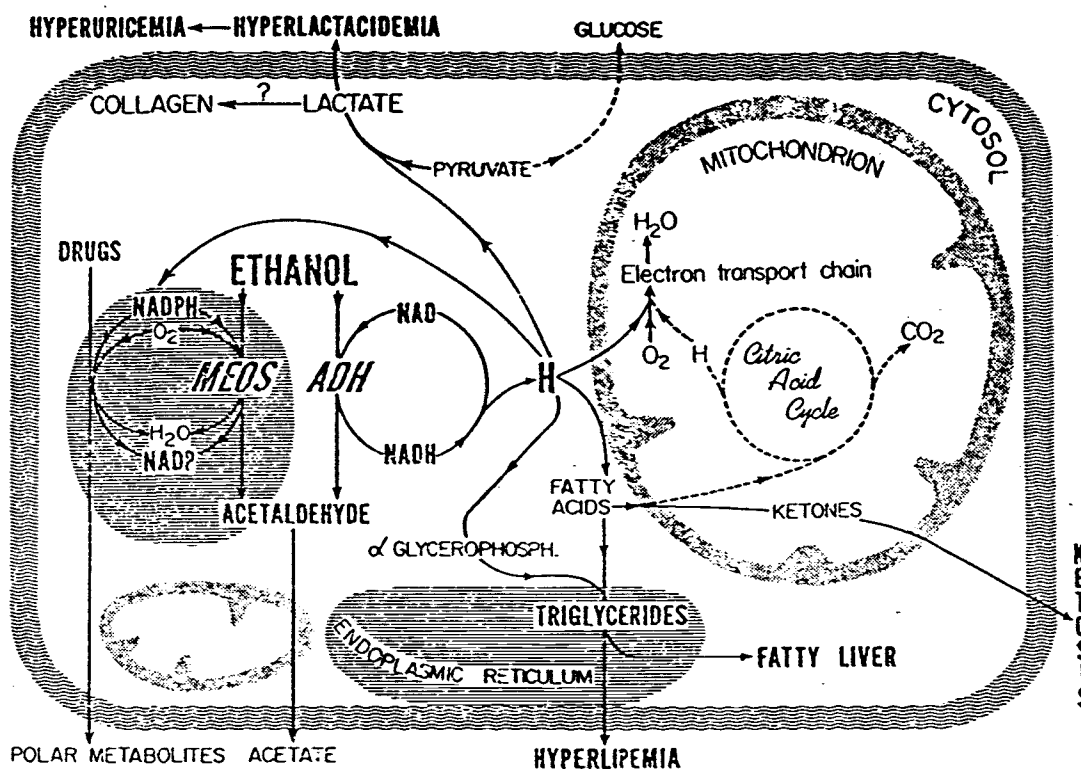


FIGURE 4.1: Metabolic Effects of Ethanol in the Hepatocyte.

Metabolism of ethanol in the hepatocyte and schematic representation of its link to fatty liver, hyperlipemia, hyperuricemia, hyperlactacidemia, ketosis, and hypoglycemia . Pathways which are decreased by ethanol are represented by dashed lines. ADH: alcohol dehydrogenase, MEOS: microsomal ethanol oxidizing system (from Lieber, 1975, p 172)

significantly lower the hepatic acetaldehyde concentrations cause a 29% and 12% increase, respectively in the ethanol elimination rate. This increase in the ethanol elimination rate was without affecting the ethanol induced alterations in the NAD^+/NADH ratio.

In rats, the LD50 for ethanol is 10 g/kg (Windholz, 1983). At such a large dose, ethanol acts through membrane perturbation and protein denaturation (Cohen and Mannering, 1973 and Cinti *et al.*, 1973). Studies of ethanol's effect on drug metabolism at this high ethanol concentration have no practical value since ethanol at these concentrations results in life threatening conditions.

