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STUDIES ON THE TOXICOLOGIC ROLE OF MAMMALIAN ALCOHOL DEHYDROGENASE

by
Walter David Watkins

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ABSTRACT

STUDIES ON THE TOXICOLOGIC ROLE OF MAMMALIAN ALCOHOL DEHYDROGENASE

by
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Chairman: Thomas R. Tephly

The first part of this dissertation was directed at evaluating the participation of hepatic alcohol dehydrogenase (ADH) in the oxidation of methanol-^{14}C and ethanol-1-^{14}C to $^{14}CO_2$ in the intact rat and monkey. This was determined by utilizing the capacity of pyrazole to inhibit hepatic ADH. Pyrazole did not influence other known enzymic systems concerned with alcohol oxidation *in vivo* during the time intervals investigated.

In the monkey, pyrazole dramatically inhibited the oxidation of ethanol and methanol. In the rat, pyrazole exerted a profound inhibition of ethanol oxidation and a modest, yet significant inhibition of methanol oxidation. These results support the concept that hepatic ADH is the major catalyst in ethanol and methanol oxidation in the monkey, and ethanol oxidation in the rat. Although the catalase-peroxidative system may still be considered the major oxidative route for methanol in the rat, these studies show that the hepatic ADH system participates in this process.

Pyrazole in doses which exhibited no apparent toxicity in rats was seriously toxic in monkeys. Standard tests for toxicity did not define the mechanism of pyrazole toxicity in the rat or monkey. No apparent relationship exists between the acute inhibitory effects of pyrazole on ADH *in vivo* and the toxicity associated with high doses of pyrazole. A combination of ethanol and pyrazole

at doses which exhibited no apparent toxicity to the monkey served to inhibit methanol oxidation to a degree observed with either compound employed alone in higher doses, suggesting possible clinical applications in the treatment of methanol poisoning in man. Also of clinical significance were the observations in vivo that pyrazole is an effective inhibitor of alcohol oxidation when administered after the alcohol; and, that pyrazole exhibits prolonged inhibitory effects.

Studies were conducted to determine whether a "microsomal ethanol oxidizing system" observed in vitro plays a significant role in the oxidation of alcohols in vivo. No role for this system could be demonstrated in the rat, as shown by experiments in vivo in which treatment with SKF 525-A which inhibits, and phenobarbital induction, which enhances microsomal hydroxylations did not alter alcohol oxidation.

The second part of this dissertation was devoted to studies on the role of retinal ADH (RADH) in the metabolism of alcohols and vitamin A. Studies were performed to assess the effect of inhibitors of hepatic ADH on the activity of RADH and vitamin A metabolism. It was determined that RADH purified from the rat and monkey does not participate in the interconversion of retinol and retinaldehyde. Such interconversion is, however, mediated by an enzyme separate and distinct from RADH and hepatic ADH, and which is located in the particulate fraction of retinal homogenates.

Studies showed that RADH resembles hepatic ADH in that both enzymes are located in the cytosol, are NAD-dependent, exhibit similar pH optima, are reactive with ethanol, 1-propanol and 1-butanol, and are inhibited by pyrazole and n-butyraldoxime. Despited these qualitative similarities between RADH and liver ADH activities,

Michaelis constants and inhibitor constants were many-fold greater for RADH than liver ADH. Also, RADH differed from hepatic ADH in that RADH is unreactive with retinol, retinaldehyde and methanol; is only slightly reactive with acetaldehyde; and is not inhibited by concentrations of 1,10-phenanthroline which inhibit hepatic ADH.

Retinol dehydrogenase activity located in the particulate fractions of retinal homogenates was shown to exhibit a pH optimum different from RADH or hepatic ADH; was more reactive with NADP than NAD; and, was unreactive with methanol, ethanol, 1-propanol, 1-butanol, formaldehyde and acetaldehyde. Furthermore, this enzyme was reactive with retinol (oxidation) and retinaldehyde (reduction) and neither of these activities was inhibited by high concentrations of pyrazole, ethanol or methanol.

Therefore, neither RADH nor retinol dehydrogenase would be expected to participate in the retinal oxidation of methanol. Furthermore, it is unlikely that administration of inhibitors of hepatic ADH would exert an effect on retinol-retinaldehyde interconversion.

Although a well-defined toxicologic role of hepatic ADH has emerged, no definitive statement can presently be made for ADH activity in other organs.

To Sharon

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABBREVIATIONS	x
INTRODUCTION	1
I. Historical Development of Research on Alcohol Dehydrogenase	2
II. The Physiological Role of Alcohol Dehydrogenase	11
III. The Pharmacological Role of Alcohol Dehydrogenase	22
IV. The Toxicologic Role of Alcohol Dehydrogenase	25
V. Inhibitors of Alcohol Dehydrogenase	39
VI. Aldehyde Dehydrogenase	45
VII. Objectives and Plan of the Present Investigation	47
MATERIALS AND METHODS	50
I. Materials	50
A. Chemicals and Reagents	50
B. Sources of Experimental Animals and Tissues	51
II. Methods	52
A. Experiments <u>in vivo</u>	52
B. Partial Purification of Rat Liver Alcohol Dehydrogenase and Rat Liver Aldehyde Dehydrogenase	53
1. Preparation of Hepatic Cytosol Fraction	53

2. Separation of Rat Liver Alcohol Dehydrogenase and Rat Liver Aldehyde Dehydrogenase 53

C. Partial Purification of Rat Retinal Alcohol Dehydrogenase and Rat Retinal Aldehyde Dehydrogenase 54

1. Preparation of Retinal Cytosol Fraction 54

2. Ammonium Sulfate Fractionation 55

3. DEAE-Cellulose Chromatography 55

D. Partial Purification of Monkey Retinal Alcohol Dehydrogenase and Monkey Retinal Aldehyde Dehydrogenase 56

E. Studies on Rat and Bovine Retinal Homogenates 56

F. Assay Procedures 57

1. Catalase 57

2. Alcohol Dehydrogenase 57

3. Aldehyde Dehydrogenase 58

4. Special Methods 58

5. Determination of Kinetic Constants 62

6. Protein Determination 62

RESULTS 63

I. Studies on the Oxidation in vivo of Ethanol and Methanol in the Rat and Monkey 63

A. Effect of Pyrazole on the Catalase-Peroxidative System 65

B. Effect of Pyrazole on Ethanol Oxidation in the Intact Rat 70

C. Effect of Pyrazole on Methanol Oxidation in the Intact Rat 72

D. Other Studies on Pyrazole Inhibition of Ethanol and Methanol Oxidation in the Intact Rat 72

E. Effect of Pyrazole on Ethanol and Methanol Oxidation in the Intact Monkey 77

F. Studies on Pyrazole Toxicity in the Rat and Monkey 80

G. Effect of Pyrazole and Ethanol on Methanol Oxidation in the Monkey 83

H. Studies on the Role of Microsomal Oxidation of Ethanol in the Intact Rat 85

II. Studies on the Retinal Alcohol Dehydrogenase System 87

A. Separation and Partial Purification of Alcohol Dehydrogenase and Aldehyde Dehydrogenase from Rat Retinas 90

B. Separation and Partial Purification of Alcohol Dehydrogenase and Aldehyde Dehydrogenase from Monkey Retinas 91

C. Studies on the Properties of Partially Purified Rat Retinal Alcohol Dehydrogenase 98

1. Substrate Specificity 98

2. The Effect of Pyrazole and Butyraldoxime on Retinal Alcohol Dehydrogenase 103

D. Studies on Partially Purified Monkey Retinal Alcohol Dehydrogenase 105

E. Studies on Partially Purified Rat Retinal Aldehyde Dehydrogenase 107

F. Studies on Vitamin A Oxidation-Reduction in Homogenates of Rat and Bovine Retinas 111

DISCUSSION 116

SUMMARY 146

BIBLIOGRAPHY 149

LIST OF TABLES

Table		Page
1	Effect of Pyrazole and Aminotriazole on Rat Hepatic Catalase Activity *in vivo*	68
2	Effect of Pyrazole on Peroxidative Oxidation of Methanol *in vitro*	69
3	Effect of Ethanol on Methanol Oxidation in the Monkey	86
4	Relative Distribution Pattern of Rat Retinal Cytosol Enzymes After Ammonium Sulfate Saturation	92
5	Procedure for Purification of Rat Retinal Alcohol Dehydrogenase and Aldehyde Dehydrogenase	94
6	Relative Distribution Pattern of Monkey Retinal Cytosol Enzymes After Ammonium Sulfate Saturation	96
7	Procedure for Purification of Monkey Retinal Alcohol Dehydrogenase and Aldehyde Dehydrogenase	97
8	Substrate Specificity of Rat Retinal Alcohol Dehydrogenase	100
9	Oxidation of Retinol in Rat Retinal Homogenates	112
10	Summary of the Characteristics of Hepatic Alcohol Dehydrogenase, Retinal Alcohol Dehydrogenase, and Retinol Dehydrogenase from the Rat	115

LIST OF FIGURES

Figure		Page
1	Effect of Pyrazole on Crystalline Catalase Activity	66
2	Effect of Pyrazole on Ethanol Oxidation in the Rat *in vivo*	71
3	Effect of Pyrazole on Methanol Oxidation in the Rat *in vivo*	73
4	Effect of Pyrazole on Ethanol Oxidation in the Rat *in vivo*	75
5	Effect of Pyrazole on Methanol Oxidation in the Rat *in vivo*	76
6	Duration of Pyrazole Inhibition of Ethanol Oxidation in the Rat *in vivo*	78
7	Effect of Pyrazole on Methanol Oxidation in the Monkey *in vivo*	79
8	Effect of Pyrazole and Ethanol on Methanol Oxidation in the Monkey *in vivo*	84
9	Effect of SKF 525-A on Ethanol and Methanol Oxidation in the Rat *in vivo*	88
10	Effect of Phenobarbital Treatment on Subsequent Oxidation of Ethanol in the Rat *in vivo*	89
11	Chromatography of Retinal Enzymes on DEAE-Cellulose	93
12	Effect of pH on the Activity of Purified Rat Retinal Alcohol Dehydrogenase	99
13	Lineweaver-Burk Plot of Purified Rat Liver Alcohol Dehydrogenase Activity	102
14	The Effects of Pyrazole and n-Butyraldoxime on Purified Rat Retinal Alcohol Dehydrogenase Activity	104

Figure		Page
15	The Effects of Pyrazole on Purified Monkey Retinal Alcohol Dehydrogenase Activity	106
16	Effect of pH on the Activity of Purified Rat Retinal Aldehyde Dehydrogenase	108
17	Lineweaver-Burk Plot of Purified Rat Retinal Aldehyde Dehydrogenase Activity	110

ABBREVIATIONS

ADH	Alcohol Dehydrogenase
AldDH	Aldehyde Dehydrogenase
AP-NAD	3-aminopyridyl-NAD
AT	3-amino-1,2,4-triazole
CM-Cellulose	Carboxymethylcellulose
DEAE-Cellulose	Diethylaminoethylcellulose
dpm	Disintegrations Per Minute
EDTA	Ethylenediaminetetraacetic Acid
Ki	Inhibitor Constant
Km	Michaelis Constant
LADH	Liver ADH
LAldDH	Liver AldDH
LDH	Lactate Dehydrogenase
NAD (NADH)	Oxidized (Reduced) Nicotinamide Adenine Dinucleotide
NADP (NADPH)	Oxidized (Reduced) Nicotinamide Adenine Dinucleotide Phosphate
POPOP	1,4-di-(2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
RADH	Retinal ADH
RAldDH	Retinal AldDH
RDH	Retinol Dehydrogenase; Retinol:NADP Oxidoreductase
SGOT	Serum Glutamic-Oxalacetate Transaminase
SKF 525-A	2-diethylaminoethyl-2,2-diphenylvalerate
TCA	Tricarboxylic Acid
TRIS	Tris(hydroxymethyl)aminomethane
Vmax	Maximal Velocity

INTRODUCTION

The broad distribution of alcohol dehydrogenases in nature is well documented in the literature. Reported sources for alcohol dehydrogenase activity include yeasts, molds, bacteria, a variety of plants and seeds, erythrocytes of lower vertebrates, the retinas of amphibia and mammals, fish and mammalian liver (1), and mammalian brain (2), kidney (3), and gastrointestinal tissue (4). Despite the ubiquitous nature of this enzyme, much is yet to be determined concerning its fundamental role in the mammalian organism. This lack of an obvious and definite role for alcohol dehydrogenase was taken as sufficient cause to review earlier studies with respect to three alternative functions that might feature this enzyme: a physiologic, a pharmacologic, and a toxicologic role. This dissertation will review and discuss the possible toxicologic role of alcohol dehydrogenase. Since other functionally allied enzyme systems coexist physically with alcohol dehydrogenase within the cell, it was not only necessary to consolidate recent information with older established knowledge of alcohol dehydrogenase, but was also pertinent to present our current knowledge on the participation of those functionally allied enzymes or enzyme systems coexistent in vivo with alcohol dehydrogenase.

In most studies, yeast and mammalian liver have served as the major sources of alcohol dehydrogenase. The emphasis of this dissertation is centered on mammalian systems, and

unless specified otherwise, subsequent considerations will relate to these systems.

I. Historical Development of Research on Alcohol Dehydrogenase

The first studies on alcohol oxidation by animal tissues appeared in the latter nineteenth century (5,6,7). However, yeast served as the primary enzyme source for initial investigations on the enzymology of alcohol oxidation.. Batelli and Stern (1909) were first to describe, in qualitative terms, certain basic properties of yeast and mammalian alcohol dehydrogenase present in acetone-dried preparations and cell-free solutions (8,9). Studies which indicated the cofactor function of NAD for yeast ADH appeared first in 1933 (10), the same year in which initial investigations of soluble preparations from yeast were reported (11). Shortly thereafter a number of important investigations on yeast ADH was published, including the stoichiometry of the reversible enzymatic oxidation of alcohols (12) and procedures for crystallization (13).

Detailed studies on animal hepatic ADH were not published until 1938 (14), at which time NAD was reported to be required as a cofactor, and certain parameters on the stability of hepatic ADH were established. A large portion of the knowledge on fundamental physical and enzymatic properties of ADH is attributable to Theorell and his many colleagues (15,1).

The original procedure for preparation of crystalline horse liver ADH was developed by Bonnichsen and Wassen (16). Modifications in this method (17) provide yields approximating

0.4 g hepatic ADH/kg equine liver. Since then, purification procedures have appeared which describe the isolation of ADH from human (18), monkey (19) and rat liver (20).

Hepatic ADH is classically known to catalyze the first step in the following NAD-dependent sequence:

$$CH_3CH_2OH \underset{\text{Dehydrogenase}}{\overset{\text{Alcohol}}{\rightleftharpoons}} CH_3CHO \xrightarrow[\text{Dehydrogenase}]{\text{Aldehyde}} CH_3COOH$$

Backlin (21) examined the equilibrium established by alcohol dehydrogenase. At a pH range of 7-10, and ionic strength $\mu = 0.1$, the equilibrium constant (Keq) at 20° was:

$$Keq = \frac{[NADH]\,[CH_3CHO]\,[H^+]}{[NAD]\,[CH_3CH_2OH]} = (8.01 \pm 0.14) \times 10^{-12} M$$

From this value it is apparent that the reduction of acetaldehyde is definitely the more favored reaction in this equilibrium. From the calculated free energy change at pH 7.4 (+ 4.9 kcal/mol), and assuming equal concentrations of NAD and NADH, this may be interpreted to mean that in the physiologic pH range ethanol concentrations will exceed the acetaldehyde concentration by a factor of approximately 5000 (22). Because ethanol is known to undergo oxidation by this system *in vivo*, the obvious question is what conditions exist *in vivo* which promote this energetically unfavorable reaction. At least two factors are known which satisfactorily answer this question. First, NADH interacts with high affinity to numerous intracellular enzymes, resulting in relatively low concentrations of this reduced cofactor in the unbound form.

The second factor embraces the second enzymatic step of this oxidative pathway, aldehyde dehydrogenase. This oxidation of acetaldehyde to acetate proceeds with a large negative free energy (-13 kcal/mol)(22), which indicates that acetaldehyde formed through ADH is continuously removed by the irreversible reaction mediated by aldehyde dehydrogenase. Experimentally obtained values for the ratio of unbound NAD to unbound NADH, and steady state concentrations of acetaldehyde have been substituted in the above equilibrium equation. From this calculation it was found that oxidation of ethanol will proceed favorably down to low blood ethanol concentrations (1.5 mg/L) (22,23).

A number of significant investigations have been performed on the physical and kinetic properties of mammalian alcohol dehydrogenases. Sund and Theorell (1) have reviewed much of the pertinent literature on the enzymatic properties of a number of alcohol dehydrogenases, including horse liver ADH. This enzyme is capable of reacting with a broad range of alcohol, aldehyde and ketone substrates. The alcohols which are reported to serve as substrates for this ADH include a variety of primary and secondary, aromatic and aliphatic compounds. Numerous experiments with ADH have shown that, in a series of primary alcohol substrates, the Michaelis constant (Km) decreases as the chain length increases. In the same manner, it has been amply demonstrated that the longer the carbon chain length of primary alcohols, the lower is the concentration at which substrate inhibition occurs (24).

Tertiary alcohols and isopropanol are not effective substrates for horse hepatic ADH (1). Ungar (25) reported the capacity of horse liver ADH to oxidize some 3-β-hydroxysteroids (but not 3-α-hydroxysteroids) to the corresponding 3-ketosteroids with NAD as coenzyme and these findings were subsequently confirmed (26,27) and extended by Waller et al. (28), who defined the substrate specificity of this enzymatic activity to the 3-β-hydroxyl group of 5-β-steroids. Reynier et al. (29) have recently discussed some of the studies on steroid-active ADH, and have demonstrated the additional steroid substrate restriction of an A/B cis-fusion. Pietruszko et al. (30) employed gel electrophoresis to separate steroid-active fractions from exclusively ethanol-active fractions of commercial hepatic ADH preparations. Soon afterwards, Theorell et al. (31) crystallized from the mother liquors of their own purified horse liver ADH preparations a separate ADH which would accept both steroid alcohols and ethanol as substrates, while the main fraction was devoid of steroid activity. Discovery of ADH isozymes is not surprising in view of the earlier work of Treble (32), who was successful in separating a horse liver homogenate into fractions possessing alcohol dehydrogenases with differing specificities toward ethanol and 2-fluoroethanol. He showed that both compounds were oxidized by the dehydrogenase found in the fraction that precipitated between 30 and 42 percent ammonium sulfate saturation, while the fraction that precipitated between 50 and 80 percent ammonium sulfate saturation contained a dehydrogenase which was capable

of oxidizing ethanol but not 2-fluoroethanol. The fact that hepatic ADH oxidizes retinol[1] to retinaldehyde[2] (20) shall be discussed at greater length in a subsequent section of this work.

Several investigators (33,34) reported horse liver ADH to be unreactive with methanol. More recently, Kini and Cooper (35) showed that although its capacity with this substrate is low, hepatic ADH is indeed capable of oxidizing methanol, but only at high substrate concentrations. The latter finding is the more plausible in light of the knowledge that ADH will catalyze the reduction of formaldehyde to methanol (36).

Detailed studies on human hepatic ADH (18,37) have shown this enzyme to possess a number of properties which are similar to the horse liver enzyme. It is important in the present work to note the contribution of Blair and Vallee (38) who found for purified human liver ADH a Michaelis constant (Km) of 1.0 mM for ethanol. When methanol served as the substrate a Km of 30 mM was obtained. In addition, the Vmax obtained with ethanol is almost ten times that observed with methanol. During their studies on human ADH, von Wartburg and his coworkers (3,39) found an "atypical" ADH variant occurring in 20 percent of a Swiss and 4 percent of a London population. Several notable differences between the "atypical" and typical enzymes were reported (39): (1) the normal pH optimum of 10.8 for ethanol oxidation is shifted to 8.5 for

[1]Retinol (All-trans), Vitamin A_1 Alcohol, Vitamin A_1

[2]Retinal (All-trans), Vitamin A_1 Aldehyde, Retinene$_1$

the atypical enzyme; (2) normal human ADH is activated by thiourea, whereas the atypical enzyme is inactivated by this treatment; (3) the inhibitory effects of o-phenanthroline are more pronounced on the normal ADH than on the atypical enzyme; and, (4) pyrazole is a more potent inhibitor of the atypical enzyme than of the normal ADH. Despite these differences, normal and atypical enzymes were reported to possess identical molecular weights as estimated by gel chromatography on Sephadex G-200, and possessed identical Michaelis constants for ethanol, acetaldehyde, NAD, and NADH. Increasingly large numbers of studies on the isozyme patterns of alcohol dehydrogenases from various mammalian sources are being reported. It should be emphasized that further studies are presently indicated to investigate the existence and function of isozymes *in vivo*. The possibility remains that some, or all, of the isozymes observed *in vitro* may be experimental artifacts resulting from trauma or alteration sustained by a single intracellular enzyme or protein complex during the preparative process (40).

Using methanol, ethanol and 2-fluoroethanol (32) as possible substrates, Makar *et al.* (19) have examined the reaction kinetics of partially purified alcohol dehydrogenases prepared from the livers of the monkey, rat and horse. Of particular interest was the observation that when methanol served as the substrate the Michaelis constants for the rat and monkey liver preparations were similar. In contrast, for the horse liver preparation under the same experimental

conditions, the Km was 2-3 times that of the rat or monkey.

Until recently, there were no published reports on the preparation or kinetic properties of purified rat liver ADH. This gap in the total knowledge of alcohol dehydrogenases was filled in 1969, when Reynier (20) described a procedure for preparing a ten-fold purification of the rat liver ADH. With this enzyme and excess NAD, reaction kinetics were performed using ethanol or retinol as substrates, and the inhibitory capacities of various pyrazole derivatives on the oxidation of these substrates were determined.

In addition to ADH, at least two additional enzyme systems have been studied which are capable of mediating the oxidation of ethanol to acetaldehyde; the catalase-peroxidative system, and a microsomal mixed function oxidase.

The biological function of catalase was believed for many years to be a protective mechanism involved in the decomposition of hydrogen peroxide. Keilin and Hartree (41,42) presented evidence for a system in which catalase served a catalytic role in the oxidation of primary alcohols to their aldehydes when low concentrations of hydrogen peroxide were supplied. These were similar to the conditions <u>in vivo</u> where certain flavin enzymes such as xanthine oxidase, amine oxidase, and D-amino acid oxidase act on their respective substrates to release low concentrations of hydrogen peroxide. These investigators presented arguments which discouraged the toxicologic role for catalase as one related solely to hydrogen peroxide reduction, and supported their

belief that catalase functions primarily in coupled peroxidative reactions. Chance (43) employed rapid reaction techniques to study the interaction of catalase with hydrogen peroxide and aliphatic alcohols from which he formulated the following reaction mechanism:

$$\underset{\text{(Rapid Combination)}}{\text{Catalase} + H_2O_2} \xrightarrow{k_1} \underset{\text{(Rapid Second Order Reaction)}}{\text{Catalase}(H_2O_2) + RCH_2OH} \xrightarrow{k_4}$$

$$\underset{\text{(Relatively Unstable)}}{\text{Catalase}(H_2O_2)(RCH_2OH)} \xrightarrow{k_5} \text{Catalase} + 2H_2O + RCHO$$

Chance observed the following rates at which various aliphatic alcohols combined with Catalase·H_2O_2(Complex I):

Substrate	k_4(L/M/s)
Hydrogen Peroxide	3.5×10^7
Ethanol	1.0×10^3
Methanol	1.0×10^3
n-Propanol	17.0
1-Butanol	2.0
Isoamyl Alcohol	0.1

Orme-Johnson and Ziegler (44) were first to report that an hepatic microsomal fraction was capable of oxidizing ethanol and methanol in the presence of NADPH and molecular oxygen. This was later confirmed by Lieber and DeCarli (45). Chronic ethanol administration produces a proliferation of the smooth endoplasmic reticulum with a corresponding induction of microsomal mixed-function oxidase reactions (46,210), and the observed microsomal alcohol oxidizing system is inhibited by

carbon monoxide (45). Furthermore, Roach *et al.* (47) have demonstrated an increase in the alcohol oxidizing capacity of this system by pretreatment with phenobarbital and benzo(a)-pyrene. These findings have been interpreted by some to indicate that a microsomal mixed-function oxidase reaction, similar to that described for the metabolism of many drugs, plays a significant role in alcohol oxidation *in vivo*. Caution should be exercised before concluding such a role exists *in vivo* for the microsomal mixed-function oxidase system, for there exist conflicting data and unanswered questions regarding this proposal. For example, no light-reversal of carbon monoxide inhibition of this system has yet been observed, and Roach *et al.* (48) found no inhibition *in vitro* of this system by SKF 525-A, a compound with a broad range of inhibitory effects on microsomal reactions (49). In addition, these same authors have found that 3-amino-1,2,4-triazole, an inhibitor of catalase (50), inhibits the observed microsomal oxidation of ethanol and that NADPH can be replaced by a peroxide-generating system. This would indicate the presence of some residual catalase in microsomal preparations, and that NADPH oxidase, a microsomal enzyme, might be a component of the "microsomal ethanol oxidizing system" serving to supply residual catalase with peroxide which could then serve to oxidize ethanol to acetaldehyde. Isselbacher and Carter (51) have recently provided evidence supporting the likelihood that NADPH oxidase is involved in the "microsomal ethanol oxidizing system" by demonstrating that

when NADPH oxidase was inhibited by potassium cholate, microsomal ethanol oxidation was inhibited by a comparable degree. In the same investigation these authors showed that, although microsomal alcohol dehydrogenase activity may be difficult to detect under the usual assay conditions in which NAD is employed as the cofactor, the use of AP-NAD, a cofactor possessing much greater affinity for LADH than NAD, enabled ready analysis of previously undetected microsomal ADH. These considerations and the fact that NADP will function as a cofactor for LADH suggested that the NADPH-dependent oxidation of ethanol by microsomes is not attributable to a distinct and membrane-bound enzyme, but results from 3 separate enzyme reactions:

1. $NADPH + O_2 + H^+ \xrightarrow{\text{NADPH Oxidase}} NADP^+ + H_2O_2$

2. $NADP^+ + C_2H_5OH \xrightarrow{\text{ADH}} NADPH + H^+ + C_2H_4O$

3. $H_2O_2 + C_2H_5OH \xrightarrow{\text{Catalase}} 2H_2O + C_2H_4O$

It is noteworthy that despite the waging controversy on the mechanism of microsomal ethanol oxidation, no reports have appeared which evaluate the quantitative significance of this system in alcohol oxidation <u>in vivo</u>.

