ABSTRACT

Thomas Richard Ward. ETHANOL METABOLISM BY THE MIXED FUNCTION OXIDASE SYSTEM. (Under the direction of Dr. Sam N. Pennington) Department of Biology, 1972.

Ethanol is oxidized into acetaldehyde by the enzyme alcohol dehydrogenase utilizing the cofactor NAD, but this system is not affected by chronic exposure to ethanol. Recently the microsomal fraction of the hepatic parenchymal cells along with NADPH and molecular oxygen has been shown to oxidize ethanol in vitro. In this study it was shown that the microsomal system is active in ethanol oxidation in vivo, and that this system is induced by chronic ethanol consumption. To study the microsomal ethanol oxidase system, pyrazole was used to inhibit alcohol dehydrogenase in vivo. Because there was no method of detecting pyrazole in whole blood a method was developed for this purpose.

The microsomal ethanol oxidase system was further studied by solubilizing the microsomes and separating the component parts using a DEAE cellulose anion exchange column. It was found that NADPH cytochrome P-450 reductase, cytochrome P-450, a hydrogen peroxide generating factor (possibly dismutase), and catalase or peroxidase to utilize the hydrogen peroxide are all needed for the oxidation of ethanol into acetaldehyde by the microsomes.

ETHANOL METABOLISM BY THE MIXED FUNCTION OXIDASE SYSTEM

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Biology

by

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December 1972

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ACKNOWLEDGMENTS

I wish to acknowledge the valuable assistance and advice rendered by Dr. Sam N. Pennington, Dr. W. James Smith, Dr. Takeru Ito, and Dr. G. Lynis Dohm.

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REVIEW OF LITERATURE

The association between alcoholism and cirrhosis of the liver was first recognized by Matthew Baillie in 1793.[1] In Western countries the incidence of cirrhosis can be directly related to the quantity of alcohol consumed. In 1836, Addison described the association between fatty liver and alcoholism.[2] The effects of alcohol on liver can be placed into two categories: (1) acute, and (2) chronic effects of alcohol consumption.

Large amounts of alcohol administered to rats in a single dose induces steatosis and ultrastructural changes in the liver. Steatosis may occur due to fat mobilization, but steatosis has also been observed without increase in circulating free fatty acids. The free fatty acid composition of hepatic tissue, however, has been found to be similar to that of depot fat.

Prolonged alcohol administration in high doses also produces fatty liver and ultrastructural changes in the liver. However in this condition the sources of fat are mainly dietary and enhanced fatty acid synthesis. This occurs both in animals on adequate and low fat diets.[3]

Ethanol metabolism is carried out in the liver by the enzyme alcohol dehydrogenase (ADH) (see Appendix I) which, with the cofactor nicotinamide adenine dinucleotide (NAD), catalyzes the initial step of ethanol metabolism by converting ethanol to acetaldehyde.[4]

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+ \qquad (1)$$

Alcohol dehydrogenase is located in the cytoplasm, primarily in the hepatic parenchymal cells. This ADH catalyzed mechanism is thought to be the primary mode by which ethanol is metabolized.[5] Yet the ADH catalyzed mechanism has certain characteristics that are difficult to explain. The pH optimum for ADH is 10.8,[6] far above normal cytoplasmic pH, and the equilibrium for this reversible reaction lies far to the left in favor of ethanol production. Also, this enzyme system is not affected by chronic alcohol consumption.[7] Furthermore, addition of excess NAD^+ does not stimulate the oxidation of ethanol *in vivo*[8] indicating that endogenous NAD is not a limiting factor. Because chronic ethanol consumption does not appear to affect ADH, two questions arise. First, how does one explain the increase in ethanol tolerance after chronic consumption, and secondly, how does liver damage develop.

At least part of the reason for induced ethanol tolerance is central nervous system adaptation.[9] Another possible explanation of the increased tolerance arose in 1965 when Orme-Johnson and Ziegler[10] first reported that liver microsomes could metabolize ethanol in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. Later investigation found that chronic ethanol consumption in man given a variety of diets[3] produced proliferation of the smooth endoplasmic reticulum (SER), and this increase was accompanied by increase in activity by the hepatic microsomal mixed function oxidase system (MFOS).[11] The proliferation of SER and MFOS is similar to the response produced by a variety of drugs.[12] In 1968, Lieber and

De Carli demonstrated that this system could be induced by ethanol consumption to cause an increased rate of ethanol metabolism in vitro.[13] Peroxidase will, with H_2O_2, also catalyze the oxidation of ethanol in vitro, but there is no evidence that this peroxidase system is active in vivo.

The second question arising from the ADH system, that of hepatic injury will now be discussed. Some possible mechanisms of hepatic parenchymal lesion that have been investigated are NAD/$NADH_2$ ratios in the liver,[14] effect on hydroxy and ketosteroids in the urine,[15] inactivation by the metabolic product acetaldehyde,[16] effect on metabolism of hepatic and plasma triglycerides,[17] effect on solvolyzable plasma steroids,[18] effect on morphology of mitochondria,[19] and effect on ultrastructure of hepatocytes,[20] as well as a number of other studies.

Damage to the liver by chronic ethanol consumption is probably due to large amounts of fatty material that accumulates[21] and the degeneration of membranes in the parenchymal cells.[22] The fatty liver could be caused by (1) increased synthesis of triglycerides in the liver, (2) decreased metabolism of triglycerides, (3) increased uptake of lipid by the liver, (4) or a disturbance of the normal route of lipid removal.

The increased synthesis and decrease in catabolism of the lipids is a possibility for some accumulation of triglycerides in the liver. Ethanol consumption decreases the NAD/$NADH_2$ ratio. This additional reducing power of the $NADH_2$ can be utilized in the terminal electron

transport system as an energy source or it can be used in the synthesis of fatty acids.[23] There is also a relationship between dietary lipids and the amount of lipid found in the liver of chronic alcoholics, but fatty liver also occurs in the presence of low amounts of dietary lipids.[24]

A disturbance in the normal route of lipid removal remains as a possibility. Lipids are found associated with a very low density lipoprotein, and there is no evidence of change in lipoprotein synthesis with chronic ethanol consumption. In addition, Young et al.[25] found that inducing the MFOS also induced phosphatidylcholine synthesis which is a component of the lipoprotein. Yet May and McCay[26,27] discovered that this same system peroxidized polyunsaturated fatty acids, which are a component of phosphatidylcholine. Later Roubal[28] found that a free radical is responsible for the peroxidation. Coon et al. have shown that this MFOS is also responsible for the ω-hydroxylation of saturated fatty acids, which are also a component of phosphatidylcholine.[29] Coon also demonstrated the presence of the O_2^- "superoxide" radical.[30]

Because the MFOS is induced by chronic ethanol consumption one would expect to find higher levels of abnormally oxidized and peroxidized lipids. These abnormally oxidized and peroxidized lipids could become incorporated into the lipoproteins and the plasma membranes and impair their functions, thereby explaining the lipid buildup and the membrane

fragility in the parenchymal cells. Di Luzio[31] found that lipid soluble but not water soluble antioxidants could prevent both the ethanol and the ethanol plus higher dietary lipids induced steatosis. Since the antioxidant must be lipid soluble, it is thought to prevent oxidation of lipids.

Thus if one can prevent steatosis by preventing abnormal increased peroxidation or hydroxylation due to the proliferation of SER and MFOS with an antioxidant, one could possibly prevent at least one biochemical lesion due to ethanol metabolism.

This same microsomal MFOS that oxidizes ethanol also hydroxylates or dealkylates many exogenous substances such as pharmacologic agents, carcinogens, and insecticides,[32] to form a more polar, and therefore more water soluble substance that can be excreted in the urine. In addition, this system also hydroxylates many endogenous substances such as steroids.[33]

This hydroxylation, or dealkylation, like that of ethanol metabolism, is an oxidative process which requires molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P-450, and NADPH cytochrome P-450 reductase. This system is inducible by repeated administration of drugs such as phenobarbital, tolbutamide, or phenylbutazone. But more importantly, this induction is mutually non-specific and enzymes of ethanol metabolism are also induced by these drugs. Many drugs like ethylmorphine, hexobarbital, chloropromazine, and ethanol are also mutually competitive and inhibit the metabolism of

each other. It appears that a single system does not metabolize all the endogenous and exogenous substances; however, there seems to be several overlapping pathways.[34] It is well known that ethanol taken in conjunction with barbiturates enhances the effects of both the alcohol and the drug; also it is known that alcoholics not under the immediate influence of ethanol are more resistant to many drugs.[35]

The microsomal mixed function oxidase system (often referred to as the microsomal ethanol oxidase system (MEOS) when referring to ethanol metabolism) is composed of three parts, NADPH cytochrome C reductase, cytochrome P-450, and phosphatidylcholine. The NADPH cytochrome C reductase (NADPH cytochrome P-450 reductase) of this system is a flavoprotein that is not associated with any metal and is capable of both one and two electron reduction.[36] Immunochemical studies indicate that NADPH-cytochrome C reductases induced by phenobarbital and 3-methylcholanthrine administration are similar.[37]

Cytochrome P-450 is a heme containing cytochrome that is so named due to a single absorption maxima at 450 nm when it is reduced and then combined with carbon monoxide (Figure 7). It has been found in man,[38] lower animals,[39] yeast,[40] and bacteria.[41] Although it is generally assumed that this cytochrome is identical or at least very similar in different organisms, immunological work done by Mitani et al.[42] indicates a lack of cross-reactivity between chick embryo and rat liver cytochrome. Also work by Hildebrandt and Estabrook[43] dealing with rabbit P-450 demonstrated that actually two cytochromes

exist. Phenobarbital induces cytochrome P-450 whereas 3-methylcholanthrine induces cytochrome P-448. Later they found that these cytochromes are actually interconvertible forms of the same cytochrome and the form found depends upon the substance used to induce the system.