Information on ethanol's affect on drug metabolism has practical value where blood alcohol concentrations are easily attained with a minimal consumption of ethanol. Rats acutely exposed at concentrations of as little as 2 g/kg showed significant inhibition (43%) of antipyrine metabolism (Chapter 1). In isolated microsomes the corresponding exposure level would be approximately 10 mM ethanol. The K_m for the metabolism of ethanol via alcohol dehydrogenase is 2 mM (Von Wartburg and Papenberg, 1966 and Plapp *et al.*, 1984) and the K_m for ethanol metabolism by the MEOS has been calculated between 10-12 mM (Teschke *et al.*, 1977). Concentrations of ethanol around saturation levels for these two enzymatic pathways would indicate that the inhibition of drug metabolism by ethanol was due to a direct membrane effect (Olsen *et al.*, 1983). Concentrations of ethanol administered near the K_m of these enzymes require a more extensive search for a mechanism of action and are considered biochemically more important than the higher concentrations of ethanol which result in nonspecific, general membrane perturbation or protein denaturation.

Acetaldehyde is the first toxic metabolite of ethanol with significantly more potent side-effects than ethanol (Windholz, 1983). In rats the immediately lethal LD50 for acetaldehyde is 1.930 g/kg (43.8 mmol/kg) (Windholz, 1983). A much smaller dose, LD90 of 18mmol/kg, (Sprince, 1985) from which the rat initially recovers only to die within the next 24 hours more closely represents the toxicity of acetaldehyde as seen in the thesis research.

A dose of 6 mmol/kg acetaldehyde was necessary to significantly increase the half-life of antipyrine (Chapter 1). The toxic manifestation of acetaldehyde in the rats severely limited both the number of rats available in each experimental group as well as the concentration of acetaldehyde which may be studied. Experimental procedures were adjusted to minimize animal loss during the course of the experiment. Problems after administration of acetaldehyde included respiratory distress, gasping, and an anesthetic-like paralysis with loss of righting reflex or response to tail-pinch test within the first 3-8 minutes. Some animals progressed from this state to death. 90% of those recovering within 20 minutes of dosing remained outwardly normal for the remainder of the experiment (5-6 hours). Only the failure of pilocarpine to stimulate salivation limited the number of animals used out of those which survived the dose of acetaldehyde. These symptoms agree with those of others working with acetaldehyde in rats (Sprince et al., 1979).

Exogenous acetaldehyde at an in vivo dose of 6 mmol/kg should amount to a hepatic subcellular concentration of 1.4 mM based on total body water distribution and equilibrium of acetaldehyde. Watanabe et al., 1986, compared blood acetaldehyde and liver acetaldehyde

concentrations in Sprague-Dawley rats (230-280 g each) one hour after intragastric administration of ethanol (2-6 g/kg) or after one hour of inhalation of acetaldehyde laden air (9-1000 mg/l air). Ethanol's metabolism to acetaldehyde resulted in blood acetaldehyde levels of 1-8 $\mu\text{g/ml}$ (.02-.18 mM) and liver acetaldehyde levels between 3-15 $\mu\text{g/g}$ liver (.34-1.0 mM). Exogenously administered acetaldehyde was capable of raising blood acetaldehyde levels proportionate to dose of 1-10 $\mu\text{g/ml}$ (.02-.23 mM), but liver acetaldehyde levels had a ceiling level of 2.5 $\mu\text{g/g}$ liver (.17mM). Rapid extrahepatic metabolism of circulating acetaldehyde (Brien and Loomis, 1983) may be one explanation for these results.

The study of acetaldehyde at in vitro concentrations greater than 3-15 $\mu\text{g/g}$ liver (.34-1.0 mM) is not biochemically meaningful toward establishing the mechanism by which ethanol inhibits drug metabolism. Other studies of exogenous acetaldehyde in vitro have also shown that high concentration of acetaldehyde (1-2 mM) were necessary to inhibit p-nitroanisole-O-demethylation (Dicker and Cederbaum, 1982 and Reinke et al., 1983). Either acetaldehyde is not an important mechanism in ethanol's inhibition of drug metabolism or exogenous acetaldehyde is not capable of producing the same inhibition of drug metabolism as that of endogenous acetaldehyde formed through the metabolism of ethanol.

Other researchers have suggested that endogenous acetaldehyde may indeed be better at the inhibition of drug metabolism than is exogenous acetaldehyde. Endogenous acetaldehyde binds stronger and in greater amounts to rat liver microsomes than an equivalent amount of exogenous acetaldehyde (Nomura and Lieber, 1981).