II. The Physiological Role of Alcohol Dehydrogenase

Despite impressive research efforts centered about ADH, much has yet to be achieved in satisfactorily elucidating the physiological function of this enzyme which exists so abundantly in the mammalian organism. A number of possible physio-

logic functions for ADH have been suggested and supported experimentally in varying degrees. Since the substrate specificity of mammalian ADH (E.C. 1.1.1.1) is rather broad (1), the possibility exists that the endogenous substrates are not necessarily those alcohols or aldehydes which are most reactive with the enzyme, but may be relatively unique biochemical entities.

Holtzer et al. (52,53) have demonstrated a role for ADH in the catabolism of fructose, where glyceraldehyde, formed through cleavage of fructose-1-phosphate, is reduced to glycerol.

Alcohol dehydrogenase may play a physiologic role in the metabolism of certain endogenous lipids, including the sterols and some derivatives, and long chain fatty alcohols and aldehydes.

A summary of investigations of certain steroids which serve as substrates for hepatic ADH was presented earlier. It is important in the context of this discussion to note that those naturally occurring steroids (3β-hydroxy-5β configuration) which can function as substrates for an hepatic ADH are of little quantitative importance in the mammal (54). The observation that lithocholic acid (3α-hydroxy-5β-cholanic acid) inhibits the oxidative reactions catalyzed by rat hepatic ADH has prompted the suggestion (29) of a possible indirect relationship between ADH activity and steroid metabolism. Steroids possessing the 3α,5β configuration may regulate ADH activity, which may in turn modify the metabolism of

vitamin A, an essential component of adrenal steroid synthesis (55,56,57). This could represent a type of feedback regulatory system for steroids. The reports (58,59,60) of "steroid-sensitive" aldehyde dehydrogenases lends further support to the possibility that the regulation of steroid and vitamin A dynamics may proceed *in vivo* through an ADH system.

Farnesol, a precursor in cholesterol biosynthesis, has been shown to serve as a substrate for ADH (61,62). Reynier *et al.* (29) have related the results of preliminary experiments (63) in which the rate of ^{14}C-elimination in the feces of rats which had been administered cholesterol-4-^{14}C was uneffected by pyrazole, an inhibitor of hepatic ADH activity.

Endogenous steroids are included in the evergrowing list of compounds which can serve as substrates for the microsomal hydroxylating enzymes. The initial products of microsomal hydroxylations are alcohols, however, the isolation of aldehyde and carboxylic acid metabolites from these microsomal preparations suggests the presence of alcohol and aldehyde dehydrogenases (64). Moir *et al.* (65) have examined the alcohol dehydrogenase of rat liver microsomal oxidative sterol demethylation. Alcohol dehydrogenase activity was extractable from their microsomal preparation with Tris-acetate buffer. Complete restoration of microsomal demethylase activity was achieved by addition of either the Tris-acetate extract or crystalline LADH. Earlier investigators (34,28) reported that liver ADH is capable of catalyzing the oxidation of long-

chain and complex cyclic alcohols, findings which are consistent with the view that liver ADH may oxidize intermediary alcohols formed during microsomal hydroxylation reactions.

Although the biochemical role for free fatty alcohols and aldehydes has yet to be established, evidence is accumulating for their metabolic activity. With a few scant exceptions much is unknown concerning the occurrence and origin of these compounds in mammals. Fatty alcohols are present in wax esters of specialized mammalian glands (66) and porcine liver (67). A recent report (68) has appeared in which small quantities of long chain fatty alcohols were measured in beef brain and heart, rat liver and mouse preputial glands, and three transplantable neoplastic tissues. Naturally occurring free and bound fatty aldehydes appear in myocardial and other tissues of a number of mammalian species, including man (69,70,71,72). NADP-dependent interconversion of fatty alcohols and aldehydes, and NAD-dependent interconversion between fatty aldehydes and carboxylic acids have been postulated (73,74) based on earlier investigations in rat liver (75,76,77) on the cleavage of O-alkyl glyceryl ethers to fatty aldehydes and glycerol. Several possible intrinsic sources for fatty aldehydes exist. Ferrell and coworkers (69,78) have presented evidence for the biosynthesis of fatty aldehydes from acetate. Radloff and Farrell (72) briefly discuss unpublished findings that crude human heart homogenates were capable of converting palmitate-1-^{14}C to labeled free fatty aldehydes. Further evidence of the metabolic

activity of the fatty compounds, although not necessarily indicative of a biochemical role, has been presented (79) in that alkenyl ethers are formed directly from the incorporation of fatty aldehydes.

Of the possible physiologic functions for ADH, probably the most generally accepted has been its enzymatic role in the metabolism of vitamin A. It has been documented for years that the primary alcohol group at C-15 of retinol (vitamin A alcohol) can undergo reversible enzymatic oxidation to retinaldehyde (vitamin A aldehyde)(80). Purified hepatic ADH in the presence of NAD is capable of mediating this reaction (81,82). Indeed, kinetic studies of this specific enzyme activity have recently appeared in the literature (20). It was assumed that this enzyme was generally involved in retinol-retinaldehyde interconversions in the liver, retina and intestine. The capacity to oxidize retinol *in vivo* was first reported by Ball *et al.* (80) for the liver and intestinal mucosa of the rat. Using bovine and frog retinas, Wald and Hubbard (83) described an NAD-dependent enzymatic reduction of retinaldehyde to retinol. Crystalline horse liver ADH, rabbit liver, and retinal rods, each in the presence of NAD, were reactive with retinol, suggesting to Bliss (81,82) that ADH (E.C. 1.1.1.1) was the enzyme generally involved in retinol oxidation. Zachman and Olson(84) measured retinaldehyde reductase activity and ADH activity in partially purified rat liver and found that the activities of these enzymes toward ethanol and retinol in the presence

of NAD remained relatively constant during fractionation, and the effects of temperature and pH were similar on both activities. Salts and detergents did effect the reduction of acetaldehyde and retinaldehyde differently, but these differences were attributed not to the enzymes involved, but rather to the micellar or soluble state of the substrates. These investigators concluded that the two activities under study were probably mediated by the same catalyst. In an effort to distinguish tissue differences in ADH, Koen and Shaw (85) examined the isozymes of alcohol dehydrogenase (E.C. 1.1.1.1) and retinol dehydrogenase (retinol: NAD oxidoreductase) in extracts of rat liver and rat retinae. The extracts employed in these studies were prepared by homogenization in distilled water, repetitive freezing and thawing, and centrifugation at either 10,000 x g (retinas) or 13,000 x g (liver). These extracts were applied to starch gels containing NAD and buffered with KH_2AsO_4-KOH. Under these conditions the liver extract was resolved into five dehydrogenase bands, four of which were presumed to be isozymes of hepatic ADH capable of oxidizing both ethanol and retinol, while the fifth band, termed "retinol dehydrogenase 3", oxidized only retinol and possessed electrophoretic characteristics notably different from the others. When retinal extracts were subjected to electrophoresis under the same conditions, four dehydrogenase bands appeared, only one of which corresponded to the nonspecific hepatic ADH isozyme. Two of the isozymes ("retinol dehydrogenases 1 and 2") were spec-

ific for retinol, and the fourth was specific for ethanol. On the basis of the isozyme patterns obtained, and additional studies on inhibition, heat inactivation, and pH effects, the authors concluded that the oxidation of vitamin A proceeds in the retina and liver by specific and distinct enzymes. Finally, they state that the "hepatic-type" ADH does not appear in the eyes and the "retinal-type" ADH does not appear in the liver.

The retina has received concentrated attention regarding a physiologic role for ADH in vitamin A metabolism. Vitamin A is currently believed to participate in the visual process according to the following scheme: In the eye, retinol derived from systemic circulation must be oxidized to retinaldehyde before it is isomerized and combined with opsins, the proteins of the retinal rods and cones, the combination of which forms in vertebrates the photosensitive pigments rhodopsin and porphyropsin in rod., and iodopsin and cyanopsin in the cones. Light exposure of the visual pigments results in cleavage to opsin and the prosthetic group, retinaldehyde, which is enzymatically reduced to retinol. This process has been reviewed in detail by Wald and Hubbard (86), who indicate that retinal vitamin A alcohol and aldehyde interconversion is catalyzed by ADH (E.C. 1.1.1.1). These authors cite as evidence experiments in which a mixture of retinol, opsin, hepatic ADH and NAD were placed in the dark, after which a visual pigment was synthesized. Exposure of this pigment to light resulted in bleaching to retinaldehyde and opsin, followed

by reduction of retinaldehyde to retinol. Investigations involved specifically with retinal ADH have been performed not in purified retinal extracts, but rather on crude preparations of the entire tissue (83,86,87,88). Serious doubt has arisen relating to the role of "hepatic-type" ADH (E.C. 1.1.1.1) in retinal vitamin A metabolism stemming from the work of Koen and Shaw (85) described earlier, and the work of Futterman (89). This latter investigator reported a water-insoluble dehydrogenase from the bovine retina which catalyzed the oxidation of retinol more effectively with NADP than NAD.

Tissues other than the liver and retina have been investigated for the capacity to oxidize or reduce vitamin A. The presence of a vitamin A-reactive alcohol dehydrogenase in the intestinal mucosa was first suggested in 1948, when Glover et al. (90) reported that orally administered retinaldehyde was readily reduced in the intestine as well as the liver. Until recently, however, this activity has not proved readily demonstrable in cell-free intestinal preparations. Fidge and Goodman (91) have reported a 13-fold purification of a preparation from rat intestinal mucosa homogenates which exhibited the characteristics of a relatively nonspecific aldehyde reductase, rather than an alcohol dehydrogenase, for it did not measurably oxidize ethanol in the presence of NAD, yet it actively reduced aliphatic aldehydes (C_2-C_{14}) and retinaldehyde. The maximal rate of retinaldehyde reduction by this preparation was slightly greater when NADPH, rather

than NADH, served as the cofactor. A similar reductase has been described for the rabbit renal cortex (92). As more reports appear on the presence or absence of gastrointestinal enzymes, including ADH, it becomes increasingly important that investigators prove the origin of these enzymes to be mammalian, rather than microbial contaminants.

From the preceding considerations, it would appear that the liver is probably the only source of hepatic-type ADH capable of catalyzing vitamin A oxidation-reduction reactions. One may yet wonder the non-visual physiologic role for ADH-catalyzed metabolism of vitamin A. As discussed earlier, it is possible that steroid metabolism may fall under some type of regulation through ADH-vitamin A control. In this same context it is interesting to note that normal mammalian steroid synthesis is decreased under conditions of vitamin A deficiency. Furthermore, administration of progesterone reportedly offsets in part the defect in reproduction observed in vitamin A deficient animals (93). The control of this process appears to pivot about the conversion of pregnenolone to progesterone, which, along with the conversion of dehydroepiandrosterone to androstenedione, is catalyzed by Δ^5 3-β-hydroxysteroid dehydrogenase (94,95). In homogenates prepared from vitamin A deficient adrenal glands, the activity of this enzyme was measurably increased by addition of retinaldehyde (56,96), retinol or retinoic acid (57). Olson (97) indicates a number of additional enzymatic processes reported to be depressed under conditions of vitamin A

deficiency: sulfate incorporation into mucopolysaccharides of colon segments (98); and a number of oxygenases including L-γ-gulonolactone oxidase (93,99), codeine demethylase (100, 101), squalene oxidocyclase (93,102,103), and p-hydroxyphenyl pyruvate oxidase. Recently, the inhibitory effect of vitamin-deficiency upon biological sulfations has been seriously questioned by several authors (104,105). Of course, further studies must be performed to determine if the observed enzymatic effects of vitamin A deficiency are indicative of the primary actions of this vitamin, or are merely measurements of effects secondary in damaged cells. It is therefore conceivable that any, or all, of the activities discussed above could be influenced in vivo by vitamin A, the intermediary metabolism of which proceeds by way of the ADH system.

Many of the "physiologic roles" for ADH have been proposed with the underlying assumption that the mammalian organism ordinarily does not absorb or synthesize significant quantities of ethanol, and there would therefore exist no normal requirement for an "ethanol dehydrogenase" system. It is this assumption that has relegated to ADH the "toxicologic" function of serving as the catalyst initially involved in the metabolism of alcohols to which the organism may be inordinately exposed. Evidence has begun to accumulate which can leave little doubt that ethanol is indeed a trace constituent of normal animal tissues (106,107,108). For example, small quantities of ethanol are measurable in the blood and tissues from subjects who have received no ethanol

for prolonged time periods (109,23). Various workers (110, 108) have suggested that most of the measurable endogenous ethanol arises from metabolic activities of the parasitic-symbiotic microbial flora normally present in the mammalian intestinal canal. In addition to a microbial origin of endogenous ethanol, recent work has strengthened the belief that endogenous ethanol is formed through normal mammalian metabolic processes. In this work, Krebs and Perkins (108) measured significant concentrations of ethanol in the blood of germ-free rats and normal rats pretreated orally with neomycin and nystatin. As discussed previously, endogenous ethanol could be enzymatically formed through alcohol dehydrogenase from acetaldehyde, for which numerous non-microbial, non-parasitic metabolic sources have either been proven or strongly suggested to exist. Juni and Heym (111) originally suggested that acetaldehyde is produced by the pyruvate decarboxylase component of pyruvate dehydrogenase. β-alanine serves as a precursor to acetaldehyde by conversion through malonic aldehyde and decarboxylation (112). Cleavage of threonine can result in the formation of glycine and acetaldehyde (113). Finally, deoxyribose phosphate can be cleaved to form glyceraldehyde phosphate and acetaldehyde (114). Krebs and Perkins (108) state, somewhat prematurely perhaps, that "It may now be taken as established that the major function of liver alcohol dehydrogenase is to remove from the circulation a potentially toxic substance, ethanol, and channel it into the pathway of energy supply". Indeed, the continual

production of endogenous ethanol has been taken by some to explain the presence of a recently reported brain alcohol dehydrogenase (2). This enzyme activity was demonstrated in the cytosol prepared from rat brain, and is reported to possess kinetic properties similar to hepatic ADH. Suggestions have been made that the cerebral enzyme serves a reserve metabolic function to ensure that this tissue remains unaffected by physiologic systemic concentrations of ethanol. In light of earlier considerations it would appear that the kinetics of this equilibrium would not favor such a role.

III. The Pharmacological Role of Alcohol Dehydrogenase

A review of research on alcohol dehydrogenase will indicate that less is known about its physiologic role than its role in modifying the biologic actions of a number of chemical agents encountered in the disciplines of pharmacology and toxicology.

The substrate specificity for hepatic alcohol dehydrogenase is relatively broad, a characteristic which may account for its participation in the biological alteration of exogenously administered drugs and toxicants. In general, the process of metabolism is not exclusively one of detoxication, for many substances are known which are rendered more toxic through metabolism. As will be discussed in the following sections, hepatic alcohol dehydrogenase is not an exception to this generality.

With the knowledge that hepatic ADH participates in modifying the biological actions of certain compounds, the

potential value of substances known to specifically inhibit hepatic ADH, such as pyrazole, becomes apparent. The actions of compounds which lose desirable biological actions through ADH-mediated oxidation may be potentiated or prolonged by an ADH inhibitor. In addition, inhibitors of ADH may serve to limit the deleterious effects of certain compounds, such as methanol or ethylene glycol, which are themselves rather inocuous, but are oxidized through ADH to toxic metabolites. It also follows that inadvertent administration of substances which modify the action of ADH may undesirably increase the activities and toxicities of other drugs or ethanol present in the organism.

The role of ADH in modifying the pharmacologic actions of certain drugs may be considered under three general categories: (1) the role of ADH in the oxidation of ethanol and other alcohols; (2) the role of ADH in microsomal oxidations; and, (3) the role of ADH in the reduction of certain drugs.

Under conditions where ADH is involved in drug metabolism, this enzyme usually serves to catalyze the oxidation of an alcohol to the more polar aldehyde, rendering that compound more readily excretable. Certainly the first drug which should be considered in this context is ethanol. Ninety to 98 percent of the alcohol that enters the body is completely oxidized, with only about 2 to 10 percent excreted, depending upon the amount initially consumed or administered (115). As discussed earlier the ADH pathway is largely responsible for the oxidation of ethanol to carbon dioxide, and is there-

fore instrumental in terminating the well-documented acute central nervous system effects of ethanol.

In addition to ethanol, numberous other alcohols employed as drugs are oxidatively metabolized by hepatic ADH. Raaflaub (116) suggested that nicotinyl alcohol, a compound used clinically as a vasodilating agent, is oxidized stepwise to nicotinic acid through ADH and aldehyde dehydrogenase. This was later confirmed experimentally by von Wartburg and Schürch (39) with purified enzymes or homogenates of human liver. The nicotinyl alcohol:ethanol oxidation ratio was reported as being a useful index in differentiating livers with normal ADH from those with atypical ADH. Another drug of clinical importance which these authors believe to be metabolized *in vivo* through ADH is 3-o-toloxy-1,2-propanediol, a compound exhibiting skeletal muscle relaxant properties.

As considered previously, hepatic microsomal enzymes are capable of oxidizing a number of compounds, such as p-nitrotoluene, to alcohols. Subsequent oxidation to aldehyde and carboxylic acid derivatives may occur through soluble alcohol dehydrogenase and aldehyde dehydrogenase, respectively (64). Similar to the metabolic fate suggested for p-nitrotoluene, recent investigations by McDaniel *et al.* (117) show that tolbutamide, an orally effective hypoglycemic agent, is hydroxylated to hydroxytolbutamide by the hepatic NADPH-linked microsomal drug-metabolizing system, after which further oxidation to the carboxylic acid proceeds through hepatic soluble enzymes. These authors presented evidence that alco-

hol dehydrogenase and aldehyde dehydrogenase may be involved in this oxidative process. Further discussion of this work will be presented in subsequent sections of this dissertation.

One well-documented example of ADH mediating the reduction of pharmacologic agents may be observed following the administration of chloral hydrate to intact animals. This dihydroxy compound is reduced by ADH, in the presence of NADH, to trichloroethanol, which is the pharmacologically active form of this drug and is less polar than the parent compound (118,119). It should be noted that chloral hydrate may be oxidized to trichloroacetic acid, although the reductive pathway is considered to be its major metabolic route (120). An additional example of a drug believed to be reductively metabolized through alcohol dehydrogenase is β-hydroxybutyraldehyde, a compound which has shown clinical potential as a sedative-hypnotic agent (39).

A large number of halogenated hydrocarbons have found varying degrees of usefulness as anesthetic or analgesic agents, such as trifluoroethylvinyl ether (121), halothane (122), and tr chloroethylene (123). The pharmacologic activities of these compounds have been suggested to be partially terminated by an oxidation-reduction pathway such as that present in the hepatic ADH system.

IV. The Toxicologic Role of Alcohol Dehydrogenase

The contribution of ADH in influencing the toxicity of exogenously administered compounds has been the underlying theme of a large number of experimental studies on this en-

zyme. One obvious toxicologic role for ADH lies in its ability to participate in the elimination of ethanol from the organism. Evidence has been discussed (124) which indicates that hepatic NAD/NADH ratios are responsible for the maintenance of the observed zero-order kinetics of ethanol oxidation. A number of experimental studies have shown that, following the administration of ethanol, there is a significant decrease in hepatic intracellular NAD/NADH ratios as a result of the transfer of hydrogen from ethanol or acetaldehyde (acetaldehyde dehydrogenase) to NAD. This change may be the primary biochemical lesion for many of the metabolic derangements attributed to the biologic effects of ethanol (125). Isselbacher and Krane (126) found that the relative increase in hepatic cellular NADH inhibits uridine diphosphate galactose 4-epimerase, a key enzyme in galactose-glucose interconversion. Ethanol had been found to interfere with the galactose tolerance test for liver function, an observation shown by these authors to be due to the inhibitory effects of resulting NADH. From the results of these studies this inhibitory effect, under appropriate conditions, is now measured and used to advantage as a sensitive index of intracellular NAD/NADH ratio change. In addition to the effect on galactose metabolism changes in hepatic redox state resulting from ethanol administration are reflected and measurable in additional ways. The lactate/pyruvate ratio and β-hydroxybutyrate/acetoacetate ratio represent the redox levels of free NAD/NADH in the cellular cytoplasmic

and mitochondrial compartments, respectively (127,128). Forsander (129) found that neither acetaldehyde nor acetate affected the lactate/pyruvate ratio, suggesting that only the first step in the oxidation of ethanol is responsible for influencing the lactate/pyruvate concentration ratio.

The influence of ADH-mediated ethanol oxidation on several biochemical processes has been studied, including the effects on aspects of lipid, carbohydrate, and amino acid metabolism.

The influence of ethanol upon the metabolism of fats has been documented for many years. Several reasons have been suggested (130) for the production of fatty liver by ethanol; increased fatty acid synthesis from ethanol derived acetate, increased mobilization of fatty acids from peripheral depots, decreased lipid transport from the liver, increased triglyceride synthesis from α-glycerophosphate and free fatty acids, and decreased fatty acid oxidation. All of these factors have been explained (125) on the basis of either the direct effect of ethanol, or indirectly from the altered NAD/NADH ratio resulting from oxidation through the ADH system. Elevated NADH concentration may increase triglyceride synthesis by effecting an increase in hepatic α-glycerophosphate, formed from dihydroxyacetone-phosphate. This elevated hepatic concentration of α-glycerophosphate would then be available to esterify free fatty acids, thus explaining the observation (131) that the plasma concentration of free fatty acids is decreased following ethanol ingestion.

Contrary to the belief that metabolism of ethanol is requisite for fatty liver production is the recent investigation of Bustos et al. (132) in which pyrazole (272 mg/kg) administered to rats prior to gavage with 4 g/kg of ethanol prevented blood ethanol disappearance and the concommitant decrease in hepatic NAD/HADH ratio, but did not however, prevent the fatty liver routinely observed after treatment with this dose of ethanol alone. These results lend support to the hypothesis that ethanol itself exerts a direct effect on hepatic utilization of fatty acids (133). Obviously, the underlying mechanism by which ethanol administration can give rise to fatty liver development is yet to be established.

A major effect of ethanol on carbohydrate metabolism is reflected in the clinical syndrome of "alcohol hypoglycemia". The seriousness of this condition if reflected in the reported (134) mortality rate of 11 percent in adults and 25 percent in children treated for this type of hypoglycemia. Studies in vivo and in vitro have furnished considerable evidence that this phenomenon results from a suppression of hepatic gluconeogenesis (134,135,136,137,138). The current hypothesis of the mechanism of this suppression is based on assumptions, recently experimentally confirmed (138), that hepatic oxidation of ethanol is NAD-dependent; and, that reducing equivalents produced from NADH during ethanol oxidation exerts an inhibition of mitochondrial oxidation of glucogenic precursors. The rapid cytoplasmic gen-

eration of NADH from ethanol oxidation results in a surge of reducing equivalents into the mitochondrion, causing a relative deficit of mitochondrial NAD. This apparent deficit may inhibit the flow of TCA cycle intermediates and glucogenic amino acids to oxalacetate, and subsequently to phosphoenolpyruvate and glucose.

The present mechanism of hepatic gluconeogenesis suppression by ethanol prompts the suggestion that the observed hypoglycemic activity of tolbutamide may occur not only through its recognized ability to stimulate islet tissue to secrete insulin, but also by virtue of the fact that tolbutamide is metabolized by microsomal enzymes to hydroxytolbutamide (117). This alcohol metabolite has been shown to serve as a substrate for alcohol dehydrogenase (117) and could itself participate in increasing the hepatic intracellular NADH/NAD ratio. This hepatocellular alteration could thereby serve to further limit gluconeogenesis by the mechanism described above for ethanol.

Ethanol apparently does not influence the metabolism of nitrogenous compounds to the extent that it affects lipid and carbohydrate metabolism. It is probably for this reason that less research emphasis has been placed on the effects of ADH-mediated ethanol oxidation upon amino acid metabolism. However, some unifying explanation of observed ethanol effects upon amino acid metabolism can be proposed on the basis of the strong effect of ethanol on the redox potential of the liver, shifting all NAD-dependent equilibria toward a more

reduced state. The oxidative deaminating dehydrogenases dependent upon NAD will thereby shift their equilibria from ketoacid formation to the generation of aminoacids. Most of the effects of ethanol on amino acid metabolism have been studied in tissues of the central nervous system. One would expect that hepatic redox potential changes would be manifest in other tissues where changes in amino acid pools could be measured. Frieden (139) has studied the effects of altered redox state on glutamate metabolism and reports that under conditions of the reduced state, glutamate dehydrogenase dissociates into inactive subunits. If this occurs in vivo, glutamate could be formed through transamination reactions but its oxidation would be inhibited, resulting in increased brain concentrations of glutamate. Ethanol administration has been reported to produce increased concentrations of glutamate, γ-aminobutyric acid, and aspartate in electrically stimulated brain slices (140) and in the brain of the intact rat (141). Studies on the effects of ethanol administration on γ-aminobutyric acid, of which glutamate is the immediate precursor, are presently inconclusive (142). The relative increase in NADH may also cause a shift in the metabolism of 5-hydroxytryptamine from the oxidative to a reductive route. Correlations between this shift and some symptoms of ethanol intoxication have been claimed (143,144).