The phosphatidylcholine requirement of the system was shown by Coon et al.[29] Their preliminary work indicates that this lipid is necessary in the reduction of the cytochrome P-450. This requirement of phosphatidylcholine in the MFOS seems paradoxical in the light of May and McCay's work[27] in which they discovered that oxidation of NADPH by liver microsomes resulted in a rapid disappearance of polyunsaturated fatty acids from the membrane phospholipids. In fact, as much as 15% of the total microsomal polyunsaturated fatty acids were consumed by the reaction within 30 to 45 minutes. Since the ester value of the phospholipid remained constant, it was evident that polyunsaturated fatty acids were being converted into some as yet unidentified moiety in situ on the phospholipid.[44]

Mechanism

The mechanism of oxidation or peroxidation of fatty acids has not been fully elucidated, but some theories have been proposed and some steps are fairly well understood. Schenkman[45] proposed the scheme of hepatic microsomal mixed function oxidase system as shown in Figure 1a. Coon found that benzphetamine hydroxylation was inhibited by dismutase.

$$2O_2^- + 2H^+ \xrightarrow{\text{DISMUTASE}} H_2O_2 + O_2 \qquad (2)$$

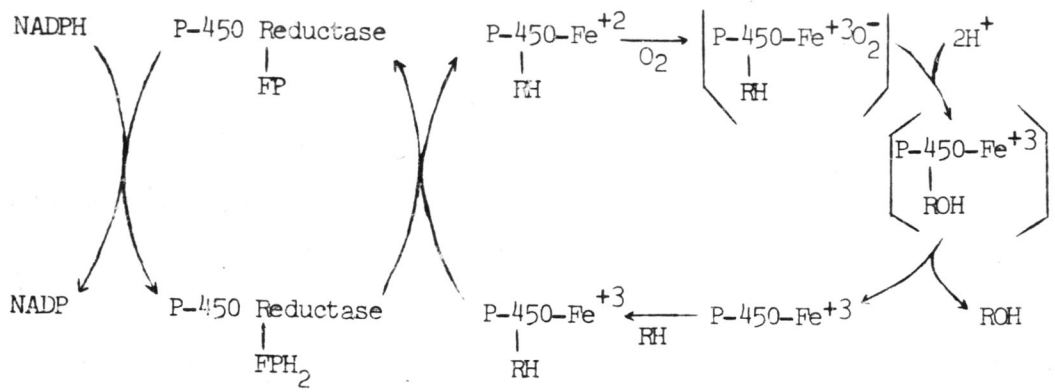
Figure 1b shows this inhibition and a possible mechanism of ethanol metabolism.

Legend for Figure 1

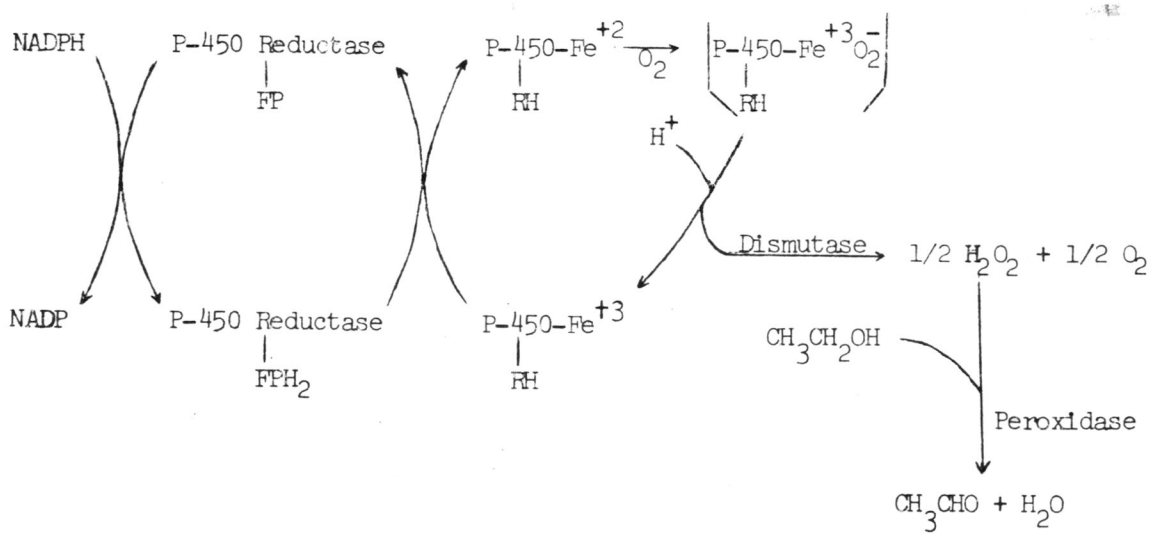
The scheme of hepatic microsomal mixed function oxidase showing the hydroxylation of a substrate "RH" is shown in a. The scheme of hepatic microsomal mixed function oxidase system (microsomal ethanol oxidase system) showing a mechanism of ethanol oxidation is shown in b. Stoichiometry of the mechanism has not been clearly elucidated yet.[30]

Note Schenkman actually proposed that the oxygen complexed with cytochrome P-450 contained two negative charges instead of one as shown, but later work by Coon indicates that there is only one negative charge.[30]

a.


NADPH
P-450 Reductase
FP
NADP
P-450 Reductase
FPH2
P-450-Fe+2
RH
O2
P-450-Fe+3O2-
RH
2H+
P-450-Fe+3
ROH
ROH
P-450-Fe+3
RH
P-450-Fe+3
RH

b.


NADPH
P-450 Reductase
FP
NADP
P-450 Reductase
FPH2
P-450-Fe+2
RH
O2
P-450-Fe+3O2-
RH
H+
Dismutase
1/2 H2O2 + 1/2 O2
CH3CH2OH
Peroxidase
CH3CHO + H2O
P-450-Fe+3
RH

The Purpose of This Study

The mixed function oxidase system has been shown to be capable of ω-hydroxylation of saturated fatty acids and the peroxidation of polyunsaturated fatty acids in the presence of O_2 and NADPH. It has also been shown *in vitro* that this system was inducible by chronic ethanol consumption. One purpose of this study, therefore, was to determine whether or not this system was functional *in vivo* for ethanol oxidation in addition to the cytoplasmic ADH catalyzed system. In order to do this, pyrazole (1,2 diazole, pyrromonazole) was used as an inhibitor of ADH.[46,47] As there was no method of determining the presence of pyrazole in the blood, a method was developed.

Another portion of this study was to determine how much overlap exists between the ω-hydroxylation system of Lu and Coon and the MEOS. In this study the method of Lu and Coon[48] in their work on ω-hydroxylation of laurate was used to solubilize the microsomes and to separate the component parts to determine if the same components were functional in the oxidation of ethanol. This was significant because if these three components (NADPH-cytochrome P-450 reductase cytochrome P-450, and phosphatidylcholine) were involved, the mechanism of an alternate method of ethanol oxidation in chronic ethanol consumption could be at least partly elucidated. After a new mechanism of ethanol metabolism has been resolved, an alternative explanation as to the cause of lipid accumulation in the liver due to chronic ethanol consumption may be offered.

MATERIALS AND METHODS

Pyrazole studies

Pyrazole has been shown to inhibit the enzyme alcohol dehydrogenase *in vivo*.[49] The mechanism of this inhibition is competitive. Pyrazole was used to study the relative contribution of alcohol dehydrogenase in alcohol metabolism in rats that were chronically exposed to ethanol. In order to interpret the results obtained, it was necessary to determine the circulating levels of pyrazole in the experimental animals, which had received pyrazole, at various times such that one might compare these levels to the rate of ethanol clearance. As there existed no simple method to determine pyrazole in animal tissue, a procedure was developed. In developing this method, the technique of LaRue[50] for determining pyrazole in melon seeds was modified. This approach used the reaction of trisodium pentacyanoaminoferrate with pyrazole to yield a product that absorbs maximally at 458 nm.

A standard curve was prepared by adding 0.1 to 1.0 µmoles of pyrazole to 0.2 ml of rat blood serum with the final volume adjusted to 0.3 ml with water. The mixture was heated at 100°C for 10 minutes, 1.0 ml of water was added, and the mixture was stirred for 10 minutes. The mixture was then centrifuged at 5,000 xg for 10 minutes. After centrifuging, 0.6 ml of the supernatant was drawn off and added to 1.0 ml of 1.2% trisodium pentacyanoaminoferrate and the solution diluted to 3.0 ml with water. One tenth ml of 10% $NaNO_2$ was then added, followed immediately by 0.1 ml of glacial acetic acid. The solution

was spun at 7,000 xg for 20 minutes and the optical density at 458 nm was read relative to a sample without added pyrazole. The results are shown in Figure 2. It was evident in the serum standard curve at concentrations of pyrazole above 1 μmole (5 μmoles/ml) that the Beer-Lambert law was not obeyed.

As a check of the possible interaction between erythrocytes and pyrazole, a recovery study was undertaken using whole blood. Obvious errors would occur if the pyrazole were to bind in some manner to erythrocytes and if a standard curve was prepared using serum from which the red blood cells were removed. To test if pyrazole binds with erythrocytes appreciably, pyrazole was added to whole blood. After allowing the blood to clot, pyrazole in the serum was determined as outlined above. The results after correcting for the dilution between whole blood and serum are given in Table I. It is apparent that only slight, if any, specific binding occurs between pyrazole and the erythrocytes.

To find the relationship between dose and circulating levels of pyrazole, 12 Holzman rats were injected I.P. with pyrazole in water at doses ranging from 0.053 μmoles/100 gm to 1.060 μmoles/100 gm. After 24 hours, blood samples were obtained by heart puncture, allowed to clot, and assayed for pyrazole in the serum as outlined above. Such a relationship is given in Figure 3, along with the overall limits of variation for three separate experiments.

Legend of Figure 2

Standard curve for pyrazole in serum prepared as outlined in the text.

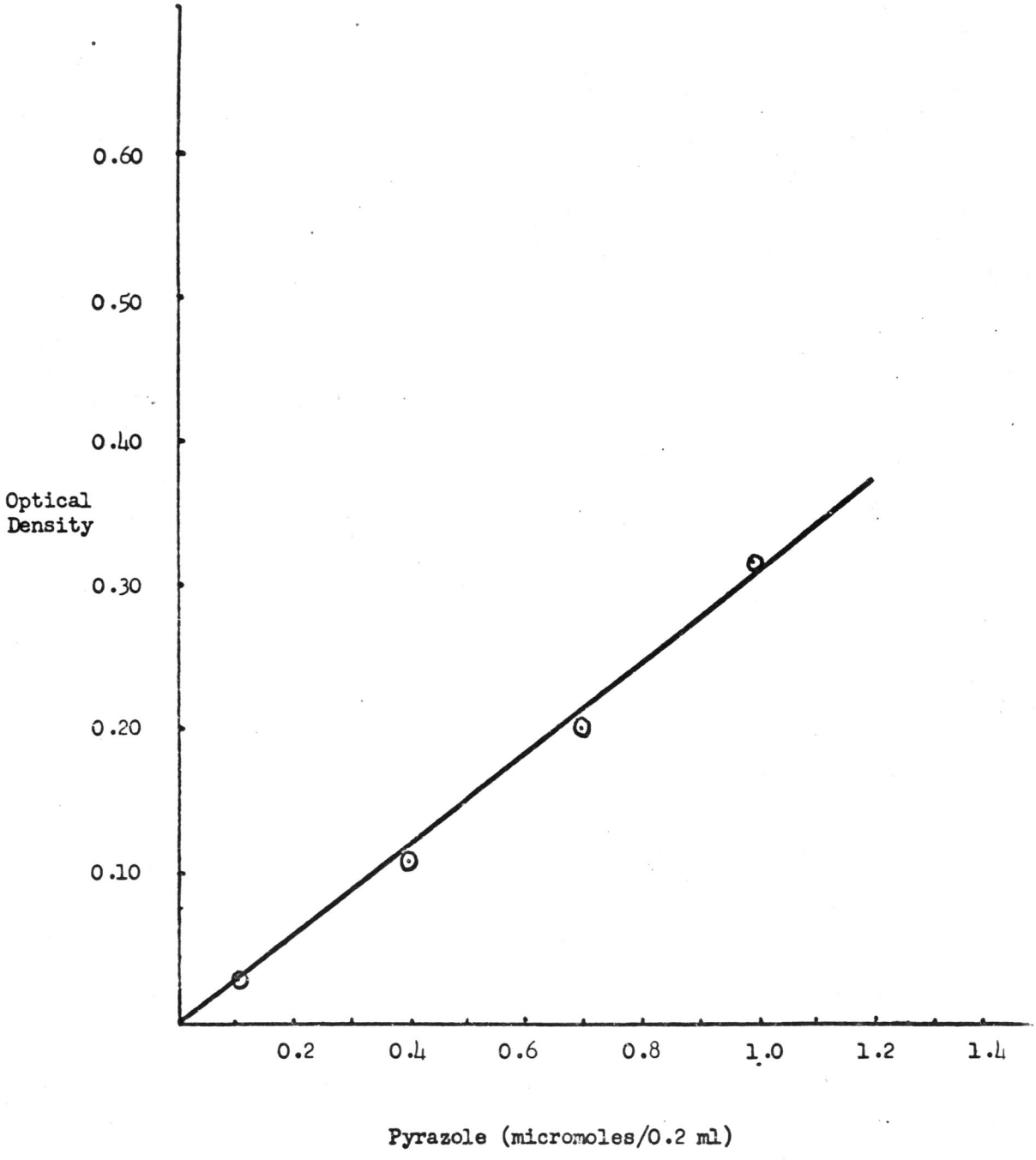

0.60
0.50
0.40
Optical Density
0.30
0.20
0.10
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Pyrazole (micromoles/0.2 ml)