Dietary Management of Ethanol Toxicity

It is widely recognized that dietary factors affect drug pharmacokinetics. Antipyrine has been the drug of choice in much of the research done in this area (Vesell, 1986). There is also extensive clinical value in finding dietary sources for the management of ethanol toxicity. Physiologic benefits include control and/or abatement of the external symptoms of acute and chronic ethanol abuse, protection against ethanol's effect on drug metabolism, and protection or minimization of alcohol induced hepatic disease states. Science and medicine benefits through potential revelations of the mechanisms of ethanol toxicity. Finding effective dietary sources for the management of ethanol toxicity has the added benefits of ready availability, often inexpensive, low inherent toxicity, and may not require extensive testing to ensure safety under the Food and Drug Administration Act. Extra benefits would be derived from the ability to use the protectants on a long-term basis for chronic heavy drinkers and/or heavy smokers.

Drug and ethanol pharmacokinetics may be influenced in many ways by dietary factors. Summarized below are some of the mechanisms through which the dietary factors selected in this research (ascorbic acid, thiamine, and cysteine) may alter the effect of ethanol on drug metabolism.

1. EFFECT ON ABSORPTION:

Orally administered dietary factors affect drug and ethanol absorption (Vesell, 1986). To avoid the complication of changes in

oral bioavailability of ethanol, dietary treatments were administered intraperitoneally, 90 minutes prior to ethanol per os. Antipyrine was administered intraperitoneally 20 minutes after ethanol. The time lag between dietary factor administration and antipyrine administration was sufficient enough that the dietary factor did not affect the absorption of antipyrine as measured by peak concentration and time to peak of antipyrine in the saliva. In the case of ascorbic acid (Susick and Zannoni, 1987) and cysteine (Beauge et al., 1976) this has been confirmed by others.

2. EFFECT ON DISTRIBUTION:

Distribution of the drug and ethanol or acetaldehyde could be affected by administration of dietary factors. Others have shown that ascorbic acid (300-600 mg/person, ie., 1.7-3.4 mmol; 4 times/day) had no effect on the pharmacokinetics of antipyrine in humans. No information on the effect of thiamine or cysteine on the pharmacokinetics of antipyrine in rats, humans or other animals could be found in the literature search. At concentrations used in this research no change was observed in antipyrine half-life, volume of distribution, or clearance by pretreatment with ascorbic acid, thiamine, or cysteine (Chapter 2). Concentrations selected for this research were based on literature review (Chapter 2).

An attempt was made to study the effect of doubling the concentrations used of each dietary factor. No effect on antipyrine metabolism was seen by doubling pretreatment with ascorbic acid from 2 mmol/kg to 4 mmol/kg. Yet, no added benefit was seen on the prevention of ethanol or acetaldehyde inhibition of antipyrine half-life. Doubling the pretreatment dose of thiamine from .24

mmol/kg to .48 mmol/kg and cysteine from 2 mmol/kg to 4 mmol/kg also did not affect the metabolism of antipyrine. Doubling the pretreatment concentrations of thiamine or cysteine did increase the toxicity of ethanol or acetaldehyde resulting in decreased salivation and increased lethal effects of acetaldehyde. Cysteine may increase ethanol and acetaldehyde toxicity through a disulfiram-like reaction which inhibits the metabolism of acetaldehyde to acetate, thereby increasing the level of acetaldehyde in the body (Messiha, 1987). Literature cites the lethal concentration of thiamine starts at approximately 124-350 mg/kg (.53-1.5 mmol/kg) (Kutsky, 1973) with symptoms similar to those of acetaldehyde. In rats, it may be that the administration of acetaldehyde and thiamine together at these concentrations may be toxic, where alone each produced no symptoms.

3. EFFECT OF NAD^+ /NADH RATIO:

It is widely recognized that ethanol causes a shift in the intracellular redox potential in favor of NADH (Thurman *et al.*, 1972). This can be measured intracellularly as shifts in the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios in favor of lactate and 3-hydroxybutyrate respectively (Ryle *et al.*, 1987). Ethanol-caused shifts in the redox potential can also be measured *in vivo* by blood lactate/pyruvate shift in favor of lactate.