A recent study by Cronholm and Sjövall (145) has explored the rather important possibility that ADH-mediated oxidation of ethanol may affect, by its influence on the

hepatic NAD/NADH ratio, a number of important hydroxysteroid-ketosteroid interconversions, including: estradiol/estrone; testosterone/4-androsterone-3,17-dione; 20α-hydroxy-4-pregnen-3-one/progesterone; and cortisol/cortisone. These authors feel that the change of ratio between 17β-hydroxy- and 17-ketosteroid sulfates may be analogous to the increased plasma lactate/pyruvate ratio associated with ethanol administration (146).

In addition to those presently described, numerous other metabolic changes accompany or follow the metabolism of ethanol and have been reviewed by several authors (130,125). As further research is completed, more of these toxic effects of ethanol will undoubtedly be found to take origin from the catalytic activity of ADH.

Methanol, unlike ethanol, serves no known therapeutic role. Although methanol is first in the series of primary aliphatic alcohols, it influences certain biological systems in a manner notably different from the other water-soluble homologs. Studies employing various non-primate animals have shown that methanol and ethanol obey Richardson's rule: the toxicity of the water soluble aliphatic alcohols increases as the number of carbon atoms increases (147). In man, the toxicity of methanol is an exception to this rule. In the early hours of methanol poisoning the symptoms in man and experimental animals resemble the well-known depressant effects of ethanol. At the time when lower animals have completely recovered (usually 12-18 hours following methanol administration), the toxic symptoms peculiar to man and some

primates (148) begin to appear, including dizziness, severe headache and nausea. During this time the alkali reserve decreases, ultimately rendering the patient severely acidotic. This condition may progress to coma and death. Retinal damage and blindness is commonly associated with the latter stages of methanol poisoning. Gilger and Potts (148) studied methanol poisoning in several laboratory species and concluded that only monkey and man exhibit similar responses with respect to ocular pathology, development of acidosis and general clinical symptoms. Frank blindness due to methanol poisoning has been reported exclusively for man.

Methanol toxicity in primates is generally agreed to result not from methanol per se, but from a metabolite or metabolites of that compound. Several observations support this view: (a) 8 to 12 hours elapse before the onset of acidosis; (b) 8 to 12 hours elapse before the onset of visual disturbances associated with methanol poisoning; and, (c) administration of ethanol, which inhibits methanol oxidation, serves to postpone the appearance of the severe signs and symptoms of methanol poisoning. Two theoretical possibilities could explain the toxicities idiosyncratic to man and monkeys. It is conceivable that primates possess biochemical mechanisms different from lower animals by which methanol is metabolized. It is equally possible that all mammalian species metabolize methanol similarly, however the metabolites may exert a toxicity selective for certain tissues or systems of primates.

An important aspect of the toxicologic role of alcohol

dehydrogenase becomes apparent when one considers the oxidation of methanol *in vivo*. Two enzymic pathways are generally accepted participants in this metabolic process: the pyridine-nucleotide dependent ADH, and the catalase-peroxidative system. Although many characteristics of these two enzyme systems were reviewed previously, it should be emphasized that with the catalase-peroxidative system *in vitro*, methanol and ethanol are about equally reactive, while 1-butanol is considerably less reactive than either of the other two alcohols. Purified hepatic ADH however, reacts with these alcohols in the following order of increasing reactivity (*i.e.* decreasing Km, increasing Vmax): methanol, ethanol, and 1-butanol.

The degrees to which these two systems participate in methanol oxidation in intact rats and monkeys has been the subject of considerable research. There is now much evidence (149,150) to show that the catalase-peroxidative system is the major route of methanol oxidation in the rat, while ADH is the primary catalyst for methanol oxidation in the monkey. This concept has been supported indirectly by investigations on the relative inhibitory effects of competitive substrates for ADH and the catalase-peroxidative system upon methanol oxidation in the rat (149) and the monkey (150). On the basis of the relative affinities of ADH and the catalase-peroxidative system for methanol, ethanol and 1-butanol described above, it would be predicted that the oxidation of methanol *in vivo* should be inhibited

about 50 percent by an equimolar concentration of ethanol if the catalase-peroxidative system were the major route of metabolism. If, on the other hand, ADH served as the major catalyst in methanol oxidation one would expect about 90 percent inhibition under the same conditions. When these experiments were performed, ethanol inhibited methanol oxidation in the rat about 50 percent (149), and, in the monkey, about 80 percent (150). Also, as would be predicted from the *in vitro* data, 1-butanol influenced methanol oxidation *in vivo* in the rat only slightly (149) but exerted dramatic inhibitory effects in the monkey (150).

The role of the catalase-peroxidative system has been evaluated directly, using the potent inhibitor 3-amino-1,2,4-triazole (AT). Intraperitoneal administration of this compound to rats has been shown (151) to reduce hepatic and renal catalase activity to about 10 percent of control values. The capacity of rat liver homogenates to oxidize methanol correlates favorably with the degree of AT-inhibition of hepatic catalase (152). Investigations have been performed to discern the mechanism of AT-inhibition of catalase (50,153). Substrates for the catalase-peroxide complex I served *in vitro* and *in vivo* to prevent the inhibition once it was established, indicating this inhibition to be competitive-unsurmountable (149). When sufficient AT was administered to the rat to achieve at least 90 percent inhibition of hepatic catalase, the rate of methanol oxidation *in vivo* was about 50 percent of control, whereas the rate of ethanol

oxidation was unaffected (149). Although AT exerts the same inhibitory effect on rat and monkey hepatic catalase *in vitro*, this inhibitor did not measurably influence methanol or ethanol oxidation in the intact monkey.

The underlying reason why the monkey fails to employ the peroxidative system in the metabolism of methanol was of interest, particularly in light of the report that the monkey liver possesses a greater concentration of catalase activity than the rat liver (19). Several factors have recently become apparent which may explain this species difference (154). Hepatic catalase is apparently capable of functioning in the peroxidative oxidation of methanol on the condition that the peroxide-generation is provided for the formation of catalase-peroxide complex I. This generation from cellular oxidases can occur anywhere in the cell, but is most apparent in the microbody (peroxisome) and cytosol. As stated above, the monkey possesses abundant catalase, however only a small proportion is measurable in the particulate cell fraction, suggesting a relative decrease in the necessary association between catalase and the peroxide-generating enzymes. Another factor contributing to the reason why the monkey fails to employ the peroxidative system in the metabolism of methanol is that, on the basis of equal catalatic activities, the peroxidative activity of monkey hepatic catalase is about one-half the peroxidative activity of the rat enzyme. A final contributing factor is that the quantity of hepatic peroxide-generating enzymes in the mon-

key is significantly less than in the rat.

Until recently no effective, specific inhibitor of hepatic ADH *in vivo* was known. Pyrazole, an imidazole isomer, was reported by Theorell and Yonetani (155) to exert a potent inhibition of this enzyme *in vitro*, and later it was shown to serve as a potent inhibitor of ethanol oxidation in the rat *in vivo* (156). Pyrazole, which will be considered again subsequently, was the first inhibitor of hepatic ADH to show promise as an experimental tool which could demonstrate directly the role of ADH in certain oxidations *in vivo*, analagous to the use of aminotriazole on the catalase-peroxidative system.

Ethylene glycol is itself not considered notably toxic, but is oxidized *in vivo* to carbon dioxide and to oxalic acid (123). Oxalate is very toxic and is probably the agent primarily responsible for the toxicities attributed to ethylene glycol poisoning in mammals. As would be expected, the extent of oxalate formation *in vivo* and ethylene glycol toxicity is related partially to the dose of ethylene glycol administered, and also to the species (157). Holzer *et al.* (158) and later Von Wartburg *et al.* (18) observed that ethylene glycol would serve as a substrate for hepatic ADH. The latter authors have observed that ethanol administration can prevent the oxidation of ethylene glycol *in vivo* and *in vitro*, and also that ethylene glycol toxicity can be abolished in the monkey and man by administration of ethanol in concentrations sufficient to compete for ADH (159). Gessner *et al.*

(157) have proposed a metabolic scheme for the metabolism in vivo of ethylene glycol, the first step of which, ethlyene glycol oxidized to glycolaldehyde, is catalyzed by ADH. It should be pointed out that glycolaldehyde and glycolic acid, an additional intermediate proposed in the metabolism of ethylene glycol, are both alcohols. These and other possible alcohol intermediates have not been investigated with the view that these compounds may also serve as substrates for ADH. The biochemical disposition of each of these compounds must be resolved before a thorough understanding of the metabolism and toxicity of ethylene glycol can be achieved.

In light of the previous discussion on methanol metabolism, it is interesting to note that ethylene glycol has served usefully in further evaluating the relative role of the catalase-peroxidative system in methanol oxidation in vivo. When the report appeared that ethylene glycol serves as a substrate for hepatic ADH, the suggestion was made (160, 161) that this compound might inhibit methanol oxidation in the monkey, but not in the rat, based on the previous knowledge of the preferred routes of methanol oxidation in these two species. Laboratory studies based on this suggestion provided results contrary to expectations. That is, ethylene glycol exerted a dramatic stimulation of methanol oxidation in the rat, and influenced methanol oxidation in the monkey only slightly (160,161). These observations are explanable on the basis of the following considerations. One of the intermediary metabolites of ethylene glycol is glycolic acid

(157). The further oxidation of this compound to glyoxylic acid and hydrogen peroxide is catalyzed by the flavin enzyme, glycolic acid oxidase (162,163). As stated previously, the catalase-peroxide complex I rather than catalase is reactive with methanol. Due to the relative abundance of catalase activity in the rat liver, the addition of peroxide arising from the oxidation of ethylene glycol increases the rate of methanol oxidation in that species. This view is further supported by the fact that stimulation of methanol oxidation in the rat is also produced by glycolaldehyde and glycolic acid (160).

The metabolic fate of a number of toxic compounds other than ethanol, methanol and ethylene glycol has either been proven or strongly suggested to proceed through alcohol dehydrogenase. One such compound is 2-fluoroethanol. Treble (32) has presented some of the historical development which led to his investigations on its metabolism. The toxicity of this compound was first reported in Germany when it was patented in 1935 for use as a rodenticide. Treble's results suggested that 2-fluoroethanol exerts its toxic effects by hepatic oxidation partially through ADH and aldehyde dehydrogenase to fluoroacetate, which serves as a direct precursor in the lethal synthesis of fluorocitrate. Fluorocitrate has been shown to serve as an inhibitor of aconitase (164). The toxic effects of other haloethanols have been shown to be significantly decreased by concommitant administration of ethanol, suggesting a common metabolic fate for these com-

pounds, including monochloroethanol (165) and trifluoroethanol (166).

V. Inhibitors of Alcohol Dehydrogenase

Many compounds have been reported to serve as inhibitors of ADH, 2 of which are of particular interest in the present work--pyrazole and n-butyraldoxime.

Theorell and Yonetani reported in 1963 that pyrazole, an isomer of imidazole, competitively inhibits the oxidation of ethanol by hepatic ADH in vitro (155). Pyrazole was shown to occupy the ethanol binding site of a ternary complex formed with ADH and NAD (167,168). Pyrazole and various 4-substituted derivatives have been recently shown to inhibit ethanol oxidation in vitro (168,169,20) and in vivo in the rat (156, 170), dog (170) and human (171), whereas this inhibitory activity is lost in those derivatives substituted at positions other than the 4-carbon (168,169,20,156). As stated earlier, Reynier (20) has shown that pyrazole inhibits the oxidation of vitamin A alcohol to vitamin A aldehyde by purified hepatic ADH. Pyrazole and its derivatives appear to possess a high degree of potency and specificity for hepatic ADH. The inhibitor constants for most of these compounds lie in the micromolar range when determined using hepatic ADH with ethanol as substrate. Theorell and Yonetani (155) have postulated that the observed stability of the ADH-NAD-pyrazole ternary complex is due to the binding of pyrazole through the zinc of hepatic ADH and nicotinamide of NAD.

Reynier (20) investigated the specificity of pyrazole

for ADH by determining its effect on other enzyme systems which also contained zinc in the enzyme structure: yeast ADH, carbonic anhydrase, carboxypeptidase, lactate dehydrogenase, glutamate dehydrogenase, α-glycerophosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and malate dehydrogenase. In addition to the zinc-containing enzymes, the influence of pyrazole upon catalase and triose-phosphate isomerase activities was tested. At pyrazole concentrations ranging from 100-1000 times that required to exert an inhibition of horse or rat hepatic ADH, no inhibition was observed in the enzymes assayed. It would appear that the pyrazole inhibition of hepatic ADH is highly selective.

Pyrazole administered *in vivo* may be described as exhibiting an extended half-life and a prolonged duration of action. Lester and Benson (172) have estimated from urinary excretion rates of pyrazole that its half-life in the intact rat is about 14 hours. In addition, the inhibition of ethanol oxidation in the rat is only slightly decreased 24 hours after pyrazole administration, and is still detectable on the sixth day following pyrazole administration (172).

Lewis and Schwartz (173) measured increased blood acetaldehyde concentrations in men who drank ethanol after previous exposure to n-butyraldoxime. Koe and Tenen (174) reported that this oxime possessed potent inhibitory effects on mouse ADH *in vivo* and *in vitro* (Ki = 1 μM), and that n-

butyraldoxime was competitive with ethanol and uncompetitive with NAD. These investigators observed inhibition of aldehyde dehydrogenase *in vivo*, but could not demonstrate this inhibition of aldehyde dehydrogenase activity *in vitro*. Dietrich and Hellerman (175), however, observed an effect on partially purified bovine liver aldehyde dehydrogenase. Recently Forsander (176) reported that butyraldoxime does not cause an increase in the acetaldehyde concentration in the intact rat liver during ethanol oxidation. He concluded that the side-effects in man associated with butyraldoxime during ethanol metabolism are probably not due to disturbances in hepatic metabolism. A number of other oximes have recently been reported to also inhibit alcohol dehydrogenase in the rat (172). In addition, certain amides were shown to serve as effective inhibitors of ethanol metabolism, but the mechanism of inhibition is apparently different from that of the pyrazoles and oximes in that, *in vitro*, the amides form a ternary complex with ADH and NADH, with the amide occupying the aldehyde binding site (167, 168). No detailed reports have appeared concerning the duration of inhibition of ethanol oxidation *in vivo* by n-butyraldoxime. However, Lester and Benson (172) have made the observation that the period of alcohol inhibition by this inhibitor is much shorter than after pyrazole. These same authors propose that the mechamisms of n-butyraldoxime inhibition and pyrazole inhibition are different, based in part on observations that butyraldoxime inhibits *in vivo* both ADH and aldehyde

dehydrogenase (174), whereas pyrazole inhibits only ADH (170,20).

Although the properties of pyrazole and n-butyraldoxime are of primary consideration in the present study, additional compounds are known which inhibit ADH, some of which possess the potential of influencing the physiologic, pharmacologic or toxicologic roles of this enzyme.

1,10-Phenanthroline inhibits hepatic ADH by a 1:1 molar complexation with the enzyme-bound zinc (1). Other organic and inorganic chelating agents are known which inhibit in the same manner, but o-phenanthroline is somewhat unique in that its inhibition of ADH is not dependent on the time of preincubation of the enzyme with the inhibitor. Alcohol dehydrogenase inhibition by o-phenanthroline is reversible by subsequent dilution or addition of Zn^{++}, Cu^{++}, or NAD (1).

Metronidazole is ordinarily clinically indicated in the treatment of trichomoniasis. Recently this drug has been employed in the treatment of alcoholism as a result of reports that it exerts "disulfiram-like" side-effects with ethanol. Disulfiram is known to inhibit aldehyde dehydrogenase and therefore results in a rise in systemic acetaldehyde levels, which causes its disagreeable reaction. Metronidazole has been shown to noncompetitively inhibit typical human ADH (177). These results would shed some doubt upon the "anti-alcohol" mechanism <u>in vivo</u> of this compound. Further doubt was cast recently by Gelder and Edwards (178), who performed controlled studies in the treatment of alcoholics and reported metronidazole to be no more effective

than a placebo.

Khouw et al. (179) have reported studies in which two phenothiazines, thioridiazine and chlorpromazine, were nearly equipotent inhibitors of horse hepatic ADH-catalyzed ethanol oxidation. The oxidation of vitamin A alcohol (retinol) to vitamin A aldehyde (retinaldehyde) by a crude bovine retinal preparation was reported to be initially stimulated and later reversibly inhibited by the same two phenothiazines (88). This latter study however, did little to differentiate nonspecific surfactant effects of these drugs from inhibitory effects resulting from specific interaction at the active center of the enzyme.

The use of dimethyl sulfoxide (DMSO) has been proposed in the treatment of rheumatoid arthritis and scleroderma, and is presently used as a solvent for topical application of several drugs (212). Perlman and Wolff (180) demonstrated that DMSO competitively inhibits the reduction of acetaldehyde by equine hepatic ADH (Ki = 5 mM). A number of pyrazolidine drugs inhibit liver ADH in either the reductive or oxidative direction. A clinically outstanding example of the pyrazolidine drugs is the antirheumatic, analgesic compound, phenylbutazone. Inhibition by this compound and its phenolic metabolite is reversed by NAD and NADH (1).

Patients undergoing treatment with orally administered antidiabetic compounds (e.g. tolbutamide and chlorpropamide) have experienced uncomfortable flushes after ingesting alcoholic beverages, resembling the classic ethanol-antabuse reaction. Evidence was presented years ago that tolbutamide

served to inhibit ADH as well as AldDH (181), although Fitzgerald et al. (182) felt that the reported "Antabuse reaction" was due to competition between acetaldehdye and metabolically-formed tolbutamide aldehyde for AldDH. McDaniel et al. (117) have recently strengthened this latter suggestion by demonstrating that tolbutamide can be hydroxylated by hepatic microsomes. Hydroxytolbutamide thus formed was shown to serve as a substrate for hepatic ADH, and in turn, the oxidation product of this reaction, probably tolbutamide aldehyde, served as a substrate for aldehyde dehydrogenase, undergoing oxidation to carboxytolbutamide. These authors did not perform kinetic studies on hydroxytolbutamide-inhibition of ADH-mediated ethanol oxidation, nor were similar studies performed on tolbutamide aldehyde-inhibition of AldDH-mediated acetaldehyde oxidation. The Km reported by McDaniel et al. (117) for the NAD-dependent oxidation of hydroxytolbutamide by dialyzed rat liver 100,000 x g supernatant (Km = 16 mM) is approximately 10 times greater than the Km determined when ethanol is used under similar conditions. It would appear unlikely that typical alcohol ingestion would be sufficient to seriously limit the formation of tolbutamide aldehyde from chronically administered tolbutamide, and could therefore establish conditions for inhibition of acetaldehyde dehydrogenase activity by virtue of its capacity to serve as a substrate alternate with acetaldehyde.

A number of compounds occurring normally in the mammal have been shown to exert inhibitory effects on hepatic ADH.

in vitro. Included in these compounds are: lithocholic acid (29), bilirubin and particularly its water-soluble conjugates (183), triiodothyronine (184) and triiodothyroacetic acid (185). Changing levels of these substances could conceivably exert an influence on the functional capacity of ADH.

VI. Aldehyde Dehydrogenase

For purposes of completeness in the discussion of the alcohol dehydrognease system, and because the present study touches on aspects of aldehyde metabolism, some consideration of aldehyde dehydrogenase shall be presented. The oxidation of aldehydes under physiologic conditions has been shown to proceed in lower mammals (186,187) and in humans (188,189) through an NAD-dependent aldehyde dehydrogenase (E.C. 1.2.1.3). The kinetic and thermodynamic participation of aldehyde dehydrogenase in ethanol metabolism was discussed previously. Some aldehyde dehydrogenases possess a high degree of substrate specificity (190,191,192), but evidence has been presented which indicates that some tissues possess AldDH which will oxidize a relatively broad group of substrates, including simple aliphatic and aromatic aldehydes (58,193,194), retinaldehyde (89,60), and the oxidative deamination products of indole (195,196), imidazole (197) and catecholamine (198, 199,194). Relatively low concentrations of steroids have been shown to influence the AldDH activities of certain enzyme preparations (58,60,196).

Aldehyde dehydrogenase, like alcohol dehydrogenase, has been a candidate for a number of pharmacologic and toxicologic

roles. For example, Truitt and coworkers (200,201) have studied the role of acetaldehyde in the pharmacologic effects observed following ethanol administration. They found that low acetaldehyde concentrations served to inhibit oxidative phosphorylation in brain mitochondria. In contrast, extremely high concentrations of ethanol did not exert such an effect. The suggested reason for this difference is based on earlier findings that pyruvate is coupled with acetaldehyde to form acetoin, and thereby deprives the mitochondrion of a major energy source (202,203). It would appear that a mechanism for rapid elimination of free acetaldehyde could be of great importance to the organism under certain conditions.

In addition to the possible role of AldDH in the metabolism of vitamin A, interesting physiologic roles may exist for this enzyme in biogenic amine metabolism. Blaschko (204) has reviewed earlier work of the 1930's which led to the proposal that aldehyde intermediates are formed in the metabolism of biogenic amines. Carlsson and Hillarp (205) later demonstrated that biogenic amines are oxidatively deaminated in the brain by monoamine oxidase. Subsequently an NAD-dependent brain AldDH was isolated which was capable of oxidizing the aldehyde derivatives of biogenic amines to the corresponding acids (206). The recent reports of a brain ADH (2) and an NADP-linked aldehyde reductase from the brain (207) suggest that the reversible steps in the metabolism of biogenic amines which has heretofore been demonstrated only in the liver, may proceed also in the central nervous system.

The capacity for control in this process would probably be greater in the brain due to the larger number of different enzymes apparently involved in this tissue.

Inhibitors of AldDH have been of practical and academic interest for years. The drinking habits of many individuals have been altered due to the disagreeable symptoms resulting from inadvertent or intentional exposure to these compounds. From an academic viewpoint, the use of a substance which will inhibit a specific step of an enzymatic process is useful in determining various biochemical roles for that particular step or sequence.

Probably the commonest inhibitor of AldDH is disulfiram, a drug used clinically in the treatment of alcoholism (208, 209). Calcium carbimide has also been used in the treatment of alcoholism, and exerts inhibitory effects almost identical to the related cyanamide (211). Numerous other substances have been reported which produce an intolerance to alcohol and yet do not affect the systemic ethanol levels: sulfonylurea compounds (tolbutamide, chlorpropamide, carbutamide); phenformin, n-butyraldoxime, furazolidone; and tolazoline (211). The enzyme specificity and kinetics of inhibition associated with these compounds have received little detailed investigation. It should be recalled, however, from previous considerations that tolbutamide and n-butyraldoxime both serve as inhibitors of hepatic ADH.

VII. Objectives and Plan of the Present Investigation

The first portion of this research project was dir-

ected at determining directly the participation of alcohol dehydrogenase (ADH) in the oxidation of methanol and ethanol in the rat and monkey. This enzyme was studied *in vivo* by observing the influence on methanol and ethanol metabolism of compounds known to inhibit hepatic ADH *in vitro*, such as pyrazole and n-butyraldoxime. An additional series of experiments was conducted to evaluate the potential usefulness of inhibitors of hepatic ADH in the clinical treatment of a number of toxicities linked to the ADH metabolic pathway, such as the treatment of methanol poisoning.

In the latter half of this investigation studies were conducted *in vitro* to evaluate the effects of inhibitors of hepatic ADH on non-hepatic tissues reported to require an alcohol dehydrogenase for normal function. The retina was chosen for these studies because this tissue has been reported to require an alcohol dehydrogenase for production of visual pigments. It is also a tissue which is known to be selectively damaged in primates following poisoning by methanol. Earlier considerations of mammalian retinal ADH were based either on studies employing the hepatic enzyme, or on studies employing crude retinal preparations. Retinal ADH and aldehyde dehydrogenase activities were purified from the rat and monkey, and a series of experiments was conducted to examine some of the kinetic characteristics of these enzymes, including susceptibility to inhibition by pyrazole and n-butyraldoxime. In addition to studying these soluble retinal dehydrogenases,

certain kinetic properties of particulate components of retinal homogenates were examined.

MATERIALS AND METHODS

I. Materials

A. Chemicals and Reagents

Isotopes were purchased from New England Nuclear Corporation and diluted with the corresponding inert alcohol and water to a specific activity of 25,000 dpm/ml. Anisole, dimethoxyethane, methanol, ethanol and 2,4-dioxane were purchased from the Mallenckrodt Company. Cab-O-Sil, POPOP (1,4-bis(2-(5-phenyloxazolyl))benzene) and PPO (2,5-diphenyloxazole) were purchased from Packard Instrument Company, Inc.

Pyrazole and 2-fluoroethanol were purchased from K and K Laboratories, Inc. n-Butyraldoxime was obtained from the Aldrich Chemical Company and 3-amino-1,2,4-triazole was purchased from Eastman Organic Chemicals, Inc. 1,10-Phenanthroline, retinol (all-trans, type X), retinaldehyde (all-trans, type XVI), pyridine nucleotides, crystalline beef liver catalase, glucose oxidase, DEAE-cellulose and CM-cellulose were purchased from Sigma Chemical Company.