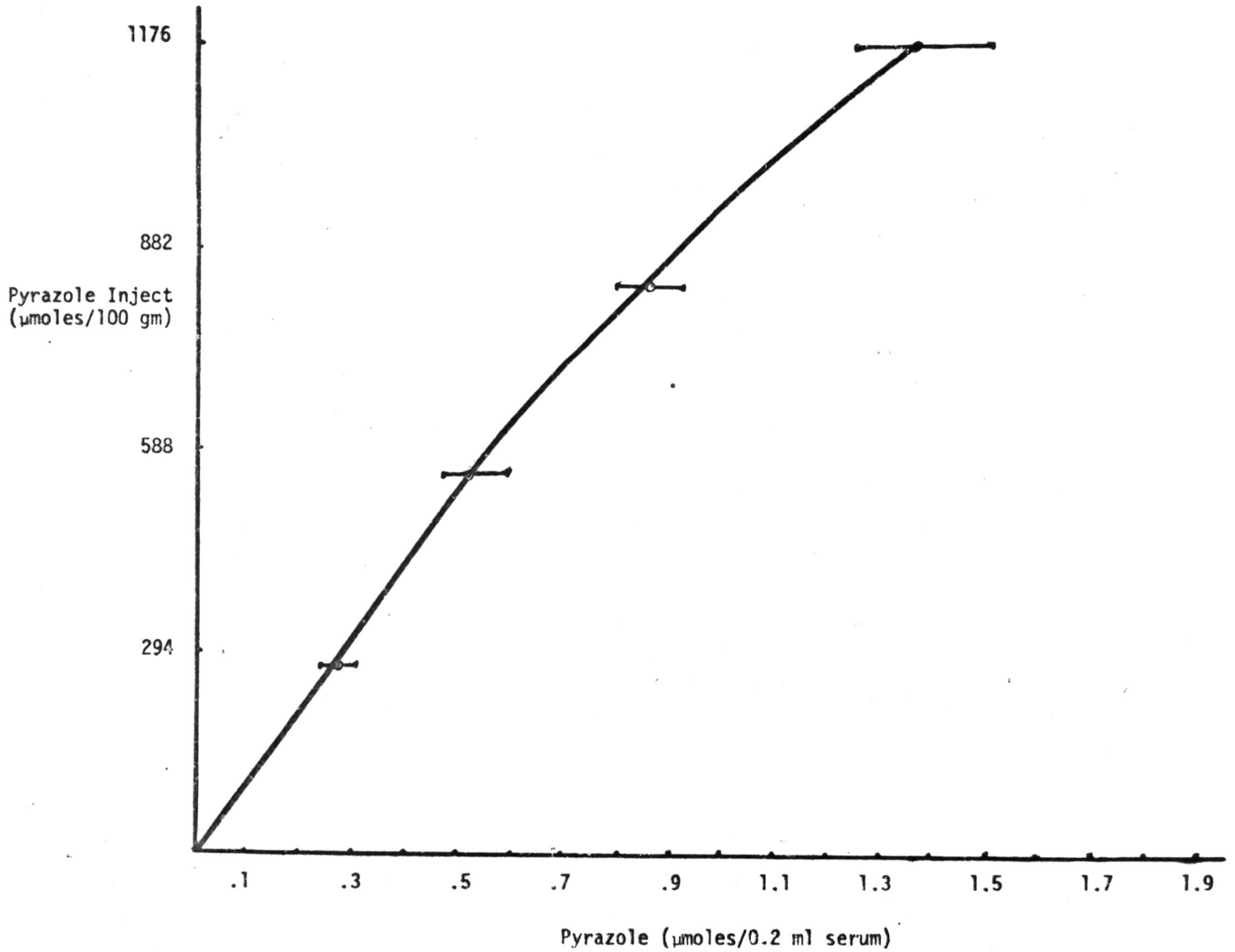
Table I

Recovery study for pyrazole in whole blood based on the standard curve prepared from serum. Pyrazole was assumed to be distributed uniformly into serum and erythrocytes, when mixed with whole blood. Under this assumption the correction was made for the expected loss of pyrazole in the serum according to known volume ratio of the serum and the erythrocytes.

Amount added/ml whole blood (Micrograms)	Amount found/ml blood serum (Micrograms)	Corrected for dilution of RBC
0.10	0.10	0.09
0.10	0.13	0.11
0.40	0.42	0.38
0.40	0.44	0.40
0.70	0.76	0.69
0.70	0.81	0.73
1.00	0.94	0.85
1.00	1.02	0.93

Legend for Figure 3

Graph showing the relationship between pyrazole injected (IP) and serum levels 24 hours later. Data represents three separate experiments at each value. Deviation is the overall variation for all three esperiments.


1176
882
588
294
Pyrazole Inject
(µmoles/100 gm)
.1
.3
.5
.7
.9
1.1
1.3
1.5
1.7
1.9
Pyrazole (µmoles/0.2 ml serum)

To check the identity of the reaction product, an absorption spectrum was obtained and is shown in Figure 4. A comparison with the standard spectrum given in reference (50) indicates that no interfering substances are found in blood of normal animals.

Blood ethanol levels

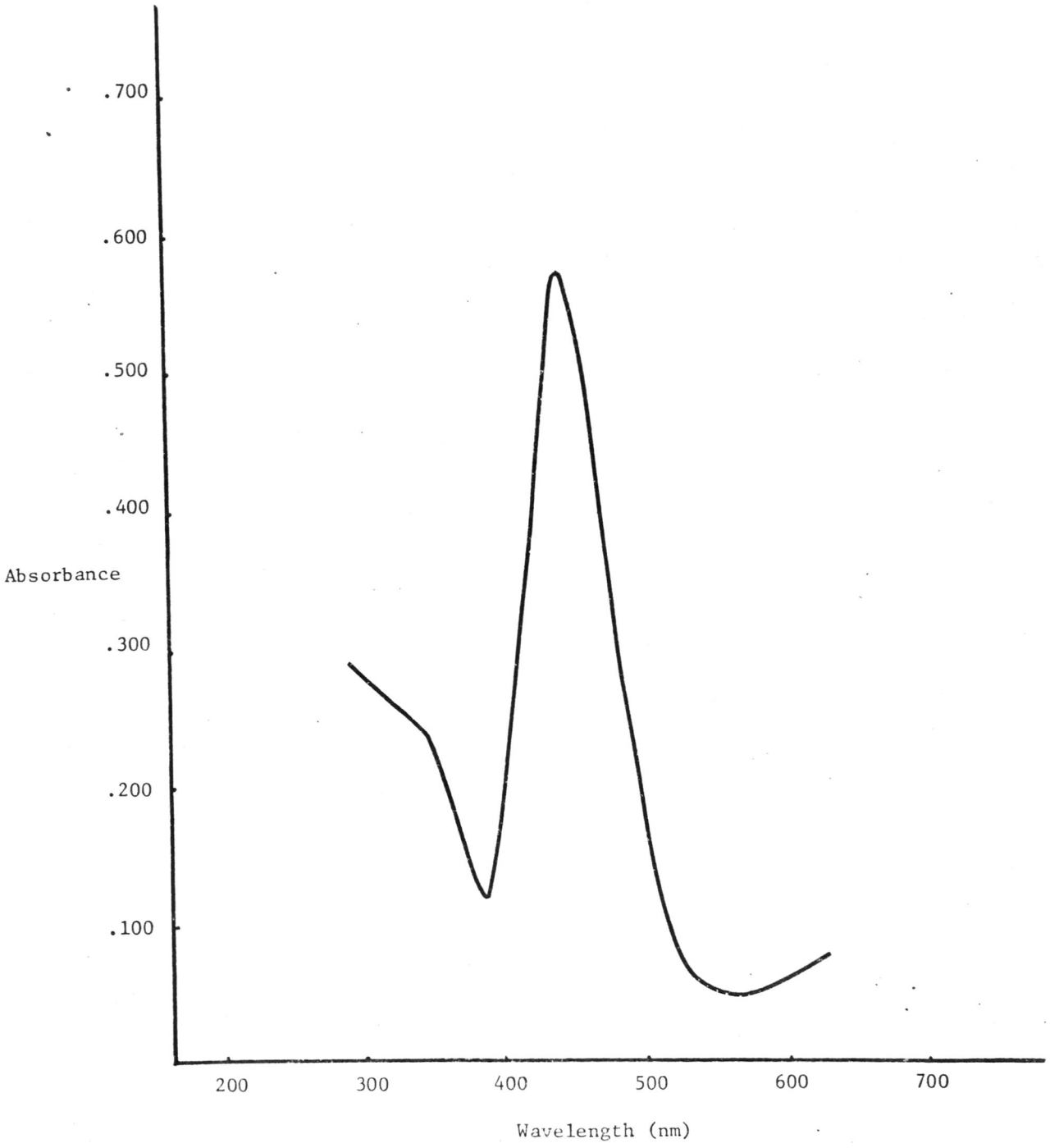
This study was designed to determine if chronic ethanol consumption in rats enhances ethanol metabolism and to study the effects of ADH inhibition by pyrazole on ethanol metabolism after chronic ethanol consumption.

Four groups of nine female Holzman rats (150-200 gm) each were used. Two groups were dosed daily by stomach tube with 3 gm/kg of ethanol mixed 50/50 (v/v) with 0.9% NaCl. Two hours prior to the final (5 gm/kg) dose of ethanol, two groups, nine control animals and nine ethanol animals, were given I.P. injections of pyrazole 18 mg/100 gm body weight in a 0.01 M aqueous solution in order to inhibit the ADH (Figure 5).