The inhibition of antipyrine metabolism is not likely to be caused by the effect of ethanol metabolism on cellular redox potential. Both cysteine (Ryle *et al.*, 1987) and ascorbic acid (Susick and Zannoni, 1987) show no effect on cellular redox changes caused by ethanol metabolism, yet both cysteine and ascorbic acid pretreatments prevented the ethanol-increased salivary half-life of

antipyrine.

4. EFFECT OF ETHANOL WITHOUT METABOLISM:

The inhibition of antipyrine metabolism by ethanol (Chapter 1) can be explained via a direct ethanol effect. Ascorbic acid pretreatment in humans caused an increase in blood ethanol clearance (Susick and Zannoni, 1987). This increase in blood ethanol clearance was without affecting the time to peak ethanol concentration or the peak concentration of ethanol in the blood. Ascorbic acid is associated with the catalytic site of cytochrome P450 (Sato and Zannoni, 1976) and an ascorbic acid-dependent ethanol metabolic pathway has been proposed (Susick and Zannoni, 1984).

The antagonism of ethanol toxicity by ascorbic acid has also been shown in mice (Ferko, 1986). In Ferko's experiments ascorbic acid antagonized the central depressant properties of ethanol without affecting the ethanol peak blood levels or ethanol's distribution. Ferko proposed that the affect of ascorbic acid was by increasing the peroxide concentration (Cederbaum et al., 1980) available to catalase and thereby increasing the metabolism of ethanol via the catalase route.

5. EFFECT OF ACETALDEHYDE:

The effect of ethanol on antipyrine metabolism may be due to the direct effect of acetaldehyde or, more likely, a combination of the direct effects of ethanol and acetaldehyde. Sulfhydryl compounds which sequester circulating acetaldehyde derived from ethanol metabolism do so by forming thiazolidinecarboxylic acid derivatives (Kera et al., 1985 and Nagasawa et al., 1987). The sequestering effectiveness of the compounds increases with increasing bond strength

between the sulfhydryl group and acetaldehyde. Sequestered acetaldehyde is ultimately diverted to urinary excretion pathways (Nagasawa et al., 1987).

Much of the toxicology of acetaldehyde as been studied within the central and peripheral nervous systems. The binding there of acetaldehyde to neurochemicals such as dopamine (Collins et al., 1988) and the resulting dihydroisoquinones and related oxidative products are believed to be the toxic agents of ethanol/acetaldehyde exposure (Collins and Cheng, 1988). One set of data even suggests that acetaldehyde-neurochemical condensation products may be the potential toxic agents in alcoholic liver disease states (Collins, 1988).

Cysteine has been found to bind nonenzymatically with acetaldehyde (Cederbaum and Rubin, 1976) by way of an intermediary Schiff base (Sprince, 1985). The thiazolidine-4-carboxylic acid formed resulted in a lowering of liver acetaldehyde concentration after ethanol administration (Ryle et al., 1987).

Thiamine, through the formation of thiamine pyrophosphate, complexes with acetaldehyde to eventually form acetyl Coenzyme A, thus inactivating acetaldehyde (Sprince et al., 1980).

Ascorbic acid is also capable of inactivating acetaldehyde (Sprince et al., 1979). It has been proposed that ascorbic acid does this by facilitating the nonenzymatic binding of acetaldehyde to serum albumin (Lumeng, 1985) through acidic adducts with lysine (Tuma et al., 1987) or endogenous sulfhydryl compounds such as cysteine (Sprince, 1985), thereby sequestering acetaldehyde for its ultimate elimination through urine pathways.

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APPENDIX

APPENDIX ONE: Oregon State University Rodent Pellet Diet (12-11-1979
through 8-15-1985)

<u>Ingredients</u>	<u>Formula (pounds)</u>
Alfalfa, gr	200
Bentonite	50
Corn, gr	546
Barley, gr	600
Soybean meal, 41%	240
Fishmeal	100
Whey, dried	200
Salt, TM	10
Vitamin A	1,000,000 IU Premixed with one of
Vitamin D	36,000 IU the ration ingredients