Lactaldehyde was synthesized by a modification of the method of Huff and Rudney (213). Threonine (2.4 g) and ninhydrin (3.6 g) were dissolved separately in water, mixed, and the final volume adjusted to about 250 ml. The threonine-ninhydrin solution was heated at 100° for 4 minutes, then cooled to room temperature, after which 3.6 g of sodium bicarbonate was added, and the resulting mixture filtered. The filtrate was clarified with neutralized acid-washed activated charcoal, concentrated at 40° with a rotary evaporator,

and allowed to stand for about 12 hours. The concentrate was further decolorized by an additional treatment with activated charcoal, and passed through a Dowex-50 resin column. Further concentration of lactaldehyde was acheived by repeating the above sequence beginning with evaporation at 40°. The formation of lactaldehyde was confirmed by observing the melting point of the purified 2,4-dinitrophenylhydrazone derivative at 150°.

Quantitative determination of lactaldehyde was achieved by measurement of the bisulfite binding capacity of the prepared concentrate. Excess 0.3 N sodium bisulfite solution was added to an aliquot of lactaldehyde concentrate, the mixture adjusted to pH 2.5-3.0 with 1 N HCl and allowed to stand for 15 minutes. Excess bisulfite was titrated with a 0.05 N iodine solution, at which point the lactaldehyde-bisulfite addition product was cleaved by addition of 3 ml of a 10 percent sodium carbonate solution. Bisulfite released from the addition product was then titrated with 0.05 N iodine solution. From the amount of standardized iodine solution necessary to titrate the released bisulfite, the lactaldehyde concentration of the original solution was calculated.

All other reagents employed in these investigations were of the highest available purity.

B. Sources of Experimental Animals and Tissues

Male Sprague-Dawley rats (250-350 g) and male rhesus monkeys (3-5 kg) were used for experiments <u>in vivo</u> and as retinal and hepatic tissue sources as required for

studies *in vitro*. All animals received commercially prepared diets and water *ad libitum* until initiation of experimentation. Bovine retinae were purchased from Pel-Freez Biologicals, Inc.

II. Methods

A. Experiments *in vivo*

Each animal was administered a single intraperitoneal injection of ethanol-1-^{14}C or methanol-^{14}C solution as a 20 percent solution of alcohol in 0.9 percent NaCl solution, then, immediately placed in a glass metabolism chamber. The basic designs of these chambers have been described previously (149,150). In this system, ambient and expired air was pulled from the metabolism chambers (about 3-4 liters/minute) through drying tubes containing magnesium perchlorate and calcium chloride. Inert and isotopic carbon dioxide was quantitatively trapped in a series of scrubbing flasks, each of which contained a solution of 2-3 N sodium hydroxide. At specified time intervals during the course of the experiment, the sodium hydroxide solution was removed from the scrubbing flasks, pooled, and replenished for subsequent $^{14}CO_2$ analysis. A 1-ml aliquot of the pooled sodium hydroxide solution was added to 15 ml of a scintillation fluid composed of: 2,4-dioxane (75% w/v), dimethoxyethane (5% v/v), anisole (12.5% v/v), POP (1.2% w/v), POPOP (0.05% w/v), and Cab-O-Sil (5% w/v). The radioactivity of these samples was determined with a Packard Tri-Carb liquid scintillation spectrometer. The efficiency of this system, determined by ^{14}C-toluene internal

standard techniques, was about 70 percent.

B. Partial Purification of Rat Liver Alcohol Dehydrogenase and Rat Liver Aldehyde Dehydrogenase

1. Preparation of Hepatic Cytosol Fraction. Rats were stunned, decapitated and bled. In each animal the thorax was rapidly opened, and the liver removed to a beaker containing ice-cold 0.9 percent NaCl solution. All subsequent operations were conducted at a temperature of 0-4°. Glass tissue homogenizers were used to prepare a 33 percent (w/v) tissue homogenate in a solution comprised of 0.005 M Na:K phosphate buffer, 0.001 M disodium EDTA and 0.0001 M dithiothreitol (Buffer A) at pH 7.0. This homogenate was centrifuged at 12,500 x g for 20 minutes, following which the supernatant from this operation was centrifuged at 105,000 x g for one hour. The microsomal pellet was discarded and the cytosol subjected to subsequent purification steps.

2. Separation of Rat LADH and LAldDH. Preliminary experiments using a wide variety of pH and ionic strength combinations indicated that LADH and LAldDH were inseparable when ammonium sulfate precipitation and DEAE-cellulose chromatography were employed. The following procedure served to accomplish this separation. LADH and LAldDH were precipitated from the cytosol with ammonium sulfate between 33 and 70 percent saturation. A pH of approximately 7.0 was maintained by the addition of an ammonium hydroxide solution. The ammonium sulfate precipitate was dissolved in a minimal amount of Buffer A and dialyzed for 12 hours against

2 changes of 100 volumes of Buffer A. The solution was then passed through a DEAE-cellulose column equilibrated also with Buffer A. Both LADH and LAldDH eluted essentially with the void volume. Fractions possessing enzyme activity were combined and this pool was applied to a CM-cellulose column equilibrated with Buffer A. LAldDH passed through the column when Buffer A was used as the eluant. Active eluates were pooled and dialyzed against Buffer A, pH 8.0. When the phosphate concentration of eluant was changed from 0.005 M to 0.1 M, LADH was released from the column matrix. The active fractions were pooled and dialyzed against Buffer A, pH 8.0.

C. Partial Purification of Rat Retinal Alcohol Dehydrogenase and Rat Retinal Aldehyde Dehydrogenase

1. Preparation of Retinal Cytosol Fraction. Each of 50 rats were stunned and decapitated and the eyes were rapidly removed and frozen at -77°. The frozen eyes were then trimmed free of any contiguous extraocular tissue. A single cut was made through the frontal portion of the eye, enabling, in one operation, a removal of the cornea, iris, lens and humors. Studies were performed with retinae which had been dissected free of connective tissue or, in most cases, with adjoining connective tissue. No enzymatic differences were observed in these preparations. Retinae were homogenized (0.5 ml buffer/retina) in Buffer B (0.005 M sodium pyrophosphate buffer containing 0.001 M disodium EDTA and 0.0001 M dithiothreitol) at pH 8.0. This, and all subsequent procedures described for this enzyme were performed

at 0-4°. The homogenate was centrifuged at 12,550 x g for 20 minutes and the resulting supernatant was further centrifuged at 105,000 x g for one hour. The microsomal pellet was discarded and most of the cytosol was subjected to the remaining purification procedures. The cytosol fraction contained all measurable capacity for NAD-dependent ethanol and acetaldehyde oxidation. A portion of the cytosol was dialyzed 12 hours against 100 volumes of Buffer B at pH 8.0 and was used for analysis of RADH and RAldDH activities.

2. Ammonium Sulfate Fractionation. Appropriate quantities of either ammonium sulfate solution saturated at 4° or solid ammonium sulfate were slowly added to the cytosol. The pH of the medium was maintained at 8.0 by the addition of small amounts of an ammonium hydroxide solution. The protein fractions precipitating in each of the saturation ranges 0-33 percent, 33-50 percent, 50-70 percent, and 70-100 percent were individually dissolved in a minimal amount of Buffer B, pH 8.0, and dialyzed against 2 changes of 100 volumes of Buffer B over a 12 hour period. Small portions of each dialyzed ammonium sulfate fraction was reserved for determination of RADH and RAldDH activities. RADH and RAldDH activities were found to reside almost exclusively in the ammonium sulfate saturation range of 33-100 percent. Protein precipitating in this range was pooled and subsequently submitted to DEAE-cellulose chromatography.

3. DEAE-Cellulose Chromatography. Protein which

precipitated after ammonium sulfate fractionation (33-100 percent) was applied to a column of DEAE-cellulose (1.8 x 20 cm) equilibrated with Buffer B at pH 8.0 and eluted with the same buffer. The flow rate was adjusted to about 30 ml per hour and 6 ml fractions were collected. Fractions possessing RADH activity were pooled and stored at -60°. Following the elution of RADH from the column, the pyrophosphate concentration of the elution buffer was changed from 0.005 M to 0.05 M. This procedure served to elute RAldDH activity, the most active fractions of which were pooled and reserved for further studies.

D. Partial Purification of Monkey Retinal Alcohol Dehydrogenase and Monkey Retinal Aldehyde Dehydrogenase

Purification of monkey RADH and RAldDH was performed using the same procedure described for the rat retinal enzymes, with the following exceptions. The primate eye was sufficiently large to allow easy dissection of the retina from the underlying connective tissue to which it was attached. Homogenization of this tissue was conducted using 4 ml of Buffer B, pH 8.0 per retina.

E. Studies on Rat and Bovine Retinal Homogenates

Rat retinal homogenates were prepared in 0.1 M sodium phosphate buffer, pH 7.6, and were examined for their ability to oxidize retinol to retinaldehyde by means of a modification of the method of Reading and Sorsby (87). Incubations were carried out in test tubes at 37° under aerobic conditions. Reaction mixtures contained, per milliliter:

homogenate equivalent to 4 mg tissue; retinol, 1.1 μmoles; NADP, 2 μmoles; and when used, pyrazole, 1 μmole; ethanol, 100 μmoles; and methanol, 100 μmoles in a final volume of 1.5 ml of 0.1 M sodium phosphate buffer, pH 7.6. Reactions were carried out in dim light, and, during a period of linear retinaldehyde formation, aliquots were taken for estimation of retinaldehyde content by the method of Mallia and Cama (214).

Similar experiments were performed with bovine retinal homogenates.

F. Assay Procedures

1. Catalase.

Catalase catalatic activity was determined by the method of Feinstein (215) and was expressed in Kat f units defined by von Euler and Josephson (216).

Catalase peroxidative activity was measured as described by Goodman and Tephly (217). Reaction mixtures routinely contained crystalline beef liver catalase (1000 units), glucose (4 mg/ml), glucose oxidase (0.2 mg/ml), methanol (0.1 M), and Na:K phosphate buffer (0.1 M, pH 6.8) in a final volume of 5 ml. Incubations were carried out at 37° for 15 minutes, after which reactions were initiated by the addition of methanol. Thirty minutes after methanol addition, the method of Nash (218) was used to determine formaldehyde formed during the reaction. Catalase peroxidative activity was expressed as mμmoles formaldehyde formed/ml/minute.

2. Alcohol Dehydrogenase. One unit of ADH activity

is defined as the amount of enzyme which catalyzes the reduction of 1 umole of NAD per minute under the conditions described. Specific activity is expressed as units per mg protein. ADH activity was routinely determined spectrophotometrically by measuring the net reduction of NAD at 340 nm on a Gilford 2000 spectrophotometer, and employing a millimolar extinction coefficient of 6.22 (219). Ambient temperature of the reaction medium was 25°. A final reaction mixture volume of 1 ml contained: enzyme; NAD, 2 µmoles; ethanol, 1 mmole; and, sodium pyrophosphate buffer, 50 µmoles, pH 10.0.

3. Aldehyde Dehydrogenase. AldDH activity was routinely determined using the method described for alcohol dehydrogenase. A final reaction mixture volume of 1 ml contained: enzyme; NAD, 2 µmoles; acetaldehyde, 100 µmoles; and sodium pyrophosphate buffer, 50 µmoles, pH 9.0.

4. Special Methods. During the course of this study a number of different techniques were employed to investigate the enzymatic oxidation of retinol to retinaldehyde:

a) The enzymatic formation of retinaldehyde was directly monitored by recording the rate of increase in absorbance at 400 nm. At this wavelength the molar absorbency index of retinaldehyde is 29,500 (20).

b) The colorimetric determination of retinaldehyde was performed by the recently reported method of Mallia and Cama (214), in which retinaldehyde is reacted with p-amino-

benzoic acid in the presence of hydrochloric acid. The resulting product exhibits maximal absorption, and is measured spectrophotometrically at 530 nm. The p-aminobenzoic acid (PABA) method was chosen over the classical thiobarbituric acid method (220) for three reasons: (1) chromagen formation occurs more rapidly in the PABA method than in the thiobarbituric acid method; (2) the color stability of the PABA method is of longer duration than in the thiobarbituric acid method; and, (3) unlike the thiobarbituric acid assay, color development in the PABA method is not retarded by the presence of water.

c) A colorimetric assay procedure for ADH was used which is based on the reduction of lactaldehyde to 1,2-propanediol, coupled to ethanol-dependent generation of NADPH (221). Alcohol dehydrogenase catalyses the oxidation-reduction of a number of alcohols and aldehydes, including lactaldehyde (222). Evidence has been presented (221) to show that when NADH is generated by oxidation to ethanol, lactaldehyde reacts preferentially with NADH bound to the enzyme, resulting in the net reaction:

$$\text{Ethanol} + \text{Lactaldehyde} \overset{\text{ADH,NAD}}{\longleftrightarrow} \text{Propanediol} + \text{Acetaldehyde}$$

From this reaction it can be seen that one mole of propanediol is formed per mole of ethanol oxidized to acetaldehyde. From this relationship it follows that measurement of ethanol-dependent 1,2-propanediol formation is a direct index of alcohol dehydrogenase activity.

A distinguishing feature of this method is the catalytic function of NAD. The assay is not dependent upon a measurement of changes in the NADH concentration of the reaction mixture, and is therefore less effected by the presence of contaminating pyridine nucleotide oxidoreductase systems. This assay procedure has been described as a sensitive method for measurement of alcohol dehydrogenase activity in the liver and brain (2).

In the present studies the activities of incubating enzyme reactions were terminated by the addition of a trichloroacetic acid solution. The concentration of 1,2-propanediol in the protein-free supernatant was determined colorimetrically by the ninhydrin-sulfuric acid method (223).

d) Certain retinal enzyme preparations were submitted to electrophoresis on analytical polyacrylamide disc gels (7 percent acrylamide), prepared using the basic formulation and procedure described by Dietz and Lubrano (224). The widely used disc electrophoretic method of Davis (225) was not employed in these studies because of concern arising from reports that only 4 of 5 lactate dehydrogenase isozymes migrate into the separating gel, the fifth isozyme ("LDH-5") remaining in the stacking gel (225). This problem was not encountered, however, when the method of Dietz and Lubrano (225) was employed. The latter method was chosen for the present studies in order to avoid analagous complications with ADH isozymes. Following electrophoretic migration the gels were ordinarily incubated under dark conditions at 37°

in a standard dehydrogenase staining medium containing in 100 ml of 0.05 M sodium phosphate buffer, pH 7.6: NAD, 60 mg; nitroblue tetrazolium, 22 mg; phenazine methosulfate, 2 mg; and enzyme substr te.

Standard gels were provided with ethanol as the substrate. Gels in which the retinol-oxidizing capacity was being investigated were incubated according to one of three schedules: (1) preliminary incubation in a medium consisting of buffer, retinol solubilized with Tween 80 (20), and NAD, followed by a second incubation in the buffered dye system only; (2) single incubation in the buffered medium containing NAD, dye system and retinol solubilized with Tween 80; or, (3) incubation identical to schedule (2) with the exception that retinol was solubilized with acetone. Control studies were performed in which each of the reaction components was withheld.

When indicated, protein bands were located in the gels by staining with Coomasie blue, employing the following procedure (226): (a) after electrophoretic migration gels were placed in 12.5 percent trichloroacetic acid solution for at least 30 minutes; (b) gels were placed in a staining solution prepared by diluting a 1 percent stock solution of Coomasie brilliant blue with 12.5 percent trichloroacetic acid solution in a ratio of 1:20; and (c) removal of unbound Coomasie blue was achieved by placing the gels in several changes of 10 percent trichloroacetic acid solution for at least 2 days.

5. Determination of Kinetic Constants. The data collected to derive the kinetic constants were submitted to statistical analysis (227) with calculations performed on the University of Michigan IBM 360/67 computer according to a FORTRAN program written by Cleland (228).

6. Protein Determination. Protein concentration was estimated by the use of the Biuret method (229), an ultraviolet absorption method (230), or the Lowry method (231). When indicated, bovine serum albumen was employed as the protein standard.

RESULTS

I. Studies on the Oxidation *in vivo* of Ethanol and Methanol in the Rat and Monkey

Initial studies in this investigation were designed to discern directly the role of alcohol dehydrogenase in the oxidation *in vivo* of ethanol and methanol in the rat and monkey. The role of ADH in methanol and ethanol oxidation has been evaluated indirectly in earlier studies in which two enzymatic systems were examined for their participation in the metabolism of ethanol and methanol: liver alcohol dehydrogenase, and the catalase-peroxidative system involving hepatic catalase and a source of peroxide generation. Employing the catalase inhibitor, aminotriazole, and competitive substrates for both liver alcohol dehydrogenase and catalase-peroxide complex I *in vivo* and *in vitro*, Tephly *et al.* (149) showed that, in the rat, the catalase-peroxidative system plays a major role in the metabolism of methanol. Ethanol metabolism in the intact rat was inhibited by other substrates favorable for LADH, such as 1-butanol, and was unaffected by aminotriazole, suggesting that ethanol was oxidized primarily by LADH in this species. Unlike the effects seen in the rat, aminotriazole does not inhibit methanol oxidation in the monkey, and experiments using alternative substrates for the LADH and catalase-peroxidative systems revealed a pattern consistent with a predominant role for alcohol dehydrogenase in both methanol and ethanol oxidation in this species (150).

Theorell and his colleagues have shown that pyrazole

competes with ethanol for the alcohol-binding site on liver ADH and that pyrazole is a very potent inhibitor of this enzyme (155,168). These investigators have also examined the inhibitory effect of pyrazole upon a number of other zinc metalloenzymes and zinc containing NAD-dependent dehydrogenases. With the exception of yeast alcohol dehydrogenase, which was only weakly inhibited, none of the enzyme activities studied were inhibited by pyrazole (20). The potent inhibitory effect of pyrazole *in vitro* on rat, monkey and human LADH was confirmed early in the present studies. These inhibitory characteristics observed *in vitro* gave indication that pyrazole might serve as a tool by which the participation of ADH in alcohol oxidation *in vivo* could be examined.

Before pyrazole could be fruitfully employed *in vivo* to distinguish directly the role of ADH in methanol and ethanol oxidation, it was first necessary to design and implement studies which would detect any effects of pyrazole on the catalase-peroxidative system.

Aminotriazole-inhibition of catalase is obtained only when a source of hydrogen peroxide is present for the formation of catalase-peroxide complex I (50,153). Aminotriazole irreversibly inhibits catalase activity when it is reacted with catalase-peroxide complex I. Further, substrates for catalase-peroxide complex I, such as methanol and ethanol, prevent the AT-inhibition of catalase *in vitro* and *in vivo*, but do not reverse the inhibition once established. These characteristics of the catalase-peroxidative system and its

inhibition by AT were utilized to determine any effects pyrazole might exert on this system. Pyrazole was tested 7 ways for interaction with hepatic catalase: (1) As a direct inhibitor of hepatic catalase activity; (2) As an inhibitor of hepatic catalase activity in the presence of hydrogen peroxide; (3) As a substance which protects against AT-inhibition of catalase in the presence of hydrogen peroxide (i.e., as a substrate for catalase-peroxide complex I); (4) As an inhibitor of hepatic catalase in vivo; (5) As a substrate for catalase-peroxide complex I in vivo; (6) As an inhibitor of catalase-peroxidative oxidation in vitro of methanol to formaldehyde; and, (7) As a substrate which protects against the AT-inhibition of catalase-peroxidative oxidation in vitro of methanol to formaldehyde (i.e., as a substrate for catalase-peroxide complex I). The results of these experiments are presented in the following section.

A. Effect of Pyrazole on the Catalase-Peroxidative System

The effect of pyrazole on crystalline bovine liver catalase activity can be seen in Figure 1. In this series of experiments, catalatic activity of crystalline bovine liver catalase was measured after preincubation with combinations of the following components: pyrazole, aminotriazole, and a peroxide-generating system composed of glucose oxidase and glucose.

From these data it can been seen that pyrazole had no effect on catalase activity in the presence or absence of

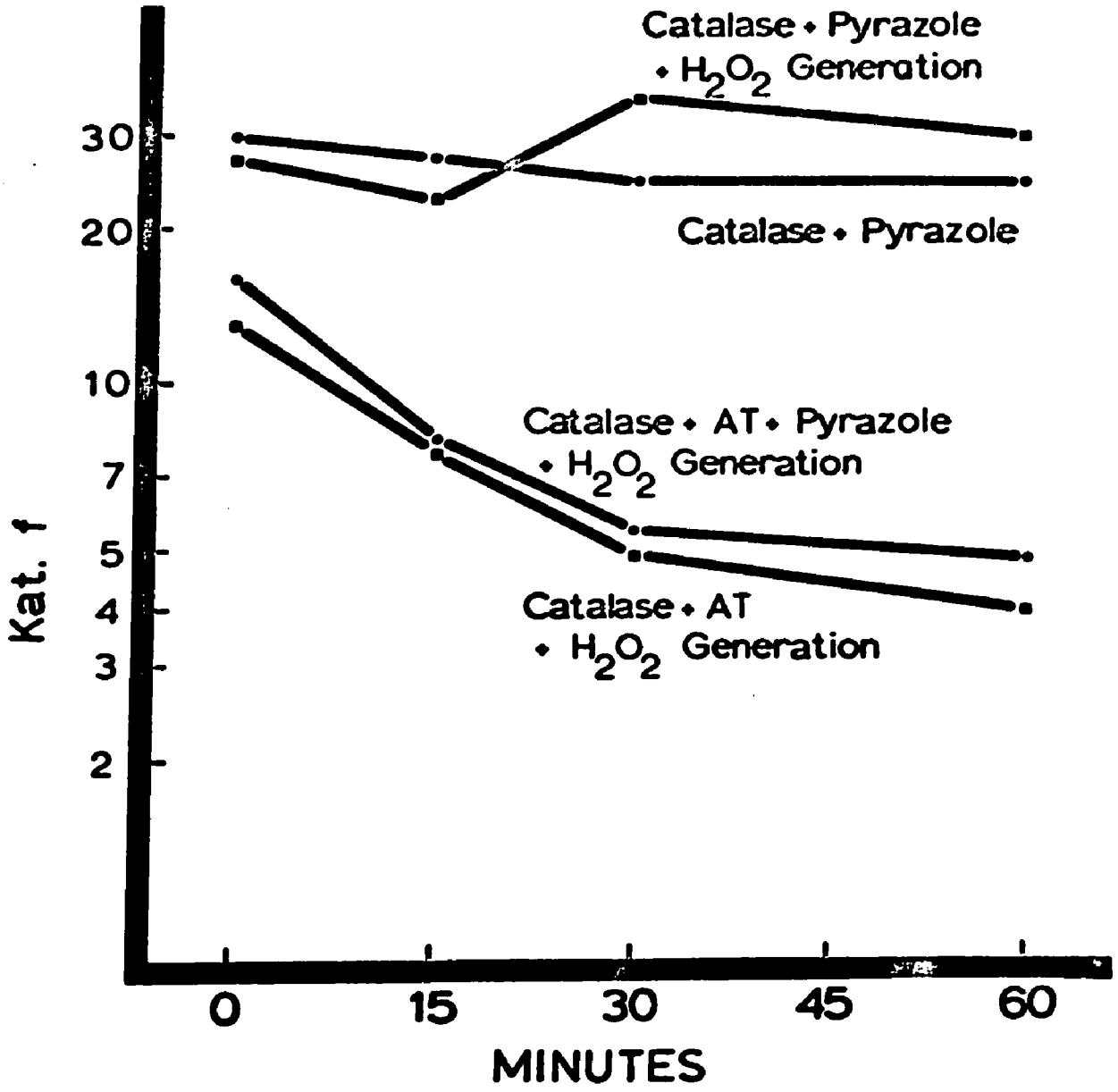


Figure 1. Effect of pyrazole on crystalline catalase activity. Samples were withdrawn at indicated time intervals and evaluated for catalase activity by the method of Feinstein (215). The following concentrations were employed where indicated: pyrazole, 10 mM; aminotriazole (AT), 30 mM; glucose, 40 mg/ml; and glucose oxidase, 0.4 mg/ml, in a final volume of 5 ml. Crystalline beef liver catalase (30 kat. f) was added at zero time, and activity represents that remaining after incubation at 37° in Na_2HPO_4-KH_2PO_4 buffer, 0.1 M, pH 7.4.

a peroxide-generating system. As expected, aminotriazole exerted a pronounced inhibition of catalase activity (Figure 1). Furthermore, Figure 1 illustrates that pyrazole had no effect on the AT-inhibition of catalase activity. Therefore, pyrazole appears not be be a substrate for catalase-peroxide complex I.

Aminotriazole has been shown to produce profound inhibition of hepatic catalase activity in vivo (151). The data presented in Table 1 describe the effects of pyrazole and aminotriazole on rat hepatic catalase activity in vivo. Combinations of pyrazole (2.94 mmoles/kg), aminotriazole (11.9 mmoles/kg) and 0.9 percent NaCl solution were administered intraperitoneally to rats. Livers were removed one hour after aminotriazole and 15 minutes after pyrazole treatment, and catalatic catalase activity was determined by the method of Feinstein (215). Catalase activity in AT-treated rats was about 18 percent of control, however the hepatic catalase activity in pyrazole-treated rats was not significantly different from controls, indicating that under these conditions, pyrazole does not inhibit catalase. Assay of rat hepatic catalase activity following combined administration of aminotriazole and pyrazole yielded activity not significantly different from that determined in rats receiving aminotriazole alone. Therefore, pyrazole did not protect catalase from AT-inhibition as is routinely observed with substrates for catalase-peroxide complex I.