Blood was taken by heart puncture of ether anesthetized animals using a size 26 needle from three different rats from each group at 8, 16, and 24 hour intervals. Therefore, blood was removed from each animal only once. The blood pyrazole levels were checked in both groups injected with pyrazole after 24 hours in order to be sure that adequate amounts of pyrazole remained and that the different groups did not metabolize or excrete pyrazole at different rates; analysis of variance proved no significant difference at .05 level of significance.

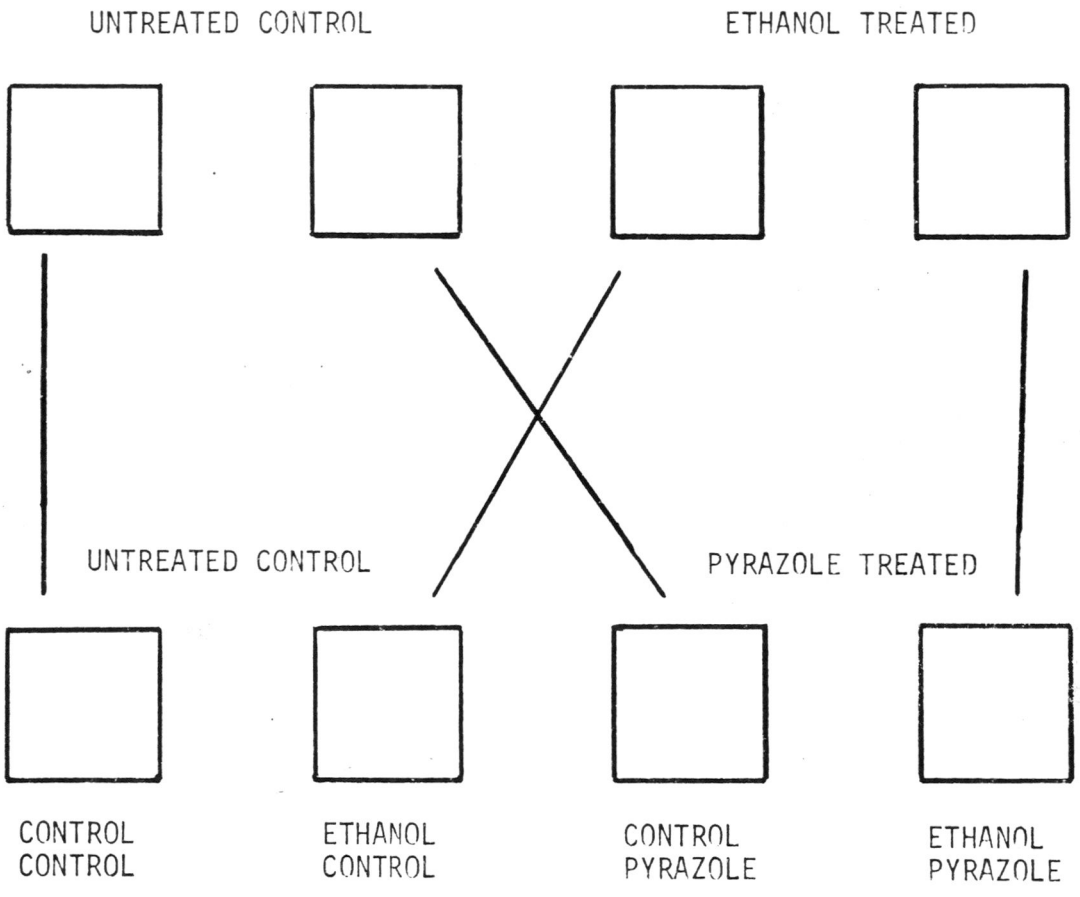
Legend for Figure 4

Absorption spectra of complex formed between pyrazole (from serum) and sodium pentacyanoaminoferrate (III).


.700
.600
.500
.400
Absorbance
.300
.200
.100
200
300
400
500
600
700
Wavelength (nm)

Legend for Figure 5

Diagram of the four groups of animals. Two groups were dosed with ethanol daily for three weeks prior to the experiment, two control groups were not exposed to ethanol. One ethanol group and one control group were injected with pyrazole, and one ethanol group and one control group were not treated with pyrazole.


UNTREATED CONTROL
ETHANOL TREATED
UNTREATED CONTROL
PYRAZOLE TREATED
CONTROL
CONTROL
ETHANOL
CONTROL
CONTROL
PYRAZOLE
ETHANOL
PYRAZOLE

The ethanol levels in the blood were determined by the following method. The blood collected by heart puncture was added to a tube containing a few crystals of EDTA. One tenth ml of blood was then added to a clean centrifuge tube along with 0.1 ml of 5% $ZnSO_4$ and 0.1 ml of 0.3 M $Ba(OH)_2$. The resulting mixture was centrifuged at 4°C at 8,000 xg for 10 minutes and 1.0 µl of the clear supernatant was injected with a 10 µl syringe into a Bendix gas chromatograph. The centrifuge tube was kept stoppered whenever possible.

Standard quantities of ethanol were injected into the gas chromatograph and the area under the peaks was calibrated and used to quantitate peak area per nanogram of ethanol injected. It was necessary to run standards at each attenuation as the electronics were not linear. Input attenuation was 10 and suppression voltage was 1000, recorder attenuation varied from "1" to "10" and one nanogram of ethanol gave a peak area of 0.71 $\pm$ .003 sq. in. at an attenuation of "5".

Solubilizing and separating component parts

Coon et al.[51] in studying the ω-hydroxylation of saturated fatty acids by the microsomal MFOS solubilized the microsomes and then separated the three component parts (NADPH cytochrome P-450 reductase, cytochrome P-450, and phosphatidylcholine) of his system using a DEAE cellulose anion exchange column. Because in vitro studies have shown that the NADPH cytochrome P-450 reductase[52] and cytochrome P-450[11] are also involved in ethanol metabolism, we attempted to resolve the ethanol metabolizing system using Coon's method and compare it to Coon's fatty acid ω-hydroxylation system.

The microsomes were prepared at 0-4°C as were all subsequent procedures until the incubations, which were carried out at room temperature. Four young adult female Holzman rats (200 gm each) were sacrificed by a sharp blow to the head and decapitated. The livers (45 gm total) were excised immediately and placed in 0.25 M cold sucrose. After the livers had been chilled, they were rinsed in sucrose and homogenized in four volumes of sucrose. The homogenate was spun at 10,000 xg for 15 minutes. The supernatant was then filtered through four layers of cheesecloth and respun at 10,000 xg for 15 minutes. The supernatant was again filtered through four layers of cheesecloth and spun at 105,000 xg for one hour.

The microsomals pellet (1.0 gm) was then solubilized in the following mixture: 8 ml of 1.0 M KCl, 8 ml of 1.0 M sodium citrate pH 7.6, 35 ml of 0.25 M sucrose, 4.1 ml of 10% sodium deoxycholate, 0.8 ml of 0.1 M dithiothreitol, and 24 ml of glycerol. This solution was stirred for 20 minutes. The solution was then centrifuged for two hours at 105,000 xg. The supernatant was placed on a DEAE-cellulose column 45 cm x 3 cm having a flow rate of 1.5 to 2.0 ml/min. The cellulose had previously been equilibrated with a solution containing 0.1 M Tris pH 7.7, 10^{-4} dithiothreitol and 0.05% sodium deoxycholate (equilibration solution).

The fine particles of the commercially available DEAE-cellulose were removed by suspending the cellulose in the equilibration solution and allowing the solution to stand for one to two hours. After the larger particles were settled out, the supernatant solution was decanted

off. This "fining" procedure was repeated twelve times to remove all the fine particles. The column was packed by pouring a suspension of the DEAE-cellulose in equilibration solution into the glass column until the cellulose was 45 cm deep. A stepwise salt gradient was used to elute the substances from the column. The equilibration solution containing either 0.1 M KCl, 0.2 M KCl, 0.3 M KCl, or 0.5 M KCl was used to elute the different fractions. One liter of each solution was used to elute each fraction (Figure 6).

The first fraction (0.1 M KCl) contained a red color that appears to be oxyhemoglobin due to its absorption spectrum (Table II), and also had a high catalase-like activity. Catalase activity was measured using a Yellow Springs Clark oxygen electrode by measuring the O_2 generation upon addition of 0.1 ml of 1.0 μM hydrogen peroxide into 2 ml of 0.1 M phosphate buffer pH 7.4 at room temperature.

The second fraction (0.2 M KCl) contained cytochrome P-450. The P-450 was concentrated and partially purified by adsorption on alumina Cγ gel that had previously been washed with 50 ml of 0.5 M potassium phosphate buffer pH 7.7. This washing was necessary for the removal of an unidentified substance which transfers with the protein to the reaction mixture and reacts with the semicarbazide during the metabolism studies to give an abnormally high optical density at 224 nm for acetaldehyde determination.

The protein solution was stirred for 15 minutes with 0.5 gm of gel, the gel was spun down at 6,000 xg for 10 minutes, and then stirred with 30 ml of 0.1 M phosphate buffer pH 7.7 for 10 minutes. The gel

Legend for Figure 6

Graph of protein eluting from the DEAE Cellulose column. The first fraction eluted with 0.1 M KCl contained a heme group and catalase-like activity. The second fraction eluted with 0.2 M KCl contained cytochrome P-450. The third fraction eluted with 0.3 M KCl contained NADPH cytochrome P-450 reductase.