Results of the third method employed to investigate the effect of pyrazole on the catalase-peroxidative system are presented in Table 2. In this procedure peroxidative

Table 1

Effect of Pyrazole and Aminotriazole (AT) on Rat Hepatic Catalase Activity *in vivo* .

Treatment	Catalase Activity*
	kat. f/g liver
None	517 ± 44
AT	28 ± 5
Pyrazole	525 ± 72
AT + Pyrazole	37 ± 3

*Rats were administered pyrazole (2.94 mmoles/kg), aminotriazole (11.9 mmoles/kg), or 0.9% NaCl intraperitoneally. Livers were removed 1 hour after aminotriazole and 15 minutes after pyrazole treatment, and catalatic catalase activity was determined by the method of Feinstein (215). Values represent the mean of three experiments ± standard error.

Table 2

Effect of Pyrazole on Peroxidative Oxidation of Methanol *in vitro*

Treatment	Methanol Oxidation*
	mμmoles HCHO formed/ml/min
None	16.5
Pyrazole	16.5
AT	0.5
Pyrazole + AT	0.0

*Peroxidative catalase activity was measured as described previously (217). The concentrations of pyrazole and aminotriazole (AT) were 0.02 M and 0.1 M, respectively. Reaction mixtures contained crystalline beef liver catalase (1000 units), glucose (4 mg/ml), glucose oxidase (0.2 mg/ml), methanol (0.1 M), and Na_2HPO_4-KH_2PO_4 buffer (0.1 M, pH 6.8) in a final volume of 5 ml. Incubations were carried out at 37°, and reactions were started by the addition of methanol after 15 min of incubation. Formaldehyde content was determined by the method of Nash (218) 30 min after the addition of methanol.

catalase activity was determined by measuring formaldehyde produced (218) in a buffered reaction system composed of crystalline bovine liver catalase, methanol, glucose oxidase and glucose. Aminotriazole and pyrazole, alone or in combination, were added to the basic reaction mixture. As seen in Table 2, pyrazole had no effect on the peroxidative oxidation of methanol, indicating once again that pyrazole exerts no inhibitory effect on this system. Also, in agreement with the results of the previous two methods, the data in Table 2 indicate that aminotriazole exerted its expected inhibition of methanol oxidation by this system (149). Pyrazole did not reverse the AT-inhibition of the peroxidative oxidation of methanol, as would be observed if pyrazole were a substrate for catalase-peroxide complex I.

From the results of these preliminary experiments, it was clear that within the limits of dose and time examined, pyrazole exerted no effect on the hepatic catalase-peroxidative system. The decision was therefore made to investigate the effect of pyrazole on the oxidation of ethanol and methanol in the intact rat and monkey.

B. Effect of Pyrazole on Ethanol Oxidation in the Intact Rat

Recently, it has been shown that pyrazole and certain derivatives inhibit ethanol metabolism <u>in vivo</u> in the rat (156,170). These studies were confirmed in the present investigation, the data from which are illustrated in Figure 2. At a dose of 2.94 mmoles/kg of pyrazole, the oxi-

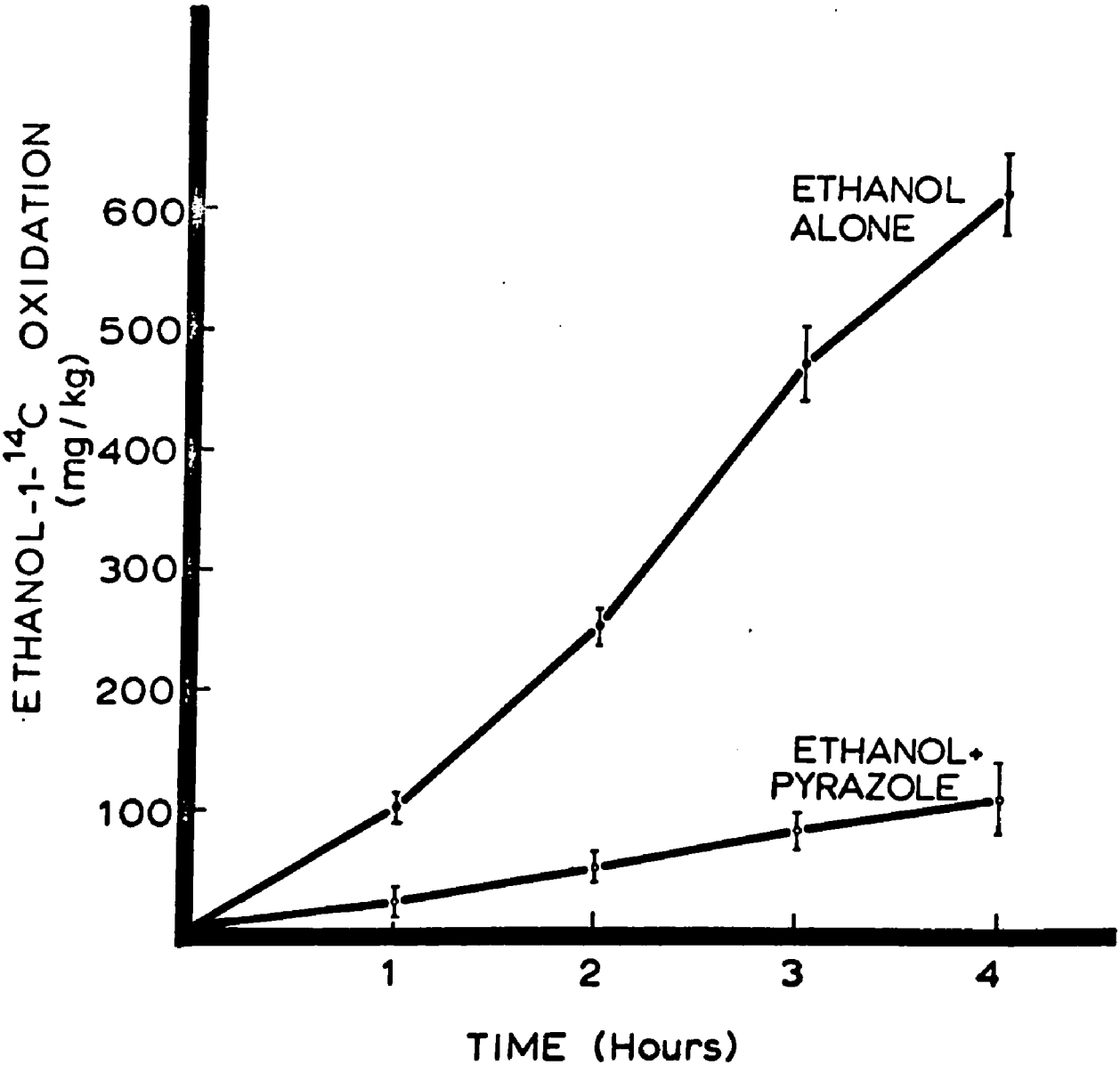


Figure 2. Effect of pyrazole on ethanol oxidation in the rat *in vivo*. Ethanol-1-^{14}C (21.7 mmoles/kg) was injected intraperitoneally. When administered, pyrazole (2.94 mmoles/kg) was injected intraperitoneally 15 min before ethanol-1-^{14}C. Each point represents the mean value of four animals ± standard error.

dation *in vivo* of ethanol (21.7 mmoles/kg) was inhibited approximately 80 percent. These results are in accord with the view that ethanol is metabolized in the rat by liver alcohol dehydrogenase (149).

C. Effect of Pyrazole on Methanol Oxidation in the Intact Rat

Figure 3 shows that pyrazole at a dose of 2.94 mmoles/kg inhibits the oxidation of methanol (31.3 mmoles/kg) in the rat to a lesser degree than the oxidation of ethanol. This was as expected, since others (149) have shown that, in the rat, methanol is oxidized to a large part by a catalase-peroxidative route. However in the absence of a direct means of evaluating the role of ADH in methanol oxidation in the rat, it was generally assumed that the catalase-peroxidative route was entirely responsible for methanol oxidation in this species. The modest, yet measurable, inhibitory effect of pyrazole upon methanol oxidation in the rat illustrated in Figure 3 strongly suggests that ADH plays a heretofore unrecognized role in methanol oxidation in the rat.

D. Other Studies on Pyrazole Inhibition of Ethanol and Methanol Oxidation in the Intact Rat

After it became apparent from the studies described to this point that pyrazole did indeed possess potent inhibitory effects upon LADH *in vivo*, the question arose as to the clinical potential of such an inhibitor in modifying the toxicities of compounds known to be metabolized through the LADH

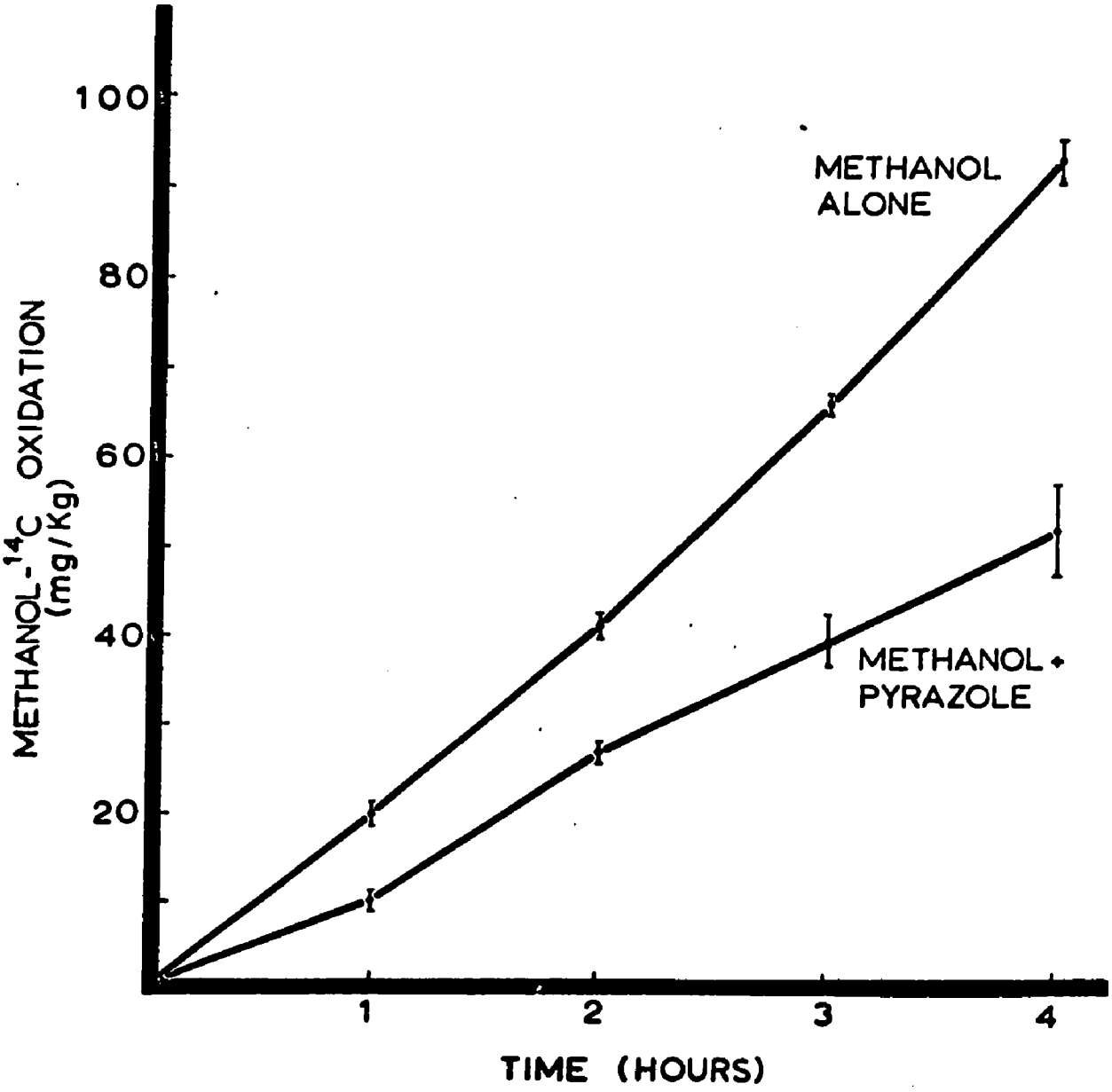


Figure 3. Effect of pyrazole on methanol oxidation in the rat in vivo. Methanol-^{14}C (31.3 mmoles/kg) was injected intraperitoneally. When administered, pyrazole (2.94 mmoles/kg) was injected intraperitoneally 15 min before methanol-^{14}C. Each point represents the mean value of four animals ± standard error.

system, such as methanol in the human.

No toxicities were observed in rats receiving pyrazole (2.94 mmoles/kg) alone, or in combination with ethanol (21.7 mmoles/kg) or methanol (31.3 mmoles/kg). Another important preliminary consideration in evaluating this clinical potential is illustrated in Figures 4 and 5. These are the results of experiments in which ethanol-1-^{14}C (Figure 4) or methanol-^{14}C (Figure 5) was administered to rats in doses of 21.7 mmoles/kg and 31.3 mmoles/kg, respectively. After a linear rate of alcohol oxidation was achieved, half of the rats were administered intraperitoneal injections of pyrazole (2.94 mmoles/kg), while the remaining half received a corresponding volume of 0.9 percent NaCl solution. Under these conditions ethanol oxidation in rats (Figure 4) was inhibited to almost the extent observed in experiments where pyrazole was administered prior to the alcohol (Figure 2). The results illustrated in Figure 5 further support the concept that methanol is oxidized in the rat primarily by the catalase-peroxidative system. However, as revealed previously in Figure 3, it is again apparent that pyrazole exerts a modest, yet definite, inhibitory effect upon methanol oxidation in the intact rat.

A final consideration concerning the clinical potential of pyrazole was the duration of inhibitory action. Figure 6 shows that ethanol-1-^{14}C (21.9 mmoles/kg) oxidation in rats is maximally inhibited (approximately 80 percent) 24 hours after administration of pyrazole (2.94 mmoles/kg), and remains substantially inhibited (approximately 65 percent)

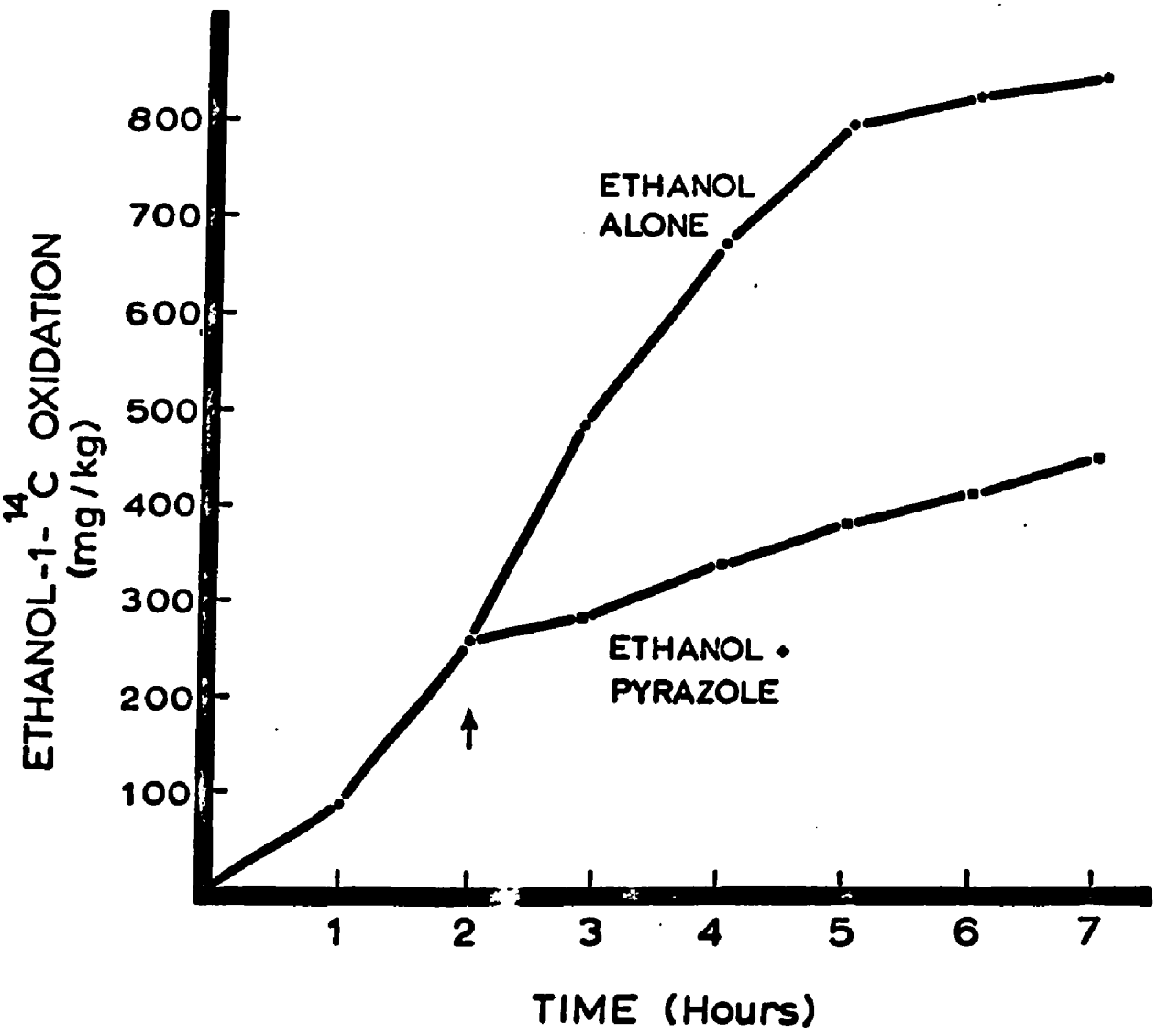


Figure 4. Effect of pyrazole on ethanol oxidation in the rat *in vivo*. Ethanol-1-^{14}C (21.7 mmoles/kg) was administered intraperitoneally. When administered, pyrazole (2.94 mmoles/kg) was injected intraperitoneally 2 hours after ethanol. Each point represents the mean value of 2 animals.

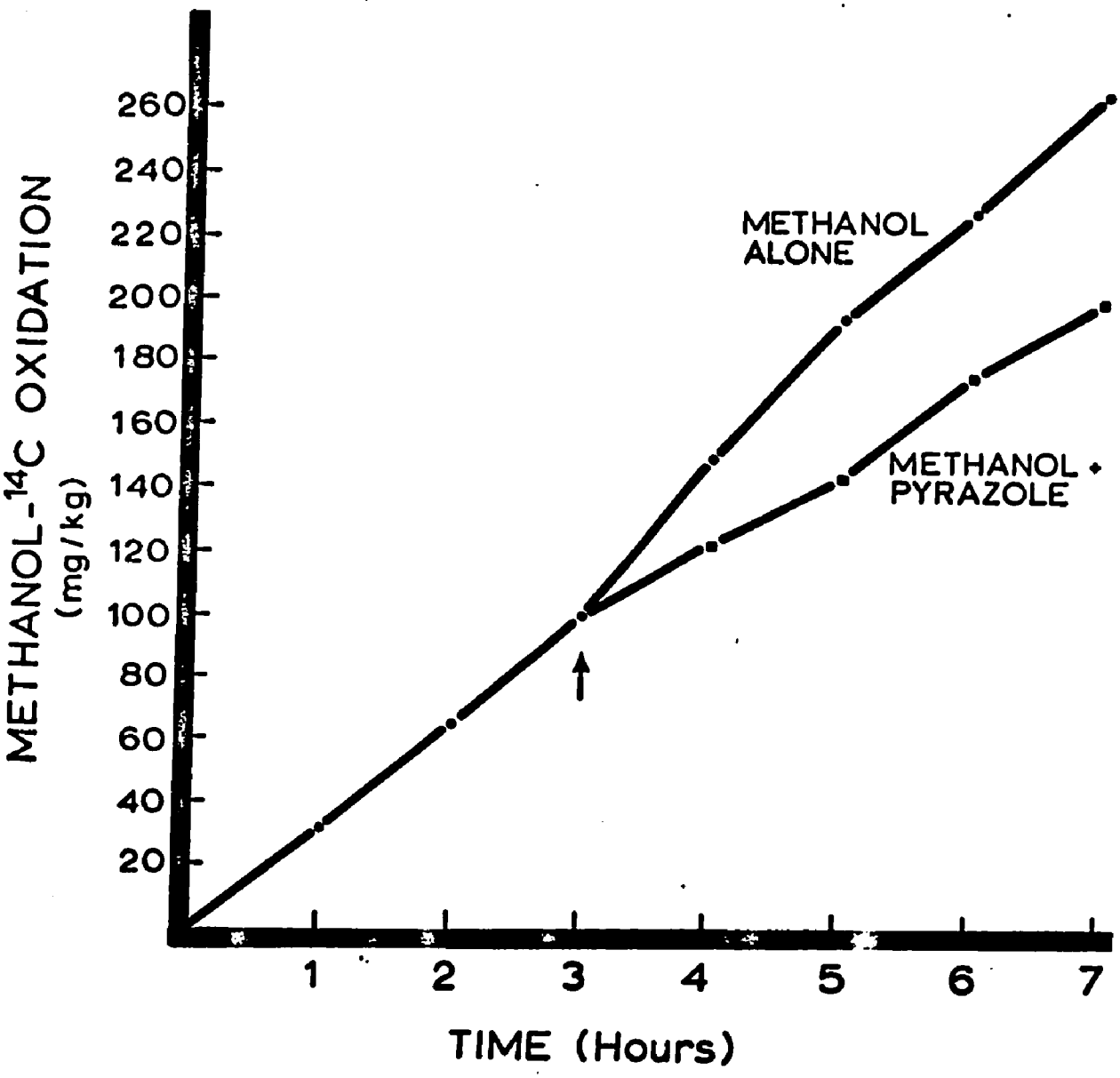


Figure 5. Effect of pyrazole on methanol oxidation in the rat in vivo. Methanol-^{14}C (31.3 mmoles/kg) was administered intraperitoneally. When administered, pyrazole (2.94 mmoles/kg) was injected intraperitoneally 3 hours after methanol-^{14}C. Each point represents the mean value of two animals.

48 hours after pyrazole administration. These findings are in general agreement with the work of others (156), who report that the inhibitory effects of a single dose of pyrazole (2.2 mmoles/kg) on ethanol oxidation in rats was still detectable on the sixth day following adminstration of the inhibitor. An estimate of 14 hours for the half-life of pyrazole has been calculated from the urinary excretion of pyrazole (172).

E. Effect of Pyrazole on Ethanol and Methanol Oxidation in the Intact Monkey

Previous studies by Makar et al. (150) indicated that whereas the peroxidative oxidation of methanol in the rat is a major pathway, its role in the monkey is minimal. Pyrazole inhibited ethanol oxidation in the intact monkey about the same extent as observed for the rat. However, Figure 7 shows that 2.94 mmoles/kg of pyrazole inhibits the oxidation of a dose of 31.3 mmoles/kg of methanol in the intact monkey by about 80 percent. While this combination of pyrazole and methanol was seemingly non-toxic in rats, monkeys treated with methanol and pyrazole as described in Figure 7 exhibited severe toxicity. This was evidenced by weight loss, ataxia, lethargy, and pallor, which often progressed to coma and death in about 3-4 days.

The unexpected toxicities associated with pyrazole administration in the monkey, and interest in the clinical potential of pyrazole prompted studies on the toxicology of pyrazole in the rat an monkey.

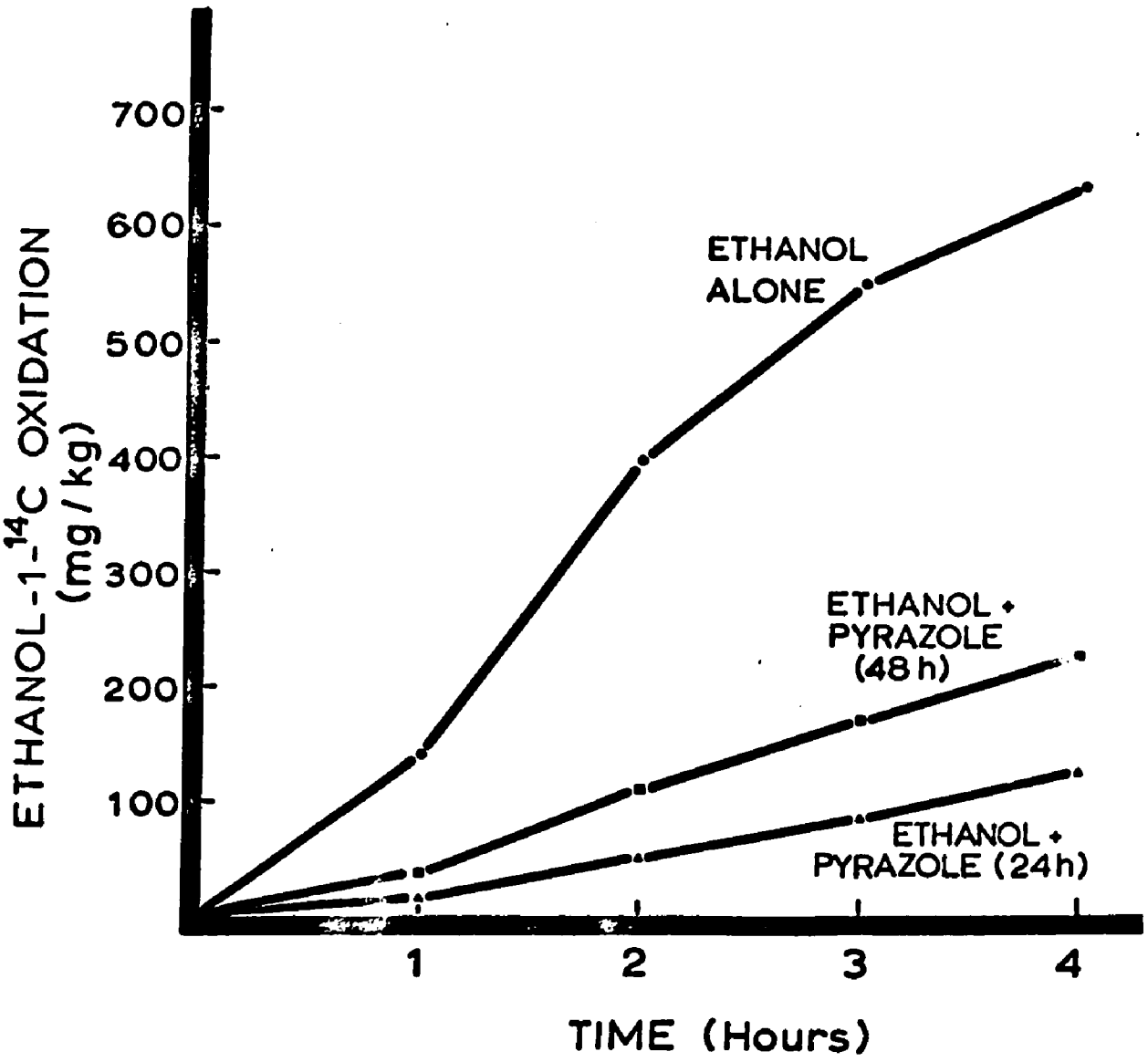


Figure 6. Duration of pyrazole inhibition of ethanol oxidation in the rat *in vivo*. When administered, pyrazole (2.94 mmoles/kg) was administered intraperitoneally. Ethanol-1-^{14}C (21.7 mmoles/kg) was administered intraperitoneally 24 and 48 hours after pyrazole. Each point represents the mean value of two animals.

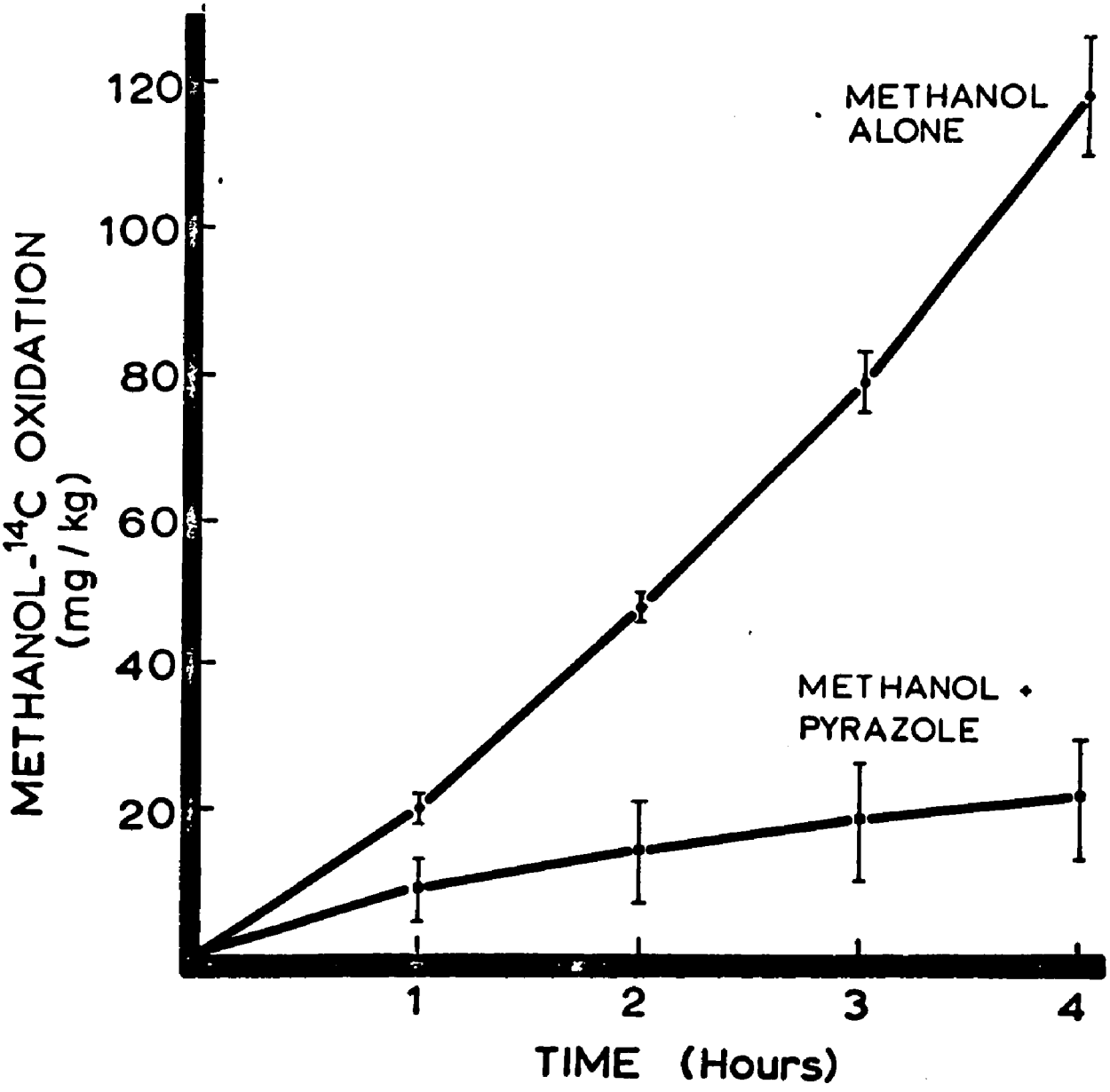


Figure 7. Effect of pyrazole on methanol oxidation in the monkey in vivo. Methanol-^{14}C (31.3 mmoles/kg) was injected intraperitoneally. When administered, pyrazole (2.94 mmoles/kg) was injected intraperitoneally 15 min before methanol-^{14}C. Each point represents the mean value of three animals ± standard error.

F. Studies on Pyrazole Toxicity in the Rat and Monkey

Preliminary toxicity studies were initiated in the rat. Pyrazole was administered intraperitoneally in a dose of 14.7 mmoles/kg--5 times the dose employed to obtain the inhibition data presented in Figures 2,3,4,5 and 6. Within minutes after receiving high doses of pyrazole (14.7 mmoles/kg), rats became comatose and remained in this condition until death occurred days later. In general, rat and monkeys both appeared to respond to high levels of pyrazole by immediate and progressive decreases in energy. Reports that certain pyrazole derivatives possess potent hypoglycemic activities in rats (232,233) prompted the suggestion that the rapid effects observed in the present study could be due to a similar mechanism. In addition, Lelbach (133) has recently reported that prolonged administration of ethanol together with pyrazole produced hepatic lesions and death in rats. Therefore, blood glucose and serum glutamate-oxalacetate transaminase (SGOT) determinations were combined with additional selected standard test procedures in an attempt to gain further insight into the toxicity of pyrazole.

Each of 5 groups of rats received a single intraperitoneal administration of one of the following: (1) methanol (31.3 mmoles/kg); (2) pyrazole (2.94 mmoles/kg); (3) pyrazole (14.7 mmoles/kg); (4) methanol (31.3 mmoles/kg) + pyrazole (2.94 mmoles/kg); (5) ethanol (21.7 mmoles/kg) + pyrazole (2.94 mmoles/kg); and, (6) 0.9 percent NaCl solution. Animals were weighed at the time of injection. Five rats from

each group were sacrificed 12, 24, 48, 72 and 96 hours following injection. The following standard procedures were performed on all animals 12, 24, 48, 72 and 96 hours after injection or until sacrifice: whole body weights were recorded, serum glucose concentrations were determined (234), and SGOT activities were determined (235). At the time of sacrifice liver and kidney weights were recorded, after which sections of these organs and myocardium were preserved in formalin solution for subsequent histopathologic evaluation. The internal organs of each animal sacrificed were visually examined for signs of tissue change or damage.

Of the indices employed in this study to evaluate the effect of pyrazole in the rat--SGOT activity; serum glucose concentration; alteration in body weight; liver weight + kidney weight : body weight ratio; liver weight : kidney weight ratio; histopathologic evaluation of hematoxylin/eosin-stained sections of liver, kidney and myocardium; and, visual examination of major organs at time of sacrifice--only the serum glucose levels were observed to vary significantly from normal controls, and this occurred only in those rats which were comatose as a result of receiving 14.7 mmoles/kg pyrazole.

Several attempts were made to reverse or prevent the pyrazole-coma by administering massive doses of glucose immediately after or prior to pyrazole (14.7 mmoles/kg) administration. This treatment appeared to delay the onset, and decrease the immediate severity of symptoms ordinarily observed with pyrazole alone, however any observed protective effects

were of only brief duration.

Studies on pyrazole toxicity in the monkey were conducted in a manner similar to those discribed for the rat, except that none of the monkeys were sacrificed, and only one monkey per group was used to test the effects of a single administration of each of the following: (1) methanol (31.3 mmoles/kg); (2) ethanol (21.7 mmoles/kg); (3) methanol (31.3 mmoles/kg) + pyrazole (2.94 mmoles/kg); (4) ethanol (21.7 mmoles/kg) + pyrazole (2.94 mmoles/kg); (5) pyrazole (2.94 mmoles/kg); and (6) untreated control. Blood was collected prior to drug administration and 4, 12, and 36 hours after drug administration, from which serum glucose and SGOT levels were determined. Liver biopsy specimens were obtained using aseptic surgical conditions on monkeys from 4 of the 6 drug groups studied: untreated control; methanol + pyrazole; ethanol + pyrazole; and, pyrazole. Biopsy specimens were obtained just prior to drug administration and 72 hours after drug administration, and were preserved in formalin solution for subsequent hematoxylin/eosin staining and histopathologic evaluation. One monkey which had received methanol-^{14}C (31.3 mmoles/kg) + pyrazole (2.94 mmoles/kg) was found minutes after death, and sections of liver, kidney, myocardium, and brain were prepared for histopatholigic evaluation.

Under the conditions established in the monkey, the toxic effects of pyrazole alone, or in combination with methanol or ethanol, were not reflected in the parameters measured in this study.

Because pyrazole in doses employed up to this point (2.94 mmoles/kg) exerted obvious adverse effects in monkeys, the decision was made to examine the effect on methanol oxidation of a lower dose of pyrazole in combination with low doses of ethanol. It was felt that such a combination of pyrazole and ethanol might serve to effectively inhibit methanol metabolism in vivo without producing toxic effects in the monkey. These studies are reported in the following section.

G. Effect of Pyrazole and Ethanol on Methanol Oxidation in the Monkey

Figure 8 shows the results obtained from two monkeys which received methanol-^{14}C (31.3 mmoles/kg) followed 4 hours later by pyrazole (0.74 mmole/kg), and 8 hours later by ethanol (5.4 mmoles/kg). The average rate of methanol oxidation in these animals was 22 mg/kg/hr, which was reduced to 11 mg/kg/hr by the injection of pyrazole. Low doses of ethanol have been shown to inhibit methanol oxidation in the monkey (150), but the inhibitory effect lasts only for a relatively short period of time due to its rapid metabolism (150 mg/kg/hr). The rate of methanol oxidation for the first hour following ethanol administration was about 5 mg/kg/hr (about 20 percent of control) which returned over the next 3 hours to the rate seen with pyrazole alone. Studies performed with monkeys receiving only methanol (31.3 mmoles/kg) showed that the rate of oxidation of this dose of methanol remains linear throughout the 12-hour period of experimentation illus-

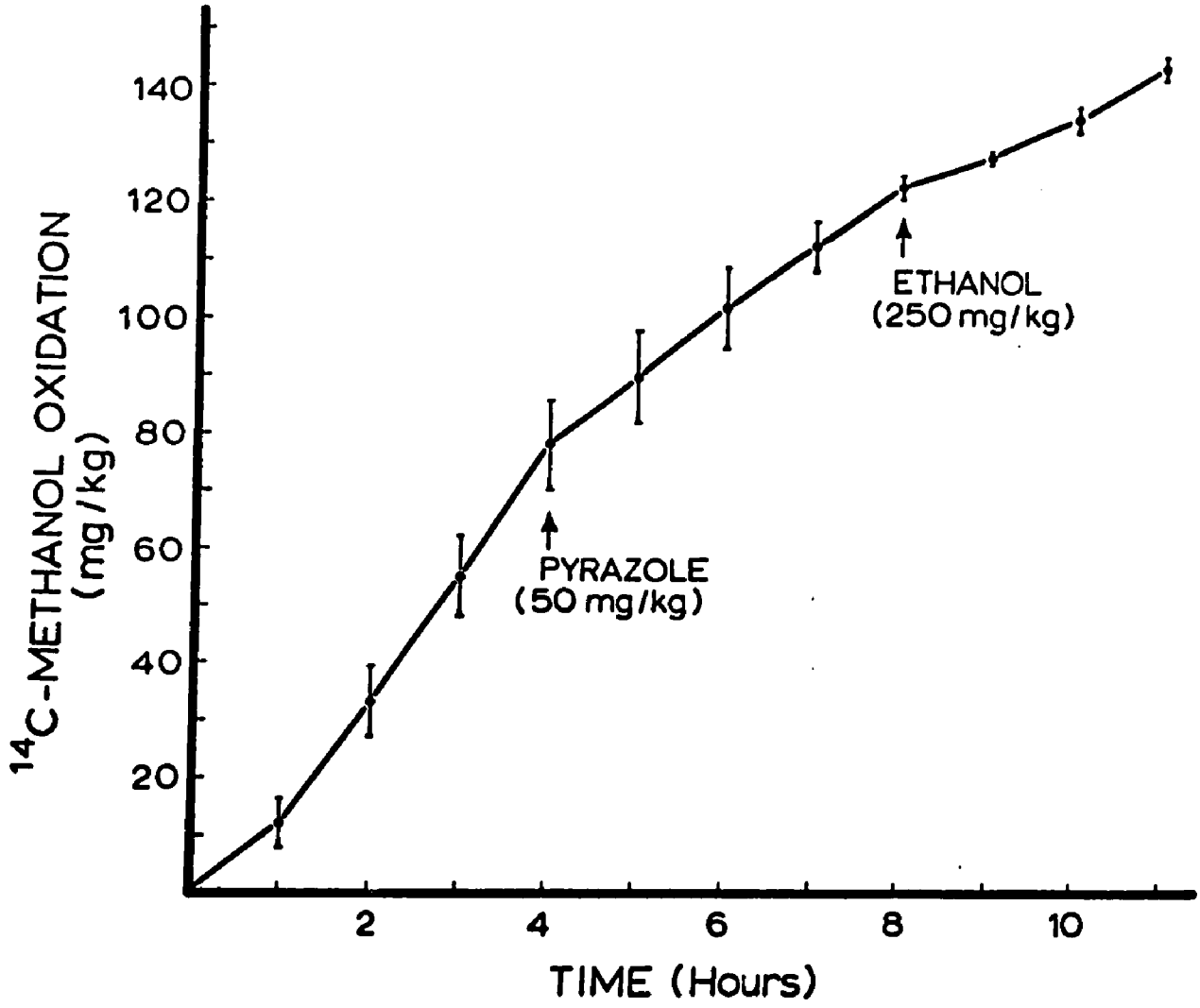


Figure 8. Effect of pyrazole and ethanol on methanol oxidation in the monkey *in vivo*. Methanol-^{14}C (31.3 mmoles/kg) was injected intraperitoneally. Pyrazole (0.74 mmole/kg) and ethanol (5.4 mmoles/kg) were injected intraperitoneally at 4 and 8 hr, respectively, following the initial methanol-^{14}C administration. Each point represents the mean value of two animals with the corresponding range at each time interval.

trated in Figure 8. Table 3 shows that in two monkeys receiving 5.43 mmoles/kg of ethanol 4 hours after 31.3 mmoles/kg of methanol, inhibition of about 50 percent was observed during the first hour following ethanol administration. The animals treated as described in Figure 8 did not display any observable toxicities. They gained weight and showed, for at least 1 month later, none of the toxic manifestations associated with the 2.94 mmoles/kg dose of pyrazole in this species.

H. Studies on the Role of Microsomal Oxidation of Ethanol in the Intact Rat

During the time in which experiments reported in this study were in progress, numerous publications appeared by Lieber and his colleagues (45,46,210). These authors suggest that the microsomal ethanol-oxidizing system observed *in vitro* was a microsomal mixed-function oxidase, similar to that described for the metabolism of many drugs, and that it might participate significantly in alcohol oxidation *in vivo*. Portions of Lieber's data (45) were submitted to further calculation in order to estimate the maximal contribution of the microsomal ethanol oxidizing system in alcohol oxidation *in vivo*. The details of these calculations will be discussed later in this dissertation. However, it appeared that the microsomal system could account for no more than 7 percent of total alcohol oxidation *in vivo*. These considerations led to a series of experiments designed to evaluate the role of the microsomal system in alcohol oxidation *in vivo*.

Figure 9 illustrates data obtained from rats which

Table 3

Effect of Ethanol on Methanol-^{14}C Oxidation in the Monkey

Experiment	Methanol Oxidation*	
	Before Ethanol	After Ethanol
	mg/kg/hr	mg/kg/hr
1	28	17
2	27	12

*The rate of methanol oxidation represents the rate over the first hour following the administration of 250 mg (5.4 mmoles)/kg of ethanol. Ethanol was injected intraperitoneally 4 hours after the injection of 31.3 mmoles/kg of methanol.

were administered SKF 525-A, a broad inhibitor of microsomal reactions, followed in 15 minutes by either ethanol-1-^{14}C or methanol-^{14}C. The measured rate of alcohol oxidation was unaffected by imposing the known inhibitory effects of SKF 525-A. In addition, Figure 10 shows that pretreatment of rats for 3 days with phenobarbital sodium (40 mg/kg) has no significant effect upon the oxidation of ethanol-1-^{14}C in vivo. Rats treated identically to those described in Figure 10 were sacrificed at the end of the third day of treatment with phenobarbital or 0.9 percent NaCl solution, the hepatic microsomes prepared, and the enzymatic N-dealkylation of ethylmorphine was measured. This measurement of microsomal mixed-function oxidase was increased three-fold beyond that measured in control animals. Therefore, these studies indicate that microsomal ethanol oxidation is not an important part of ethanol oxidation in vivo.

II. Studies on the Retinal Alcohol Dehydrogenase System

The experiments described to this point gave reason to believe that pyrazole (or other related inhibitors of hepatic alcohol dehydrogenase) might possess a clinical potential by virtue of modifying the metabolism of various endogenous and exogenous compounds, or by modifying intracellular ratios of NAD/NADH.

This latter portion of the dissertation was centered about an underlying concern that administration of inhibitors of hepatic alcohol dehydrogenase to intact mammals might adversely influence non-hepatic tissues believed to require

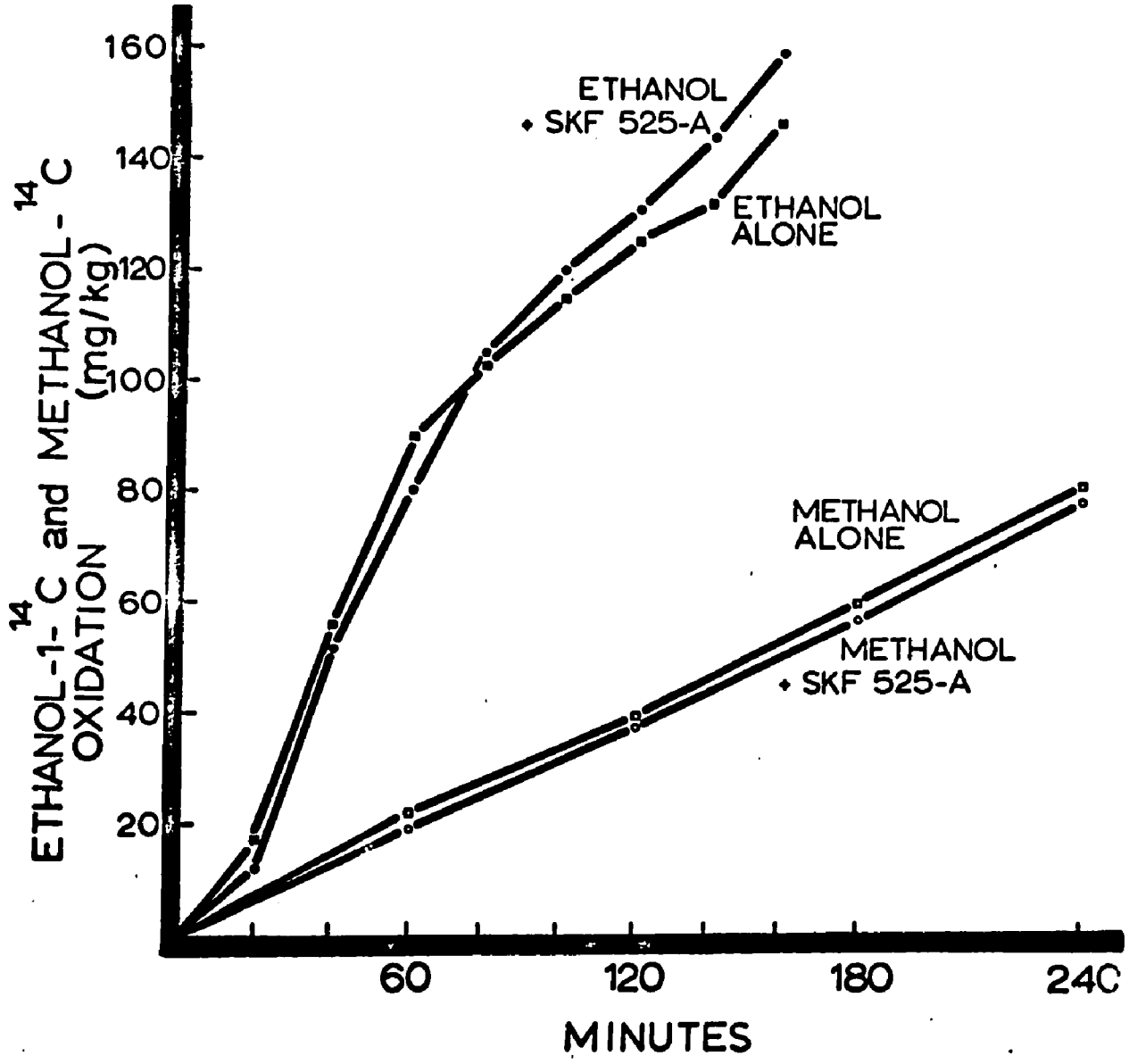


Figure 9. Effect of SKF 525-A on ethanol-1-^{14}C and methanol-^{14}C oxidation in the rat in vivo. SKF 525-A (130 μmole/kg) was administered intraperitoneally 15 min before intraperitoneal administration of ethanol (5.4 mmoles/kg) or methanol (31.3 mmoles/kg). Expired $^{14}CO_2$ was collected at timed intervals as described in Methods. Each point represents the mean value of four animals.