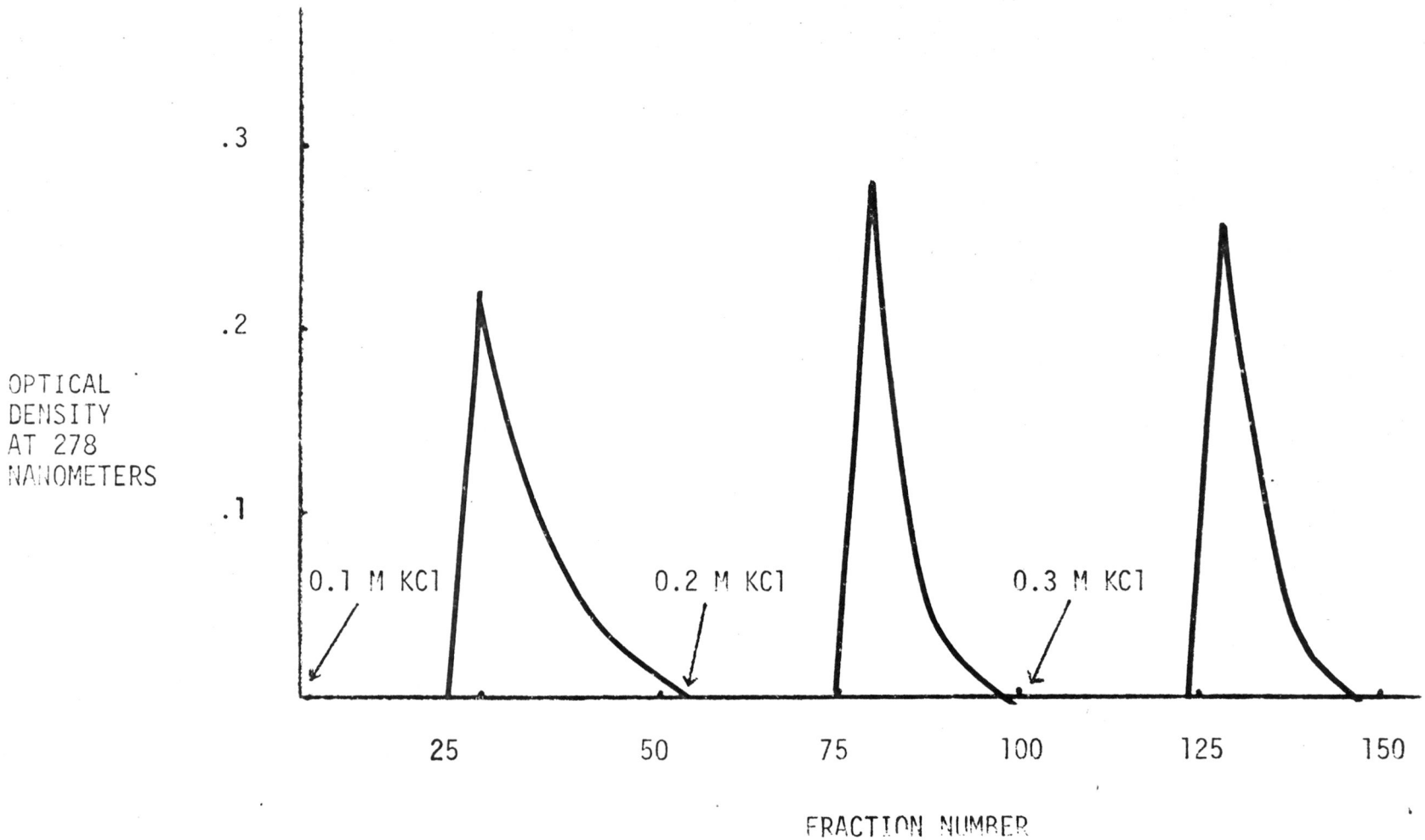

OPTICAL DENSITY AT 278 NANOMETERS
.3
.2
.1
0.1 M KCl
0.2 M KCl
0.3 M KCl
25
50
75
100
125
150
FRACTION NUMBER

Table II

Absorption of Visible Light by Hemoglobin and Derivatives

	Absorption bands, wavelengths in nanometers		
Hemoglobin	430	555	
Oxyhemoglobin	412-415	540-542	576-578
Methemoglobin (pH 7)	405-407	500	
Fraction #1	415	541-543	576-577

was then centrifuged again at 6,000 xg for 10 minutes, the supernatant was discarded. The P-450 was eluted from the gel by stirring for 15 minutes with 15 ml of 0.5 M phosphate buffer pH 7.7 and the gel was removed by centrifuging for 10 minutes at 10,000 xg. The concentration of cytochrome P-450 was 0.27 $\pm$ .04 nanomoles/ml (using the extinction coefficient 91 cm^{-1} $mmole^{-1}$).

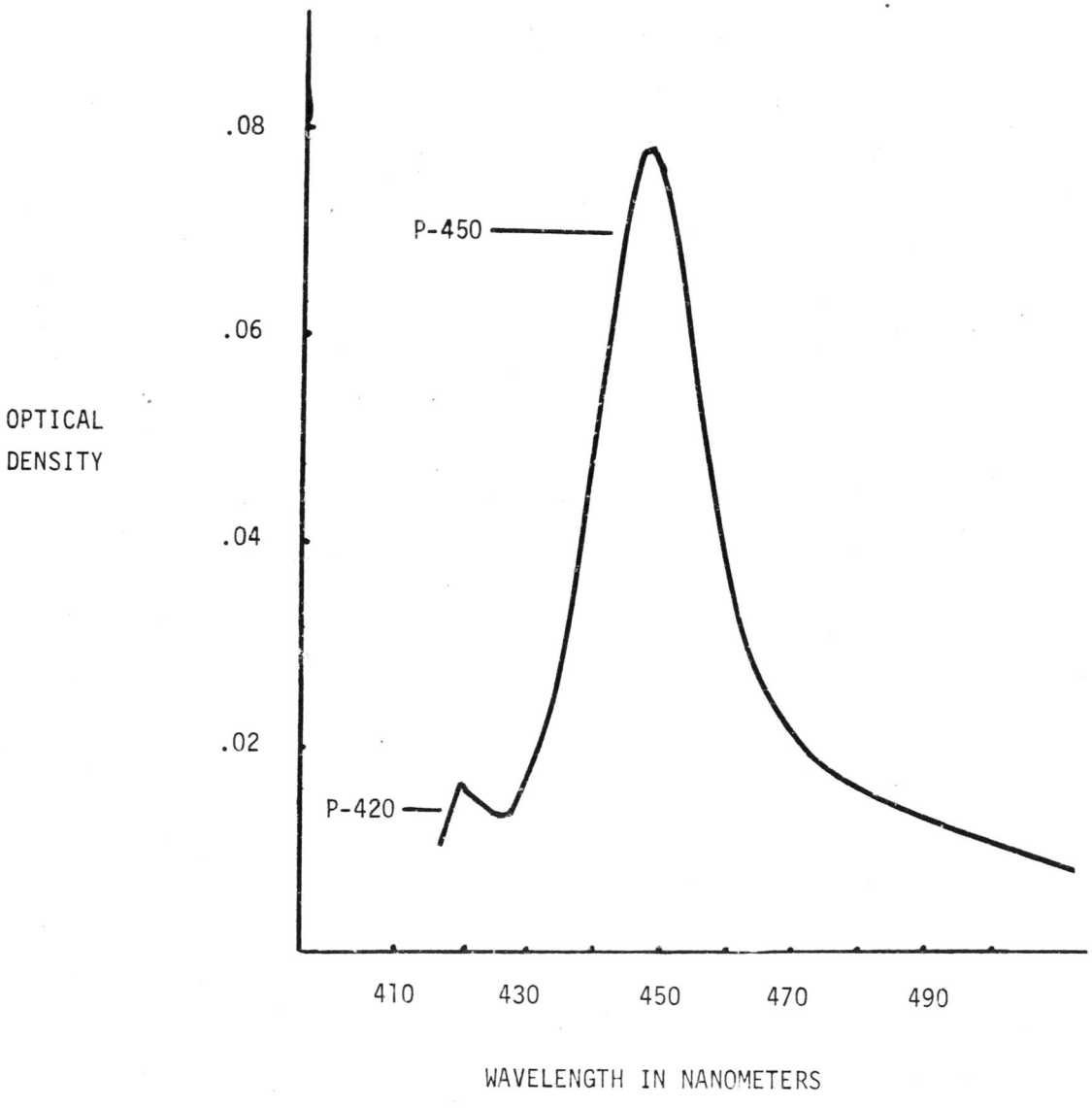
Cytochrome P-450 remains stable for two weeks in a frozen state, but thawing and refreezing cause the P-450 to be converted or deteriorate into an inactive form (P-420). P-420 has a single absorption peak at 420 nm when reduced and bound with carbon monoxide (Figure 7).

The third fraction (0.3 M KCl) contained NADPH-cytochrome P-450 reductase. This fraction was also concentrated and partially purified using alumina Cγ gel that had been previously washed with 0.5 M potassium phosphate buffer pH 7.7. The protein was adsorbed on the gel by stirring for 20 minutes with 0.5 gm of gel. The solution was centrifuged for 10 minutes at 6,000 xg, and the supernatant was discarded. The gel was then resuspended in 50 ml of 0.02 M potassium phosphate buffer pH 7.7 and stirred for 10 minutes. This solution was spun at 6,000 xg for 10 minutes, and the supernatant discarded. The reductase was eluted by stirring the gel for 10 minutes with 10 ml of 0.25 M potassium phosphate buffer pH 7.7 and spinning it for 10 minutes at 10,000 xg.

Reductase was analyzed by measuring the reduction of cytochrome C. The reaction mixture contains 0.3 μmoles of NADPH, 0.12 μmoles of cytochrome C, the reductase sample, and 0.10 M phosphate buffer pH 7.4

Legend for Figure 7

Absorption spectra of cytochrome P-450 and P-420 complexed with carbon monoxide.


.08
.06
.04
.02
OPTICAL
DENSITY
P-450
P-420
410
430
450
470
490
WAVELENGTH IN NANOMETERS

to a total volume of 3.0 ml. The reaction was monitored at 340 nm and at 550 nm. The average reductase activity was found to be 7.1 ± 0.9 µmole of cytochrome C reduced/ mg protein/minute.

The fourth fraction (0.5 M KCl) contained a heat stable phosphatidylcholine, among other phospholipids. An ether extraction was used to separate the lipid from the aqueous solution coming off the column. One hundred ml of redistilled ether was stirred with the 0.5 M KCl fraction overnight. The ether was evaporated under N_2 at room temperature and the residue was resuspended in 10 ml of water and stored in a frozen state until needed.

In several experiments the second, third and fourth fractions were eluted together. First the oxyhemoglobin fraction was eluted with 0.1 M KCl as before, then 0.7 M KCl was used to elute the remaining substances. This elutant contained the combination of cytochrome P-450, NADPH cytochrome P-450 reductase, and phospholipid (the last three fractions off the column). This solution had NADPH cytochrome P-450 reductase activity of 11.2 ± 0.8 µmole of cytochrome C reduced/ mg protein/minute, and 0.35 ± 0.08 nanomoles cytochrome P-450/ml, and a total protein concentration of 5.3 mg/ml.

After all the fractions were eluted, the column was then washed with one liter of 0.7 M KCl in the equilibration solution to remove any other substances remaining on the column. One liter of the equilibration solution was then run through to remove the salt. The column was then ready for another run. After two or three runs, the

cellulose was removed from the column and repacked. This repacking was necessitated by the gradual slowing of the flow rate and darkening of the first one centimeter of cellulose. The darkened area was removed and the same cellulose was used to repack the column.

An incubation flask was made by building a center well (1.5 ml) into a 25 ml Erlenmeyer flask. The incubations were carried out in the main chamber of the stoppered Erlenmeyer flask. The incubation mixture contained various combinations of components of the mixed function oxidase system (see Table III), 0.36 μmoles of NADPH, 0.15 mmoles of ethanol, and 0.1 M potassium phosphate buffer pH 7.4 to bring the volume to 3.0 ml. The center well contained 0.6 ml of 0.15 M semicarbazide hydrochloride in 0.10 M potassium phosphate buffer pH 7.0.[53] The semicarbazide reacted with the acetaldehyde that was produced in the main chamber and diffused into the center well to form a compound that has an absorption peak at 224 nm.[54] The concentration of the acetaldehyde bound to the semicarbazide was determined by removing 0.4 ml of solution from the center well and adding it to 1.0 ml of water. The absorption spectrum of the product of the reaction was the same as produced by the acetaldehyde standard (Figure 8). An acetaldehyde standard curve was drawn by placing acetaldehyde in the main chamber of the flask and allowing it to diffuse into the center well overnight (Figure 9).