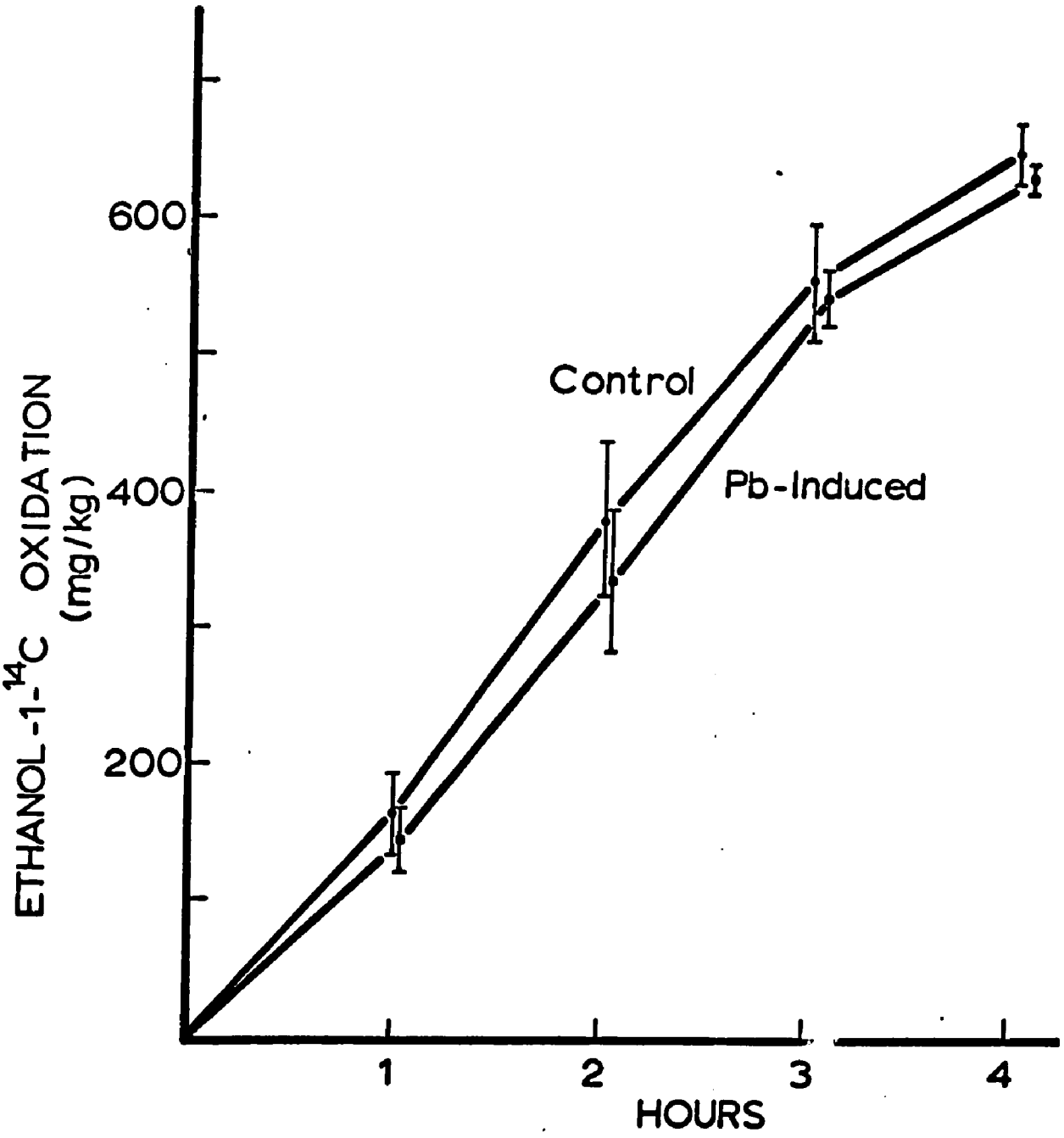


Figure 10. Effect of phenobarbital (40 mg/kg) treatment (3 days) on subsequent oxidation of ethanol-1-^{14}C in the rat *in vivo*. Ethanol-1-^{14}C oxidation was determined as described in Methods. Each point represents the average value of four animals ± standard error of the mean. The dose of ethanol was 21.7 mmoles/kg, injected intraperitoneally.

an alcohol dehydrogenase for physiologic function. For years it has been generally accepted that the interconversion of retinol and retinaldehyde in the retinal visual cycle was mediated by an alcohol dehydrogenase possessing characteristics identical to hepatic ADH. This concept was subject to question however, when Koen and Shaw (85) reported studies on ADH-isozymes from hepatic and retinal preparations. These authors concluded from their investigations that the oxidation of retinol proceeds in the liver and retina by specific and distinct enzymes. In light of this report, it was felt that the effects of inhibitors of LADH on alcohol dehydrogenase(s) in the retina would best be evaluated using a partially purified retinal enzyme preparation. Therefore attempts were made to partially purify and characterize retinal ADH activity. Also, during these purification procedures retinal aldehyde dehydrogenase activity was monitored for 2 reasons. If such activity were present in the retina, its enzymatic characteristics would be of interest as they related to the retinal ADH pathway. Secondly, any existing AldDH activity would have to be separated from ADH activity due to mutual interference in the standard assay procedures for either pyridine nucleotide-dependent dehydrogenase.

A. Separation and Partial Purification of Alcohol Dehydrogenase and Aldehyde Dehydrogenase from Rat Retinas

Retinal NAD-dependent oxidation of ethanol (RADH) was found to reside exclusively in the supernatant fraction obtained after centrifugation of the retinal homogenate at

105,000 x g for one hour. This is the primary cellular locus (cytosol) of hepatic ADH. The relative solubilities of RADH and retinal aldehyde dehydrogenase (RAldDH) during ammonium sulfate saturation of the rat retinal cytosol is shown in Table 4. It can be seen that most of the RADH precipitates in the 33-70 percent ammonium sulfate saturation range whereas most of the RAldDH activity precipitates between 70-100 percent ammonium sulfate saturation.

Figure 11 illustrates the elution pattern of RADH and RAldDH activity obtained after DEAE-cellulose chromatography of the cytosol fraction of retina or the 33-100 percent ammonium sulfate fraction of retinal cytosol. The pattern shows a complete separation of RADH and RAldDH activity. This was surprising since it is not possible to resolve hepatic ADH from AldDH with DEAE-cellulose chromatography of hepatic cytosol or a 33-100 percent ammonium sulfate precipitate of rat hepatic cytosol. Chromatographic separation of hepatic ADH and hepatic AldDH usually requires the use of CM-cellulose chromatography (20). Table 5 summarizes the purification procedure conducted on the rat retinal cytosol used in these studies. The procedure allowed for a complete separation of RADH and RAldDH and a 7.4 and 7.3-fold purification, respectively, relative to the original cytosol preparation.

B. Separation and Partial Purification of Alcohol Dehydrogenase and Aldehyde Dehydrogenase from Monkey Retinas

As observed for the rat retinal enzymes, the monkey retinal NAD-dependent oxidation of ethanol (RADH) was found

Table 4

Relative Distribution Pattern of Rat Retinal Cytosol Enzymes After Ammonium Sulfate Saturation

$(NH_4)_2SO_4$ Saturation (%)	RADH[+]	RAldDH[#]
0-33	1%*	2%
33-50	18%	10%
50-70	79%	2%
70-100	2%	86%
Total	100%	100%

*Percent total activity in precipitate

[+]Retinal alcohol dehydrogenase activity

[#]Retinal aldehyde dehydrogenase activity

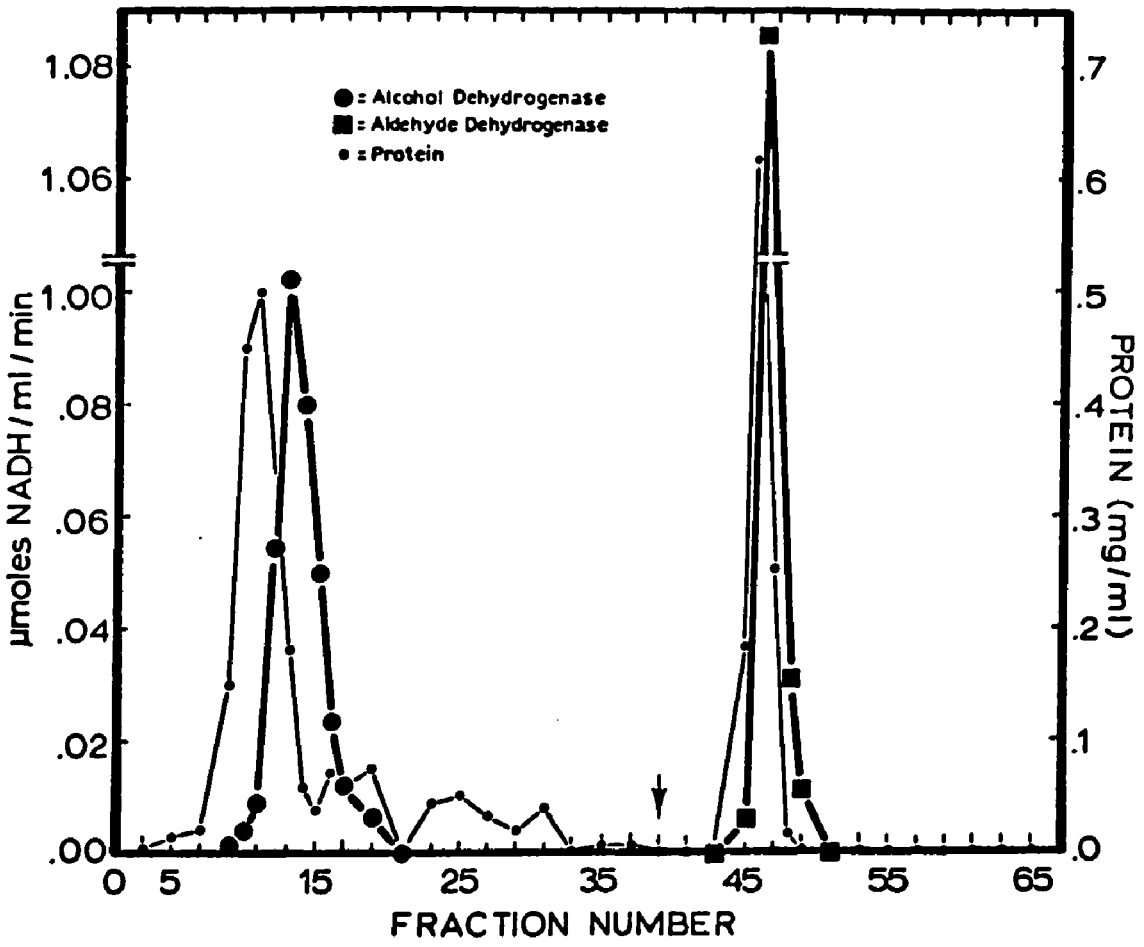


Figure 11. Chromatography of retinal enzymes on DEAE-cellulose. The dialyzed precipitate following 33-100% ammonium sulfate saturation was applied to a column of DEAE-cellulose and elution was begun with 0.005 M sodium:potassium phosphate buffer, pH 8.0 containing 1 mM Na_2EDTA and 0.1 mM dithiothreitol. At the point indicated by the arrow, the buffer concentration was increased 10-fold (0.05 M). Enzyme activity was assessed as described in Methods.

Table 5

Procedure for Purification of Rat Retinal Alcohol (RADH) and Aldehyde Dehydrogenase (RAldDH)

Procedure	Volume (ml)	Concentration (units/ml)	Total Units	Protein (mg/ml)	Specific Activity	Yield	Purification
RADH Activity							
Cytosol	20.0	.257	5.1	7.1	.036	100	1
Ammonium Sulfate (33-100%)	16.8	.233	3.9	4.1	.057	76	1.6
DEAE-Cellulose	53.0	.0354	1.9	0.045	.207	37	7.4
RAldDH Activity							
Cytosol	20.0	1.318	26.4	7.10	.186	100	1
Ammonium Sulfate (33-100%)	16.8	1.133	19.1	4.10	.276	72	1.5
DEAE-Cellulose	33.6	.426	14.4	.315	1.35	55	7.3

exclusively in the supernatant fraction obtained after centrifugation of the retinal homogenate at 105,000 x g for one hour. Procedures identical to those used to separate and purify the rat retinal dehydrogenases were employed to achieve satisfactory separation and purification of the monkey retinal enzymes. The relative distribution of RADH and RAldDH during saturation of the monkey retinal cytosol with ammonium sulfate is shown in Table 6. Monkey RADH precipitated at higher ammonium sulfate saturation limits than the corresponding rat enzyme. Although rat and monkey RAldDH were both precipitated at relatively high ammonium sulfate saturation, the rat enzyme.precipitated within a narrower saturation range than the rat enzyme. Table 7 summarizes the results of the entire purification procedure when conducted on the monkey retinal cytosol used in these studies. This procedure varied from that used for the rat retinal enzymes in that the 50-100 percent ammonium sulfate precipitate of the monkey retinal cytosol was submitted to DEAE-cellulose chromatography, whereas the 33-100 percent fraction was used in preparing the rat enzyme. The elution pattern observed during DEAE-cellulose chromatography of the monkey retinal enzymes was essentially the same as presented in Figure 11 for the rat enzymes.

Two notable points became apparent on reviewing the data summarized in Table 5 and Table 7. The specific activitiy of RADH obtained from the monkey retinal homogenate ranged from approximately 10-50 percent of that obtained from the corresponding rat tissue. Comparison of the specific activities of RAldDH obtained from the monkey and rat shows

Table 6

Relative Distribution Pattern of Monkey Retinal Cytosol Enzymes after Ammonium Sulfate Saturation

$(NH_4)_2SO_4$ Saturation (%)	RADH[+]	RAldDH[#]
0-33	3%*	6%
33-50	3%	6%
50-70	71%	56%
70-100	23%	32%
Total	100%	100%

*Percent total activity in precipitate

[+]Retinal alcohol dehydrogenase activity

[#]Retinal aldehyde dehydrogenase activity

Table 7

Procedure for Purification of Monkey Retinal Alcohol (RADH) and Aldehyde Dehydrogenase (RAldDH)

Procedure	Volume (ml)	Concentration (units/ml)	Total Units	Protein (mg/ml)	Specific Activity	Yield	Purification
RADH Activity							
Cytosol	15.0	.0225	.34	5.2	.0043	100	1
Ammonium Sulfate (50-100%)	11.5	.0205	.24	.74	.028	80	6.5
DEAE-Cellulose	17.1	.0105	.18	.052	.127	60	29.0
RAldDH Activity							
Cytosol	15.0	.0322	.48	5.2	.006	100	1
Ammonium Sulfate (50-100%)	11.5	.0297	.34	.735	.0404	71	6.5
DEAE-Cellulose	8.5	.0145	.128	.108	.134	26	22.0

that the monkey enzyme ranges from 1-10 percent of the specific activity measured for the rat. Further, comparison of the total units of activity of ADH and AldDH obtained from the retinae of these two species shows the monkey retinal preparations to possess about 5-10 percent and 1 percent, respectively, of the total units of enzyme activity measured in rat retinae. The second notable point is the fact that, under the same isolation procedures, 3-4 times greater enzyme purification was obtainable from the primate tissue than from the rodent tissue.

Later results and discussion will show that the differences just described for the primate and rodent retinal enzymes are the only significant enzymatic differences observed in these studies.

C. Studies on the Properties of Partially Purified Rat Retinal Alcohol Dehydrogenase

1. Substrate Specificity

The pH optimum for RADH was determined to be very similar to that observed for the hepatic enzyme. The effect of pH on RADH activity is illustrated in Figure 12. Maximal activity was found to occur from pH 10.0-10.5 and all subsequent analyses of this activity were performed at pH 10.0. Table 8 shows data obtained when a series of substrates known to be reactive with hepatic ADH were employed using an RADH preparation. The Michaelis constant for NAD in the presence of excess ethanol was 0.4 mM. This cofactor was chosen for subsequent studies of this enzyme activity,

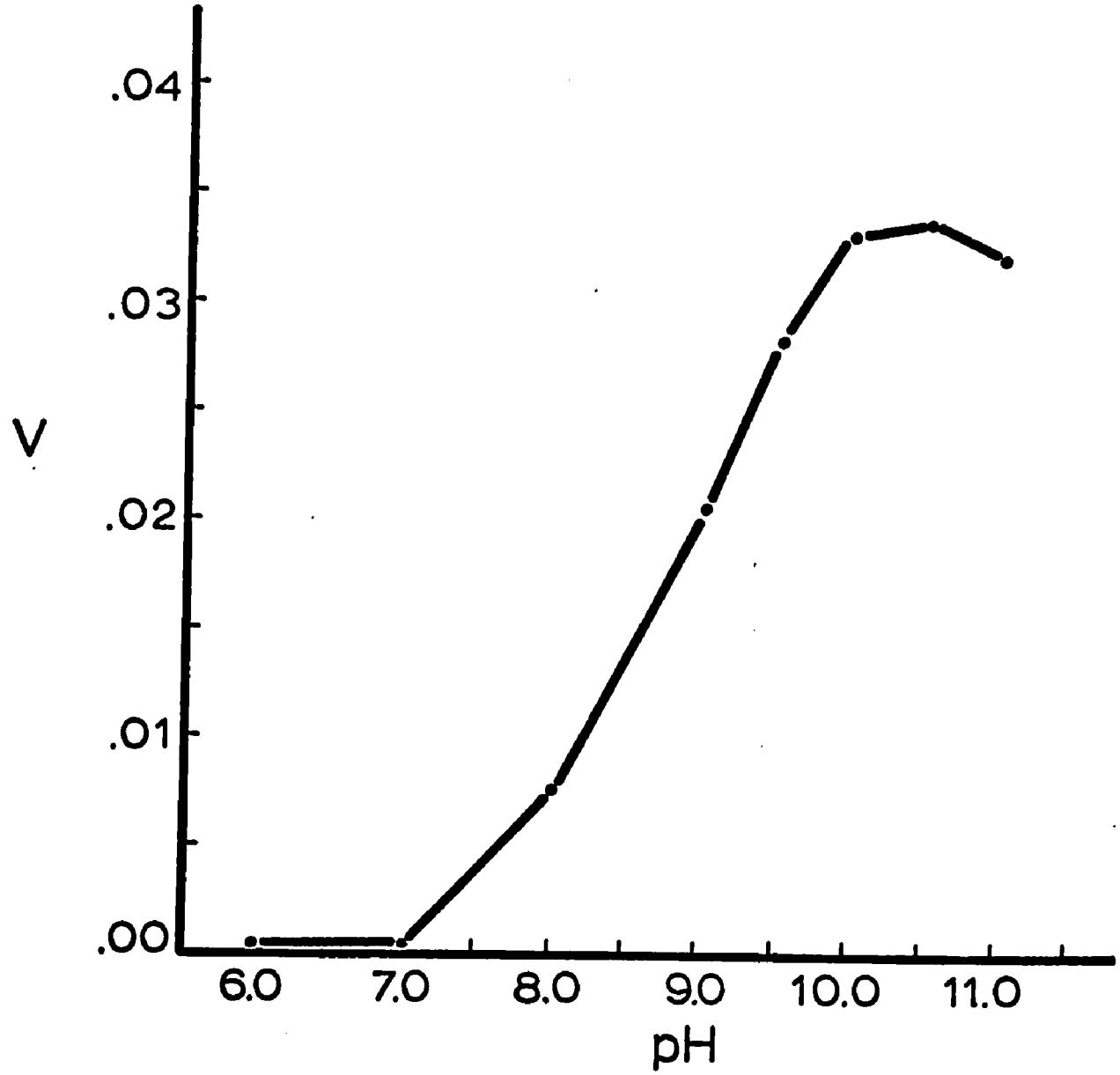


Figure 12. Effect of pH on the activity of purified rat retinal alcohol dehydrogenase. Reactions were carried out at 25°; reaction mixtures contained in a final volume of 1 ml: enzyme; NAD, 2 μmoles; ethanol, 1 mmole; and, sodium:potassium or sodium pyrophosphate buffer, 50 μmoles, adjusted to the desired pH with HCl or NaOH. V = μmoles NADH/ml enzyme/min.

Table 8

Substrate Specificity of Rat Retinal Alcohol Dehydrogenase

Substrate	Michaelis Constant (M)
NAD	0.0004
Ethanol	0.40
1-Propanol	0.12
1-Butanol	0.02
2-Fluoroethanol	Slightly Reactive*
Acetaldehyde	Slightly Reactive*
Methanol	No Reactivity
Retinol	No Reactivity

Alcohol dehydrogenase activity was determined as described in Methods. When the Michaelis constant of NAD was determined, 1.0 M ethanol was the co-substrate. When acetaldehyde was tested, NADH, 2 mM, was employed in reaction mixtures and reactions were carried out from pH 6.5 to pH 9.0. Phosphate (0.05 M potassium phosphate) or sodium pyrophosphate (0.05 M) buffers were used. Retinol was solubilized according to the method used for studies with hepatic ADH (20).

Each value represents the mean of at least 3 determinations.

*Concentrations of 2-fluoroethanol and acetaldehyde were 2.0 M and 0.1 M, respectively.

for, although NADP and acetyl-pyridyl NAD would serve as co-substrates, NAD was found most effective. Methanol at concentrations up to 2.0 M and retinol at concentrations up to 0.01 M were unreactive with RADH. Recently, Reynier (20) showed that hepatic ADH was an effective catalyst for the oxidation of retinol to retinaldehyde. This author's studies have been confirmed in the present study. The data used to calculate the Michaelis constant (Km = 3 μM retinol) for this enzyme activity are presented in Figure 13. Methanol has been shown to serve as a substrate for hepatic ADH although its Michaelis constant with the hepatic enzyme is about 20 times that of ethanol (150). Ethanol, 1-propanol and 1-butanol do serve as substrates for RADH although their Michaelis constants are about 100 to 1000-fold higher than those reported for hepatic ADH (33), and as observed characteristically with the liver enzyme, the Michaelis constant decreases with increasing length of the carbon chain. 2-Fluoroethanol has been employed as a substrate for hepatic ADH in order to dissociate several types of hepatic ADH (32). RADH was reactive with 2-fluoroethanol only at extremely high concentrations (2.0 M). Another notable property of RADH was its relatively poor reactivity with acetaldehyde as substrate under conditions in which acetaldehyde functions effectively as a substrate with the hepatic enzyme.

To establish further that RADH was unreactive with retinol, 5 additional assays were employed. These included: (1) the direct spectrophotometric measurement of the formation of retinaldehyde (20); (2) a colorometric assay of the

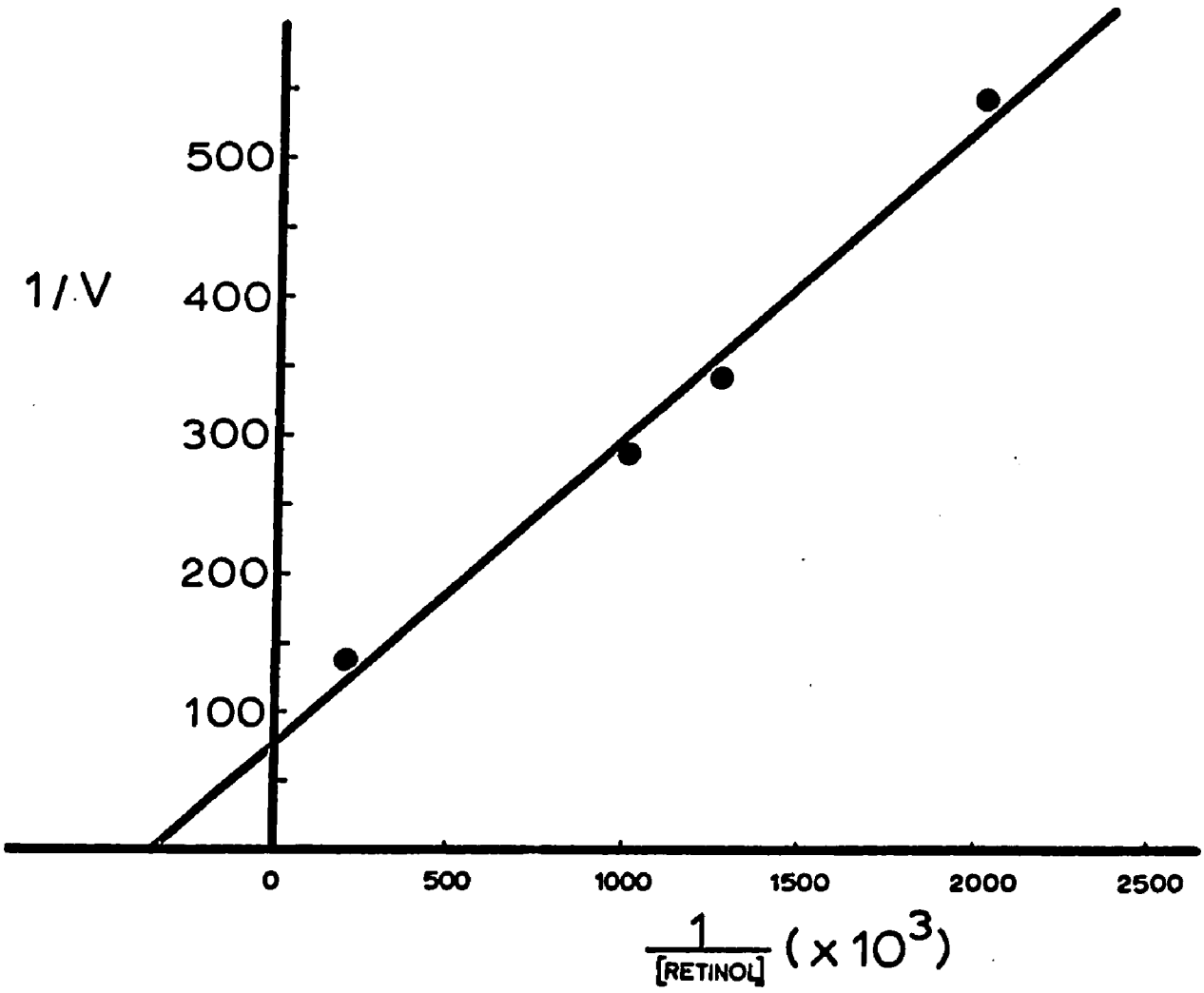


Figure 13. Lineweaver-Burk plot (227) of purified rat liver alcohol dehydrogenase activity. Reactions were carried out at 25°, pH 10.0. Substrate = retinol, V = reaction velocity expressed as μmoles retinaldehyde formed/mg protein/min.

retinaldehyde-paraaminobenzoic acid complex (214); (3) the coupling of RADH to lactaldehyde whereby the formation of propanediol was linked to the oxidation of alcohol substrate (221); (4) kinetic studies on the possible inhibition by retinol of ethanol oxidation; and, (5) activity stains of RADH on polyacrylamide gels using retinol as substrate. In each case Tween-80 and acetone were employed in various concentrations in order to facilitate the solubilization of retinol. This technique has been employed by others (85,87). In each case there was no suggestion of the interaction of retinol with RADH, although ethanol did yield measurable activity when employed as a substrate. Further, none of these procedures showed retinol to possess any capacity to inhibit the oxidation of ethanol by RADH. It should be noted that activity was observed when hepatic cytosol was used with retinol. Therefore, retinol is not a substrate for RADH, although it does react with hepatic ADH.