All protein concentrations were determined by the Biuret method. A standard curve was drawn using known amounts of bovine serum albumin (Figure 10).

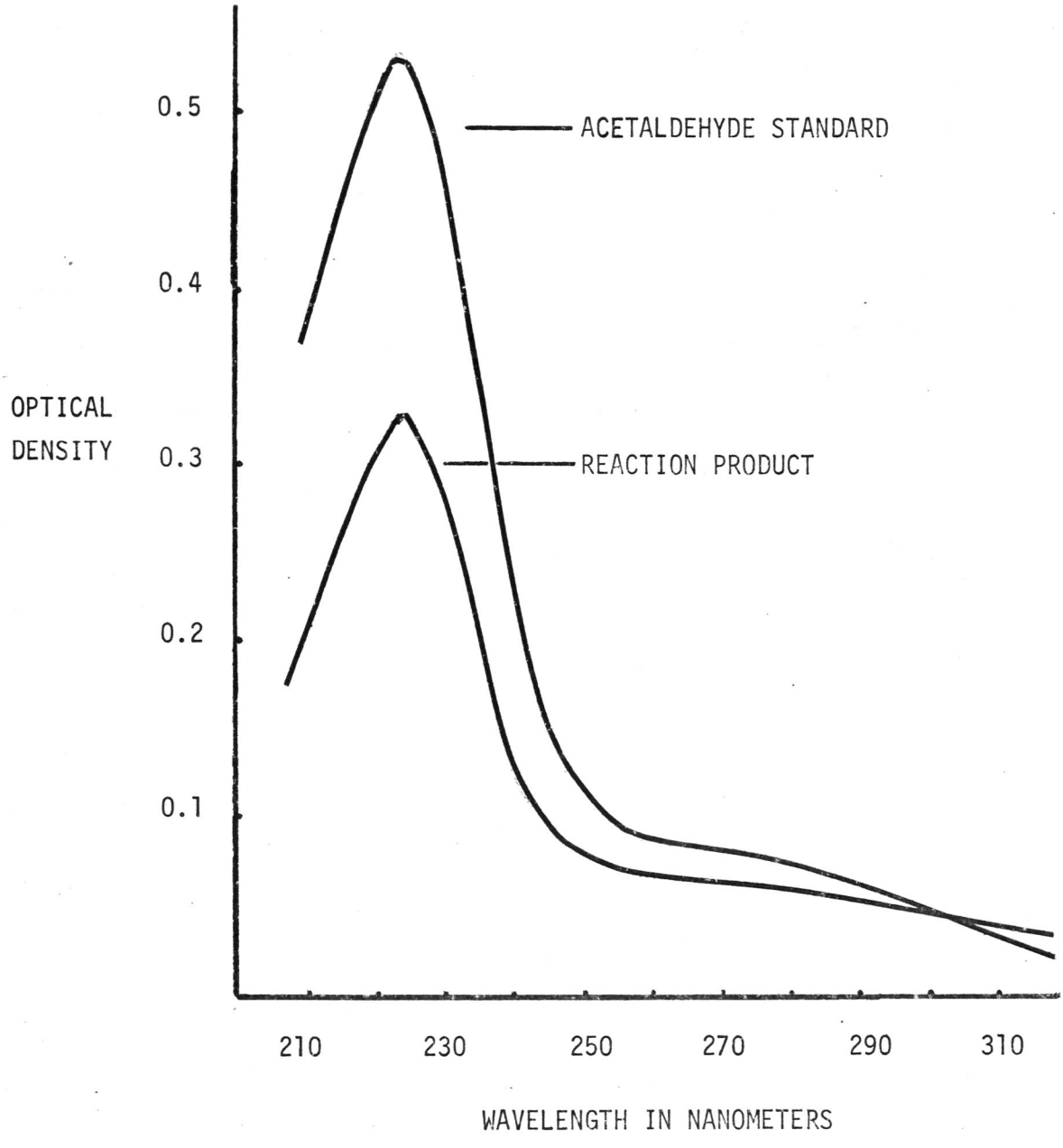
Table III

The incubation mixture comtained 0.36 micromoles of NADPH, 0.15 millimoles of ethanol, 0.1 milliliter of one of the components listed below and enough 0.1 M potassium phosphate buffer pH 7.4 to bring the total volume to 3.0 milliliters.

Components Added	Micromoles of Acetaldehyde Produced
whole microsomes (30.0 ± 3.6 mg/ml)	.351 ± .031
solubilized microsomes (11.1 ± 1.0 mg/ml)	.316 ± .023
oxyhemoglobin fraction (0.7 ± 1.0 mg/ml)	.016 ± .010
cytochrome P-450 (1.2 ± 0.3 mg/ml)	.013 ± .005
NADPH cytochrome P-450 reductase (2.0 ± 0.4 mg/ml)	.009 ± .005
phospholipid	.011 ± .006
cytochrome P-450, reductase, phospholipid	.009 ± .005
cytochrome P-450, reductase, phospholipid, oxyhemoglobin	.000 ± .010
"last three fractions" (5.3 ± 0.1 mg/ml)	.030 ± .015
"last three fractions" + oxyhemoglobin fraction	.170 ± .016
"last three fractions" + catalase (2.0 mg/ml)	.180 ± .009
"last three fractions" + peroxidase (2.0 mg/ml)	.166 ± .011

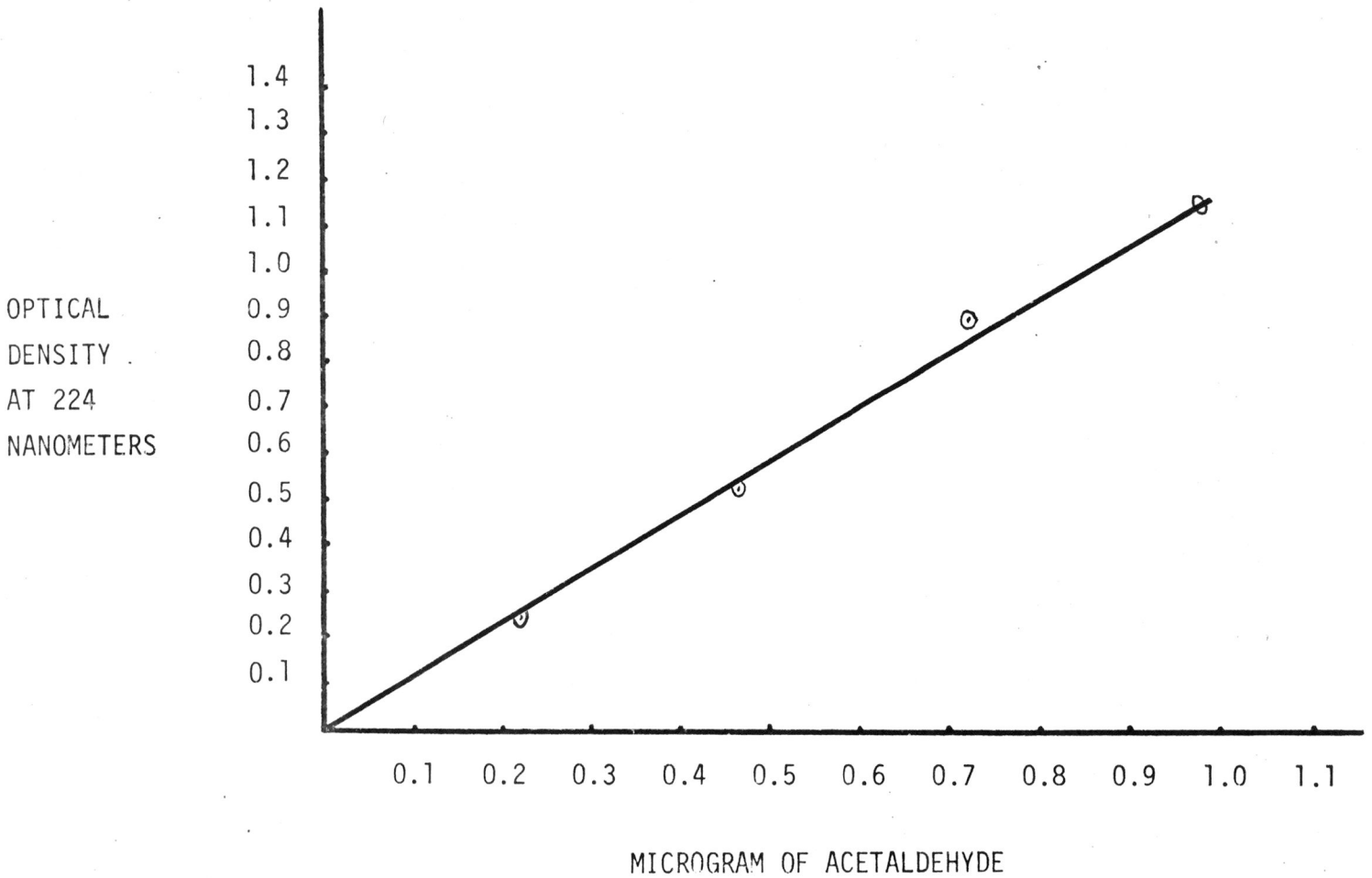
Legend for Figure 8

Absorption spectra for acetaldehyde and the product of the reaction in the incubation flask complexed with semicarbazide.


0.5
0.4
0.3
0.2
0.1
OPTICAL
DENSITY
ACETALDEHYDE STANDARD
REACTION PRODUCT
210
230
250
270
290
310
WAVELENGTH IN NANOMETERS

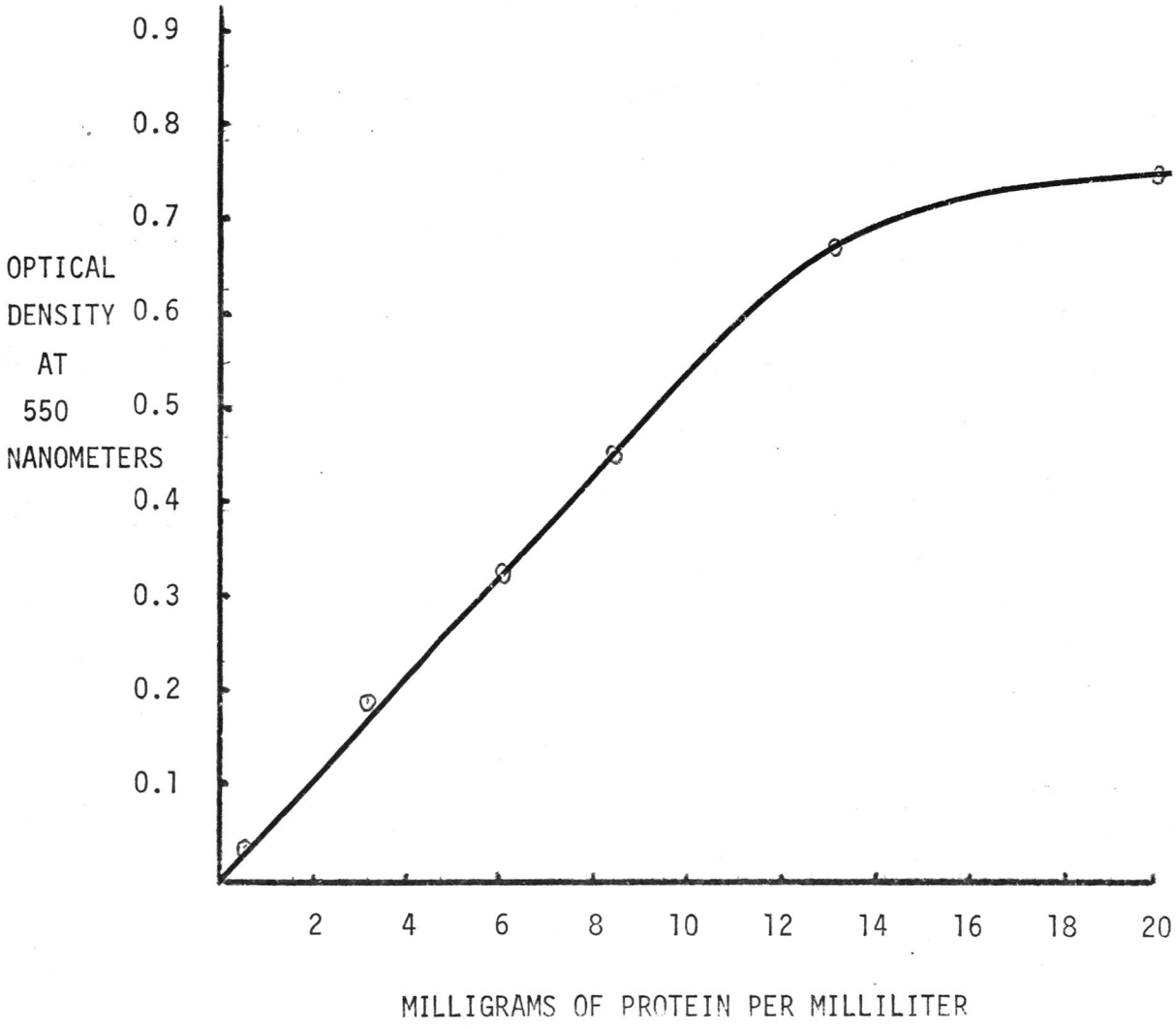
Legend for Figure 9

Standard curve of acetaldehyde complexed with semicarbazide prepared as described in the text.


OPTICAL DENSITY AT 224 NANOMETERS
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
MICROGRAM OF ACETALDEHYDE

Legend for Figure 10

Standard curve of protein concentration (BSA) as determined by the biuret method.


0.9
0.8
0.7
OPTICAL
DENSITY 0.6
AT
550 0.5
NANOMETERS
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
18
20
MILLIGRAMS OF PROTEIN PER MILLILITER

RESULTS AND DISCUSSION

Pyrazole determination

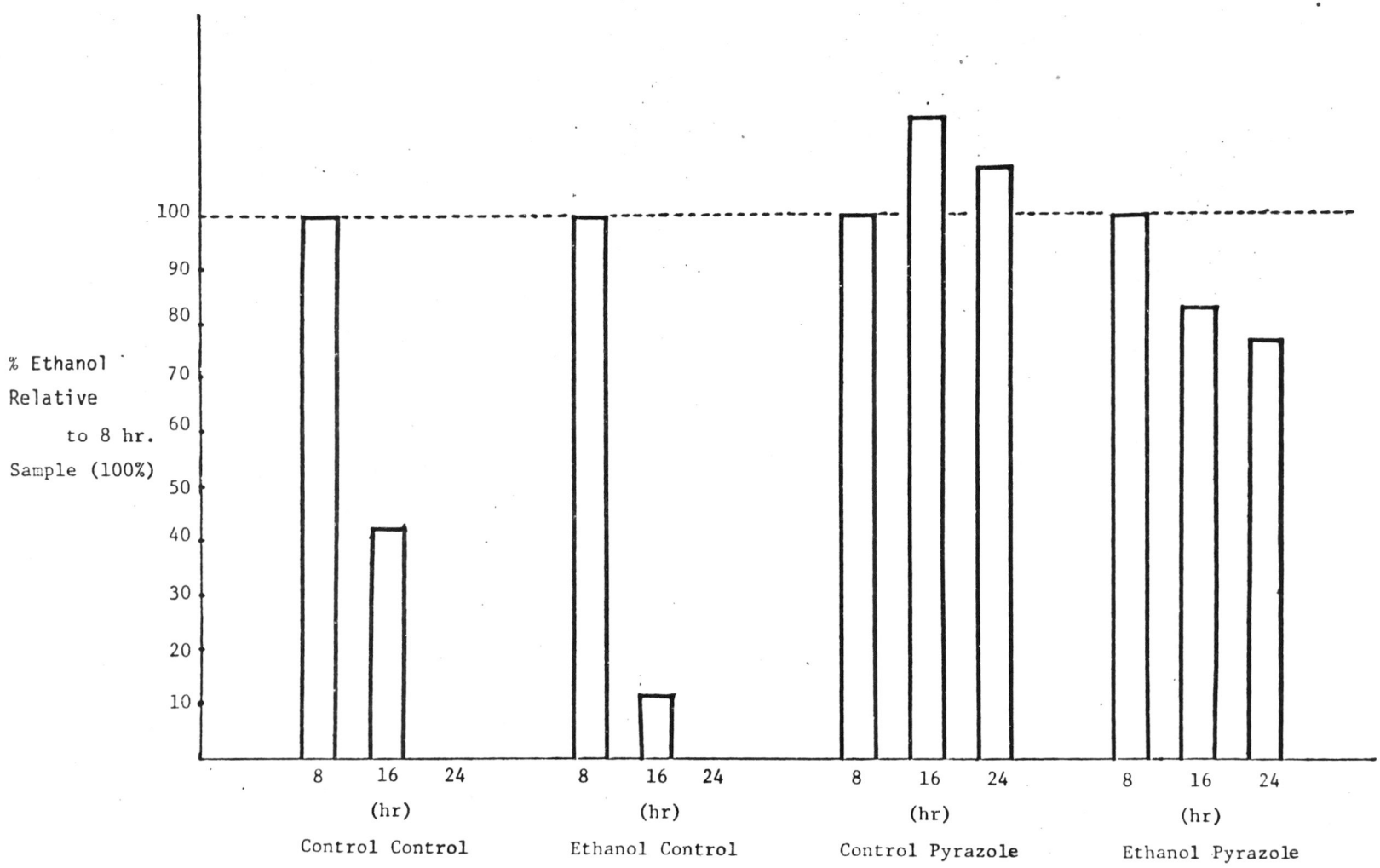
In our study of ethanol metabolism Holzman rats were injected I.P. with 18 mg/100 gm pyrazole in order to inhibit ADH. In order to interpret our results in the ethanol metabolism studies we had to be certain that there was no difference in blood pyrazole levels between experimental groups after 24 hours. Pyrazole was assayed as a reaction product with pentacyanoaminoferrate (III). This method will detect levels of pyrazole down to 100 μmoles in a total sample volume of 0.2 ml.

Ethanol determination

As stated previously, one factor not understood about the chronic alcoholic was the enhanced tolerance to ethanol since ADH is not increased upon chronic alcohol consumption. Earlier, Lieber and De Carli had shown that the microsomal MFOS did metabolize ethanol *in vitro* and that this system was induced by chronic ethanol consumption. By comparison of normal to ethanol treated rats both uninhibited and inhibited with pyrazole, it was found that chronic exposure to ethanol did enhance ethanol metabolism. The data presented in Figure 11 indicates that in the normal animals the MFOS did not play a significant role in ethanol metabolism *in vivo* but in the ethanol dosed animals there was a significant increase in the clearance of ethanol both in the uninhibited system and the ADH inhibited system. The difference within the control ccntrol, ethanol control and the ethanol pyrazole groups was significant at the $P>0.05$ level, but the differences within the control pyrazole was not.

Legend for Figure 11

Graph of percent ethanol remaining in the blood relative to eight hour sample in the four experimental groups of animals. "Control Control" signifies normal animals not injected with pyrazole. "Ethanol Control" signifies ethanol dosed animals but not injected with pyrazole. "Control Pyrazole" signifies normal animals injected with pyrazole. "Ethanol Pyrazole" signifies ethanol induced animals injected with pyrazole.


% Ethanol
Relative
to 8 hr.
Sample (100%)
100
90
80
70
60
50
40
30
20
10
8
16
24
(hr)
Control Control
Ethanol Control
Control Pyrazole
Ethanol Pyrazole

One possibility that existed was that the ethanol induced the rate of pyrazole clearance by either metabolism or excretion, thus allowing for an increase in ethanol metabolism by ADH. Pyrazole blood levels checked in both groups after 24 hours were found to be 2.5 μmoles/0.2 ml of blood serum, and that no significant difference was present.

It should be noted here that pyrazole at the levels used will also inhibit 48% of the MFOS activity,[55] but this inhibition was not nearly so complete as the inhibition of ADH.[56]

Therefore, it has been shown that ADH was not responsible for the increased rate of ethanol metabolism *in vivo* and that some alternative system, possibly MFOS was responsible.

Solubilization and separation of the component parts

It was apparent that the MFOS was induced by both drugs and ethanol, and it would oxidize both drugs and ethanol. The question that then arose was how much overlap was there between the microsomal drug oxidase system and the microsomal ethanol oxidase system. Lieber and DeCarli had shown that cytochrome P-450 and NADPH cytochrome P-450 reductase were increased upon chronic ethanol consumption, and that these components had also been found in the microsomal drug oxidase system. Coon and Lu separated the three component parts of the ω-hydroxylation system (NADPH cytochrome P-450 reductase, cytochrome P-450, and phosphatidylcholine) that utilized NADPH and molecular oxygen. Following their procedure, we found that although the solubilized microsomes would convert ethanol into acetaldehyde; the three components separated and

partly purified then added back together would not (see Table III). This indicated that there were one or more components in the ethanol oxidizing system that are not common with the ω-hydroxylation system.

If dismutase were present in vivo, then H_2O_2 would be generated by the ethanol induced system (Figure 1b), which could in the presence of peroxidase oxidize ethanol into acetaldehyde. Previous workers have indicated that they believe that H_2O_2 was an intermediate in ethanol oxidation, because a hydrogen peroxide generating system could replace the NADPH and molecular oxygen, although H_2O_2 added directly cannot.

To determine if H_2O_2 was indeed an intermediate in the ethanol metabolism, we prepared an incubation mixture that contained everything that was eluted from the column after the first (oxyhemoglobin) fraction. To this was added the oxyhemoglobin fraction (which contained catalase-like activity), catalase, or peroxidase in various experiments (see Table III). Although the "last three fractions" would not catalyze the oxidation of ethanol into acetaldehyde, the "last three fractions" with catalase, peroxidase, or the oxyhemoglobin fraction would catalyze the reaction indicating that H_2O_2 was an intermediate. This oxidation being catalyzed by catalase seems strange in the light that this reaction by definition was a peroxidation type reaction; nevertheless, other workers have also reported that catalase will, with H_2O_2, oxidize ethanol into acetaldehyde.[55]

Also noted was the fact that P-450 reductase, and phospholipid that have been partially purified, together with the unpurified oxyhemoglobin fraction still did not oxidize ethanol. This indicated

that somewhere in the partial purification of each of the three fractions some component, possibly dismutase, was lost, thereby making it impossible for the formation of H_2O_2 from the "superoxide" radical thus blocking the reaction.

In summary microsomes plus NADPH and molecular oxygen catalyze the oxidation of ethanol into acetaldehyde. This oxidation required cytochrome P-450, NADPH cytochrome P-450 reductase, some form of H_2O_2 utilizing enzyme (catalase or peroxidase), plus some factor that catalyzes the formation of H_2O_2, probably dismutase.

LITERATURE CITED

1. Baile, M. The Morbid Anatomy of Some of the Most Important Parts of the Human Body, p. 141. London: J. Johnson, St. Paul's Churchyard, and G. Nicol, Pall Mall (1793).

2. Addison, T. Gary's Hosp. Rep., 1: 476 (1936).

3. Lane, B. P. and Lieber, C. S. Amer. J. Pathol., 49: 593 (1966).

4. Marshall, E. K., Jr., and Frity, W. F. J. Pharmacol. Exp. Ther., 109: 431 (1953).

5. West, Edward S., Wilbert R. Todd, Howard S. Mason, and John T. Van Brazzen. Textbook of Biochemistry. The Macmillan Company (1966).

6. von Wartburg, J. P. and Papenbury, J. Psychosomat. Med., 28: 405 (1966).

7. Greenberger, Norton J., Cohen, Richard R., and Issebacker, Kurt J. International Academy of Pathology, Vol. 14 (1965).

8. Majihrowicz, Bercaw, B. L., Cole, W. M., and Gregory, D. H. Quarterly Journal of Studies on Alcohol, Vol. 28, No. 2 (1967).

9. Rubin, Emanuel, Lieber, Charles S. Annals of Internal Medicine, Vol. 69, No. 5 (1968).

10. Orne-Johnson, W. H. and Ziegler, D. M. Biochim. Biophys. Res. Comm., 21: 78 (1965).

11. Rubin, E., Hutter, F., and Lieber, C. S. Science, 159: 1469 (1968).

12. Gupta, R. Ke, and Robinson, W. G. Biochim. Biophys. Acta, 118: 431 (1966).

13. Lieber, C. S. and DeCarli, L. M. Science, 162: 917 (1968).

14. Baxter, R. C. and Henseley, W. J. Biochem. Pharmacol., 18: 233 (1969).

15. Cronholm, T., Sjovall, J., and Sjovall, K. Steroids, 13: 671 (1969).

16. Ammon, H., Estler, C., and Heim, F. Biochem. Pharmacol., 18: 29 (1969).

17. Bezman-Torcher, A., Nestel, P., Felts, J., and Havel, R. J. Lipid Research, 7: 248 (1966).

18. Cronholm, T. and Sjovall, J. Biochim. Biophys. Acta, 152: 233 (1968).

19. Kiessling, K. and Pilstrom, L. Acta Pharmacol. et Toxicol., 24: 103 (1966).

20. Lane, B. and Lieber, C. Amer. J. Pathol., 49: 593 (1966).

21. Kufer, C. S. and Frier, E. D. Trans. Amer. Acad. Physicians, 57: 283 (1942).

22. Iseri, G. A., Gottlieb, L. S. and Lieber, C. S. Fed. Proc., 23: 579 (1964).

23. Lieber, C. S., Rubin, E., and DeCarli, L. M. Biochemical and Clinical Aspects of Alcohol Metabolism. C. C. Thomas Publisher, Springfield, Illinois (1969).

24. Lieber, C. S. and Spritz, W. J. Clin. Invert., 45: 1400 (1966).

25. Young, David L., Powell, Geraldine, and McMillan, W. O. Journal of Lipid Research, 12 (1971).

26. May, Hubert E. and McCay, P. B. The Journal of Biological Chemistry, 243(9): 2288-2295 (1968).

27. May, Hubert E. and McCay, P. B. The Journal of Biological Chemistry, 243(9): 2296-2305.

28. Roubal, W. T. Lipids, Vol. 6, No. 1 (1971).

29. Coon, M. J. and Lu, A.Y.H. International Symposium on Microsomes and Drug Oxidation, Bethesda, Md. Feb. (1968).

30. Strobel, H. W. and Coon, M. J. Journal of Biological Chemistry, 246: 24 (1971).

31. Di Luzio, N. R. Experimental and Molecular Pathology, 8: 3 (1968).

32. Rubin, Emanuel and Lieber, C. S. Annals of Internal Medicine, 69: 5(1968).

33. Sik, Charles J. Science, 163: 1297-1300 (1969).

34. Sladek, N. E. and Mannering, G. J. Molecular Pharmacology, 5: 2 (1968).

35. Gillette, J. R. Advances in Pharmacology, 4 (1965).

36. Kamin, H., Masters, B. S., Gibson, Q. H. and Williams, Charles H., Jr. Federation Proceeding, 24: 5 (1965).

37. Glazier, Robert I., Schenkman, John B., and Gartorelli, Alan C. Molecular Pharmacology, Vol. 7, No. 6 (1971).

38. Alvares, A. P., Schilling, S., Levin, W., Kuntzman, R., Brand, L., and Mark, L. C. Clin. Pharmacol. Ther., 10: 655 (1969).

39. Klingenberg, M. Arch. Biochem. Biophys., 75: 376 (1958).

40. Togawa, K., Ishidate, K., Kawagushi, K., and Hagihora, B. Abstr. 7th Int. Congr. Biochem., Tokyo, 887 (1967).

41. Appleby, C. A. Biochim. Biophys. Acta, 747: 399 (1967).

42. Mitani, F., Winchester, R. J. Alvares, A. P., Poland, A. P., and Kappas, A. Molecular Pharmacology, 7: 6 (1971).

43. Hildebrandt, A. G. and Estabrook, R. W. Microsomes and Drug Oxidation. Academic Press (1969).

44. May, Hubert E. and McCay, Paul B. The Journal of Biological Chemistry, 243: 2288-2295 (1968).

45. Schenkman, John B. Molecular Pharmacology, 8: 178-188 (1971).

46. Theorell, H. Experientia, 21: 553 (1965).

47. Goldbery, L. and A. Rydberg. Biochem. Pharmacol., 18: 1794 (1964).

48. Lu, Anthony Y. H. and Coon, M. J. Journal of Biological Chemistry, 243(6) (1968).

49. Morgan, J. and N. V. Luzio. Proc. Soc. Exp. Biol. Med., 134: 462 (1970).

50. La Rue, T. Anal. Chem., 37: 245 (1965).

51. Lu, Anthony Y. H., Junk, Karen W., and Coon, Minor J. Journal of Biological Chemistry, 244: 13 (1969).

52. Lieber, C. S. and DeCarli, L. M. Science, Vol. 170, p. 78-80 (1970).

53. Lieber, C. S. and DeCarli, L. M. Science, 162: 917-918 (1968).

54. Supta, N. K. and Robinson, W. G. Biochim. Biophys. Acta, 118: 431 (1966).

55. Carter, E. A. and Isselbacker, K. J. Annals New York Academy of Science, p. 283-295.

56. Lieber, C. S. and DeCarli, L. M. Journal of Pharmacology and Experimental Therapeutics, Vol. 181 (2) (1972).

APPENDIX A

Table of Abbreviations

ADH	Alcohol dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
MFOS	Mixed function oxidase system
I. P.	Interperitoneum
EDTA	Ethylenediamine tetraacetic acid
DEAE	Diethyl amino ethyl
Tris	Tris (Hydroxymethyl) aminomethane
RBC	Red blood cell

APPENDIX B

The following chemicals were produced by the Fisher Scientific Company, Fair Lawn, New Jersey: KCl (A.C.S. Lot 714195); glycerol (white, U.S.P. Lot 792118); NaCl (A.C.S. Lot 711749); KH_2PO_4 (A.C.S. Lot 714365); K_2HPO_4 (A.C.S. Lot 712096); $ZnSO_4$ (A.C.S. Lot 702464; $Ba(OH)_2$ (A.C.S. Lot 712594); trisodium pentocyanoaminoferrate (Lot 784979); Na citrole (Lot 712632); H_2O_2 (A.C.S. Lot 74879).

The following chemicals were produced by Sigma Chemical Company, St. Louis, Mo.: dithiothreitol (Lot 92C-0140); sodium deoxycholate (Lot 42C-1090; DEAE cellulose anion exchanger (capacity 0.85 mg/gm, medium mesh Lot 30-2400); alumina C gel (prepared 11-9-71, Lot 111 C-8240); NADPH (Type I chemically reduced Lot 71C-7370); cytochrome C (oxidized form, Lot 24B-7150); catalase (Lot 29B-8121); peroxidase (Lot 40C-2610).

The ethanol was from Commercial Solvents Corporation, Terre Haute, Indiana and was U.S.P. grade. The semicarbazide hydrochloride (Lot 1-1675) and the $NaNO_2$ (A.C.S. Lot 33736) were from J. T. Baker Chemical Company, Phillipsburg, New Jersey. The sucrose was from Savannah Food and Industries, Savannah, Ga. Eastman Organic Chemical Company supplied the acetaldehyde (Lot 468) and the pyrazole (Lot 9876).

A Bendix model 2100 gas chromatograph was equipped with a 6 ft by 1/4 in O.D. Teflon DuPont column packed with "Poly Pak 2" (Hewlett-Packard Co., Avondale, Pa.) and operated at 135°C. The carrier gas was nitrogen (50 ml/min); injector port temperature was 135°C; the detector (flame ionization) was operated at 220°C using hydrogen

(50 ml/min) and oxygen (400 ml/min). The original electrometer was replaced with a Bendix Model Mark I solid state electrometer. A Barber-Coleman Model 8300-57000 recorder was used to record the data. The instrument was calibrated using known amounts of ethanol in water. A Gelman No. 26748 planimeter was used to calibrate the area under the peaks. The 10 microliter syringe used to inject samples into the gas chromatograph was made by Sienco Scientific, Inc., Houston, Texas.

In the pyrazole studies, a Beckman DB-G spectrophotometer (Fullerton, Calif.) was used with a Beckman 100 m.v. log/linear recorder.

In the ethanol metabolism studies a Perkin-Elmer Coleman 124 spectrophotometer (Hitachi Tokyo, Japan) was used with a Perkin-Elmer Coleman 56 recorder (Hitachi Tokyo, Japan).

A Fisher accumet Model 320 pH meter was used to determine pH of solution.

The catalase assays were carried out using a Yellow Spring Incorporated oxygen electrode Model 53 equipped with a Perkin-Elmer Coleman Model 56 recorder.