2. The Effect of Pyrazole and Butyraldoxime on Retinal Alcohol Dehydrogenase

Pyrazole and n-butyraldoxime have been shown to be potent inhibitors of hepatic ADH (156,174). The competitive inhibition of RADH-mediated ethanol oxidation by pyrazole and n-butyraldoxime is illustrated in Figure 14. The inhibitor constant observed for pyrazole was 0.5 mM, and the inhibitor constant for n-butyraldoxime was determined to be about 2.0 μM.

The inhibitor constant for pyrazole with rat hepatic ADH when ethanol is the substrate was reported to

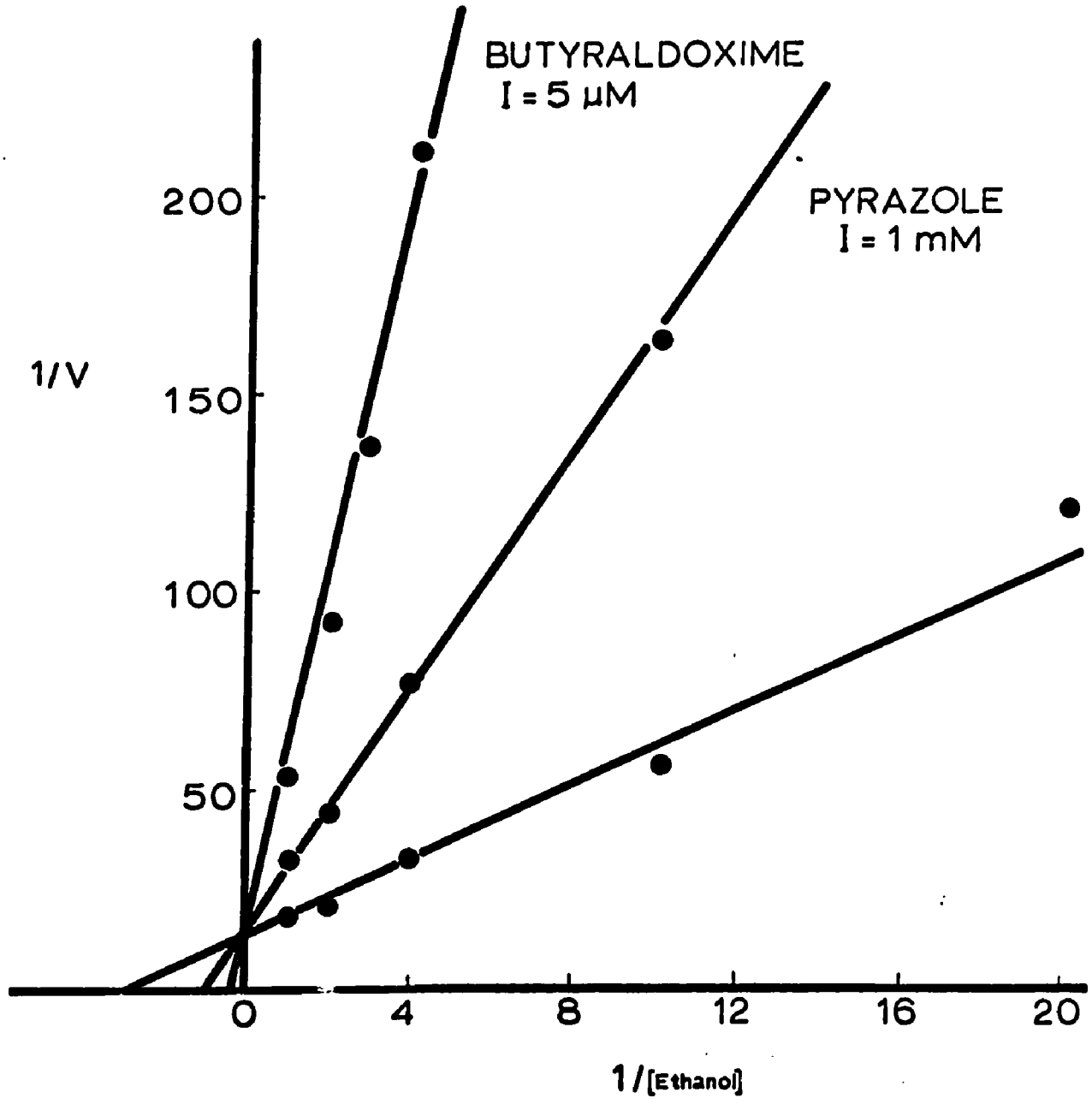


Figure 14. The effects of pyrazole and n-butyraldoxime on purified rat retinal alcohol dehydrogenase activity. Reactions were carried out at 25°, pH 10.0. Substrate = ethanol, I = Inhibitor concentration, V = reaction velocity expressed as µmoles NADH formed/mg protein/min.

be about 4 μM (20). This value was further confirmed in the current investigation. In addition, the inhibitor constant for n-butyraldoxime with rat hepatic ADH and ethanol as the substrate has been calculated to be approximately 8 μM. 1,10-Phenanthroline is a zinc chelating agent which is a potent inhibitor of hepatic ADH (1). This inhibitor exerts its effects presumably by chelating zinc known to participate in the function of this hepatic enzyme. Inhibition by 1,10-phenanthroline of rat retinal ADH was observed only at extremely high inhibitor concentrations (10 mM). This suggests that either RADH is not a zinc-containing enzyme and that the retinal ADH is different than hepatic ADH, or that the zinc contained in the retinal enzyme is somehow protected from the effects of this chelating agent.

D. Studies on Partially Purified Monkey Retinal Alcohol Dehydrogenase

Other than the minor physical differences observed during the purification procedures involved in the preparation of the rat and monkey dehydrogenases, no other significant differences were observed between the retinal enzymes of these two species. That is, far greater differences existed between the enzymes of retinal and hepatic tissues than existed between rodent and primate species. Figure 15 illustrates a standard Lineweaver-Burk plot of kinetic data obtained from monkey RADH. Under these conditions the Km for ethanol was 0.4 M. The corresponding Km determined for the rat RADH was 0.5 M. The Ki value obtained for pyrazole with monkey RADH

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under these conditions was about 30 μM, while the corresponding Ki for the rat RADH (Ki = 0.5 mM) was about 16 times that calculated for monkey RADH. Pyrazole served as a competitive inhibitor of both rat and monkey enzymes.

In addition to the relative similarities in rat and monkey RADH just described (Figures 14 and 15), monkey RADH possessed other characteristics in common with rat RADH. Monkey RADH was definitely unreactive with methanol in concentrations up to 2 M, with retinol in concentrations up to 0.01 M, and with acetaldehyde (in the presence of NADH) in concentrations up to 1 M. As with the other alcohol dehydrogenases studied, this enzyme exhibited progressively more reactivity with ethanol, 1-propanol, and 1-butanol.

E. Studies on Partially Purified Rat Retinal Aldehyde Dehydrogenase

Studies were performed on rat RAldDH in order to investigate the possible participation of this enzyme in the retinal oxidation of ethanol or methanol, and to observe whether or not this enzyme might play a role in the retinal metabolism of retinol and retinaldehyde.

The effect of pH upon the activity of partially purified rat retinal aldehyde dehydrogenase is illustrated in Figure 16. Maximal activity was measured when the reaction medium was adjusted to a pH ranging from pH 9.0 to 9.5.

RAldDH was unreactive with formaldehyde in concentrations up to 0.1 M, and with retinaldehyde in concentrations up to 0.01 M. Acetaldehyde served as a substrate for RAldDH,

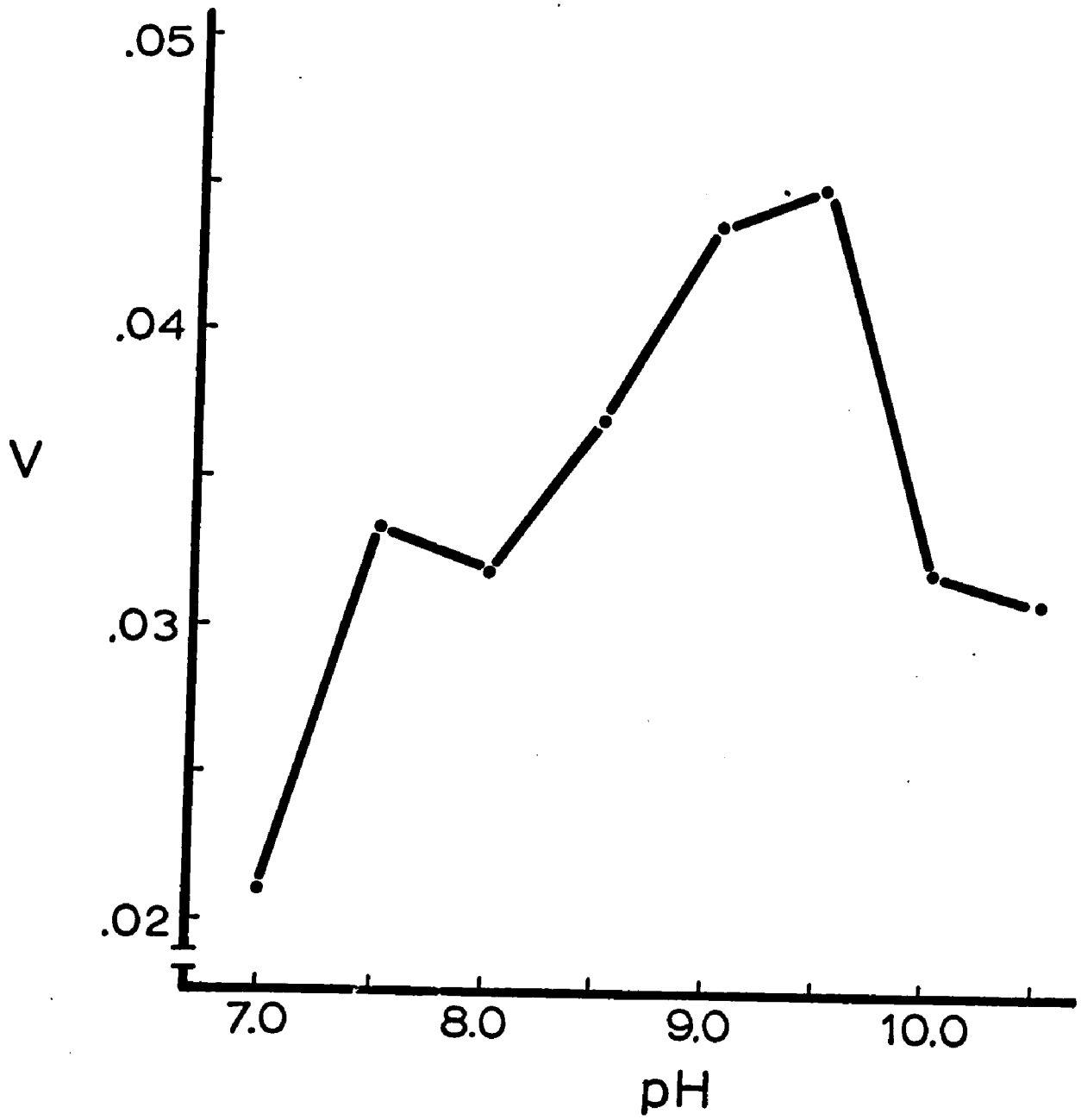


Figure 16. Effect of pH on the activity of purified rat retinal aldehyde dehydrogenase. Reactions were carried out at 25°; reaction mixtures contained in a final volume of 1 ml: enzyme; NAD, 2 μmoles; acetaldehyde, 10 μmoles; and sodium: potassium or sodium pyrophosphate buffer, 50 μmoles, adjusted to the desired pH with HCl or NaOH. V = μmoles NADH formed/ml enzyme/min.

although, analagous to RADH alcohol oxidation, substrate concentrations were necessarily high, relative to the hepatic enzyme, before this NAD-dependent oxidation would measurably proceed. Figure 17 illustrates a Lineweaver-Burk plot of kinetic data used to calculate the Michaelis constant for this reaction of approximately 30 mM.

Koe and Tenen (174) reported that n-butyraldoxime serves not only as an inhibitor of hepatic alcohol dehydrogenase, as confirmed in the present study using partially purified rat retinal and hepatic ADH, but also was reported to serve as an inhibitor of hepatic aldehyde dehydrogenase. This property was investigated in the present study using partially purified RAldDH and rat hepatic aldehyde dehydrogenase. n-Butyraldoxime was not observed to influence the NAD-dependent oxidation of acetaldehyde mediated by either rat retinal ADH or rat hepatic ADH, as determined by spectrophotometric analysis of the enzymatic formation of NADH. However, when control experiments were performed, it was observed that n-butyraldoxime itself was capable of serving as a substrate for either of the aldehyde dehydrogenases employed in these studies. Under conditions used routinely to evaluate aldehyde dehydrogenase activity in this investigation, the Michaelis constant for RAldDH with n-butyraldoxime as substrate was calculated to be approximately 10 mM. This would indicate that n-butyraldoxime functions at least as favorably as acetaldehyde, and possibly better than acetaldehyde as a substrate for retinal aldehyde dehydrogenase. Qualitatively similar results were observed for the corres-

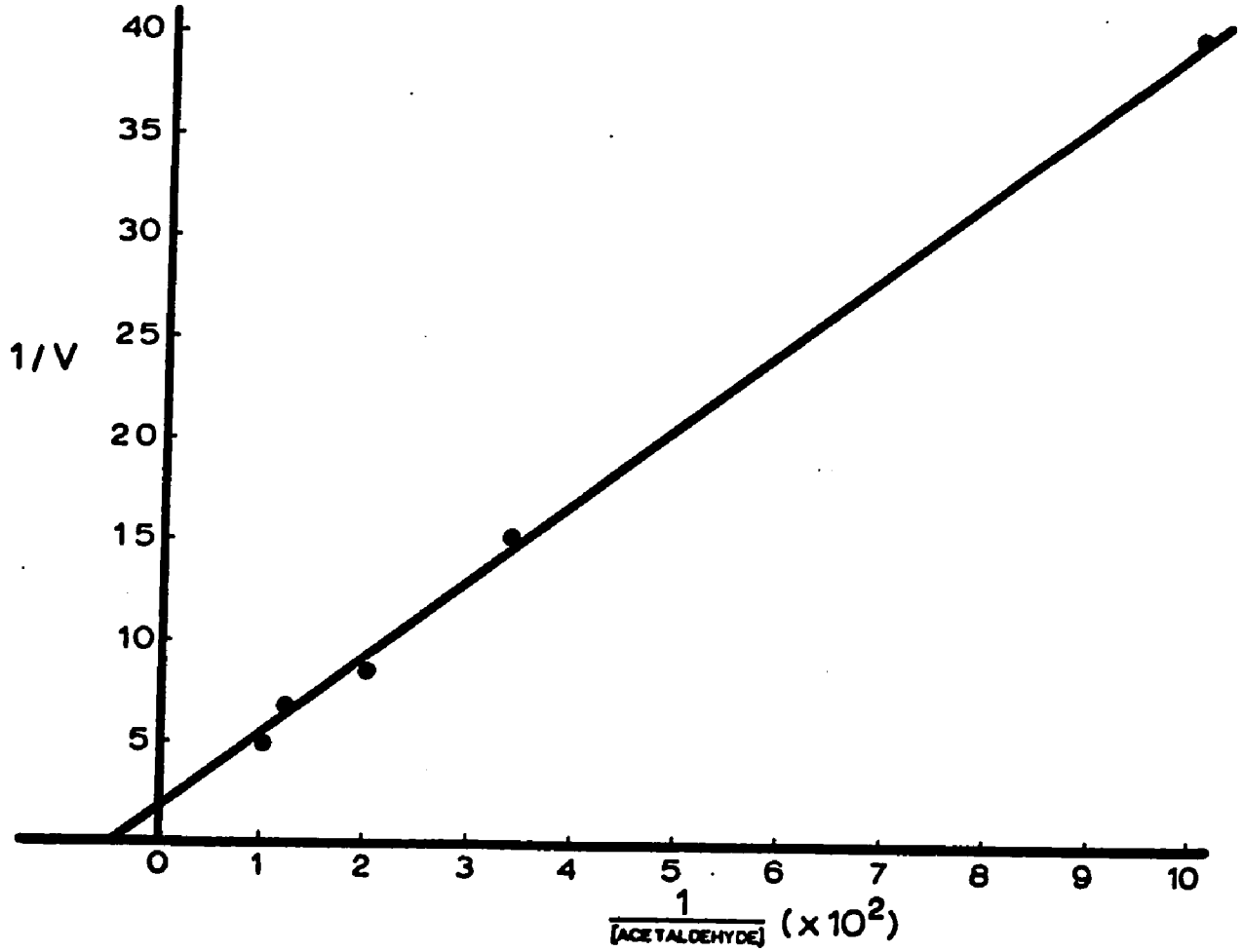


Figure 17. Lineweaver-Burk plot (227) of purified rat retinal aldehyde dehydrogenase activity. Reactions were carried out at 25°, pH 9.5. Substrate = acetaldehyde, V = reaction velocity expressed as μmoles NADH formed/mg protein/min.

ponding hepatic enzyme.

F. Studies on Vitamin A Oxidation-Reduction in Homogenates of Rat and Bovine Retinas

Koen and Shaw (85) have reported that certain isozymes present in a 10,000 x g supernatant from rat retinal homogenates yield activity on starch gel in the presence of NAD and retinol. No such activity was observed in the present experiments in which partially purified alcohol dehydrogenases from the retinas of rats, monkeys and beef were investigated. Neither was retinol observed to undergo NAD- or NADP-dependent enzymatic oxidation in the presence of supernatant obtained following centrifugation of rat, monkey, or beef retinal homogenates at 105,000 x g for 1 hour. In light of these results, and since the enzymatic preparations of Koen and Shaw (85) could have contained certain particulate cell fractions, it was decided to examine retinol oxidation in homogenates of rat and bovine retinas. Although this enzymatic oxidative activity was not observed to proceed through enzymes present in the retinal cytosol or its fractions, Table 9 presents data showing that rat retinal homogenates enriched with 2 mM NADP were capable of mediating retinol oxidation to retinaldehyde. Values reported (Table 9) for the enzymatic formation of retinol or retinaldehyde represent the measured difference between reaction mixtures containing all reported reaction components with the addition of either standard retinal homogenate or boiled retinal homogenate. This use of a boiled enzyme control was necessitated by the complex nature of such crude retinal preparations. NADP

Table 9

Oxidation of Retinol in Rat Retinal Homogenates

	Retinol Oxidation (micromoles retinaldehyde/g retina/hour)
Control	1.8
Pyrazole	1.8
Ethanol	1.7
Methanol	2.1

Rat retinal homogenates were incubated with retinol (1.1 mM), NADP (2 mM), and, when used, pyrazole (1 mM), ethanol (100 mM) and methanol (100 mM) in a final volume of 1.5 ml, pH 7.6, 0.1 M potassium phosphate buffer. Reactions were carried out in air at 37° for 60 minutes during which time product formation was linear.

Each value represents the mean of 2 experiments.

was used in this study because, relative to NAD, its use led to about twice the retinol oxidizing activity observed with NAD. These data are in agreement with the observations of Futterman (89), who showed that NADP is an active co-substrate in the function of visual cell outer segment preparations.

The results presently reported for the rat and bovine retinal homogenates consistently were qualitatively identical in both species. However, when the capacity to oxidize or reduce vitamin A was calculated, the specific activity of the rat retinal preparation exceeded that of the bovine retinal preparation by an approximate factor of 10.

Table 9 shows that pyrazole (1 mM), ethanol (100 mM), and methanol (100 mM) were ineffective as inhibitors of the oxidation of retinol by retinal homogenates. Studies of the reduction of retinaldehyde in the presence of NADPH were performed under optimized conditions. Analagous to the results observed in the oxidation of retinol by these preparations, neither pyrazole (10 mM), acetaldehyde (10 mM), ethanol (0.1 M), nor formaldehyde (1 mM) exerted significant inhibitory effects on this reductive process.

Further investigations were conducted in order to pursue possible physical and enzymatic relationships between RADH and the "retinol dehydrogenase" activity observed in crude retinal homogenates. Standard differential centrifugation and sucrose gradient techniques (236) were employed in order to isolate retinal microsomes, outer segments, mixed fragments (mitochondria, microbodies, nuclei, plasma membranes,

cell debris), cytosol, and a 3x-washed (cytosol-free) precipitate prepared from crude retinal homogenate centrifuged at 105,000 x g for 1 hour. No attempt was made to ascertain the absolute composition of the cell fractions thus prepared. Each of these cell preparations was examined for its capacity to mediate the oxidation of retinol ("retinol dehydrogenase") and for its capacity to mediate the oxidation of ethanol (RADH). "Retinol dehydrogenase" specific activity was greatest in the retinal outer segment fraction, but was definitely measurable in the microsomes, mixed fragments, and 3x-washed precipitate. This suggests that either the particulate enzyme is dispersed in a variety of subcellular organelles, or that the procedures employed to separate these components were not totally successful in eliminating cross-contamination of the various cell components. No "retinol dehydrogenase" activity was measurable in the cytosol fraction. On the other hand, RADH activity was measurable only in the cytosol fraction.

As segments of the studies on hepatic ADH, RADH, and retinal "retinol dehydrogenase" were completed, a number of characteristics became apparently distinctive for each of these enzymes when compared with the other two dehydrogenases. Table 10 was compiled to show typical differences observed <u>in vitro</u> in the mammalian "alcohol dehydrogenases" investigated in this study.

Table 10

Summary of the Characteristics of Hepatic Alcohol Dehydrogenase (LADH), Retinal Alcohol Dehydrogenase (RADH), and Retinol Dehydrogenase (RDH) from the Rat

	LADH	RADH	RDH
Solubility	Soluble	Soluble	Insoluble
pH Optimum (Approx.)	10	10	7.5
Substrates (Michaelis Constant)			
Methanol	15 mM	Unreactive	Unreactive
Ethanol	2 mM	0.5 M	Unreactive
1-Propanol	1 mM	0.12 M	Unreactive
1-Butanol	0.2 mM	0.02 M	Unreactive
Retinol	3 μM	Unreactive	Reactive
Formaldehyde	Reactive	Unreactive	Unreactive
Acetaldehyde	Reactive	± Reactive	Unreactive
Retinaldehyde	Reactive	Unreactive	Reactive
Inhibitors			
Pyrazole	4 μM+	.5 mM+	Not Inhibitor#
n-Butyraldoxime	5 μM+	2 μM+	
1,10-Phenanthroline	Inhibitor	Not Inhibitor	
Favored Cofactor (NAD or NADP)	NAD	NAD	NADP
Favored Reaction	Aldehyde Reduction	Alcohol Oxidation	Aldehyde Reduction

Details of individual assays are described in Methods.

+Inhibitor Constant, Substrate = Ethanol

#Substrate = Retinol

DISCUSSION

The first portion of this study was directed at determining directly the role of alcohol dehydrogenase (ADH) in the oxidation *in vivo* of ethanol and methanol in the rat and monkey. Earlier studies have considered two enzymatic routes for the oxidation of aliphatic alcohols in the rat and monkey *in vivo*: a pyridine nucleotide-dependent ADH pathway, and the catalase-peroxidative system involving hepatic catalase and a source of hydrogen peroxide generation. Alternate substrates for hepatic ADH and catalase-peroxide complex I, and the catalase inhibitor, aminotriazole, had been employed previously in the rat, showing that in this species the catalase-peroxidative system plays a major role in methanol metabolism (149). Substrates favorable for hepatic ADH served to inhibit ethanol oxidation in the rat *in vivo*, while aminotriazole exerted no influence on this process, suggesting that ethanol was oxidized in the intact rat primarily through the liver ADH pathway. In the monkey, however, aminotriazole does not inhibit methanol oxidation, and experiments using alternate substrates for the hepatic ADH and catalase-peroxidative systems revealed a metabolic pattern consistent with a predominant role for ADH in the oxidation of both methanol and ethanol (150).

Although aminotriazole served to demonstrate directly the role of the catalase-peroxidative system in methanol and ethanol oxidation in the rat and monkey (149,150), no effective, relatively specific inhibitor of hepatic ADH was known

until recently. The potent inhibitory effects of pyrazole on hepatic ADH *in vitro* were first reported by Theorell and Yonetani (155), and only very recently were reported the potent inhibitory effects of pyrazole and its derivatives on ethanol oxidation in the intact rat (156,170), dog (170) and human (171).

Considerable investigation has been centered about determining the mechanism by which pyrazole exerts its inhibitory effect on equine hepatic ADH (155). Recent publications suggest that the same inhibitory mechanism exists for liver ADH from the human (169) and rat (20). Pyrazole competes with ethanol for the alcohol binding site on liver ADH, forming an inactive ternary complex composed of pyrazole-NAD-ADH. The formation of this complex has been ascribed to a bond formation between the negatively charged pyrazole ion and the 4-carbon of the pyridinium ring of NAD, occurring at, or near the alcohol binding site of the enzyme. Zinc atoms present at the active centers of liver ADH are currently considered to promote the dissociation of H^+ from pyrazole. The resulting pyrazole anion then interacts with NAD (155). Reynier (20) has examined the influence of pyrazole upon the activities of a number of zinc-containing enzymes, including: yeast ADH, carbonic anhydrase, carboxypeptidase, lactate dehydrogenase, glutamate dehydrogenase, α-glycerophosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and malate dehydrogenase. Pyrazole in concentrations 100 to 1000 times that required to inhibit liver ADH exerted no inhibitory effects on the enzymatic activities examined,

with the exception of yeast ADH--an enzyme ordinarily absent in the mammal. In addition, experiments were performed during the current study in which partially purified rat hepatic aldehyde dehydrogenase activity (AldDH) was found to be unaffected by 10,000 times (10 mM) the concentration of pyrazole necessary to inhibit liver ADH.

The work described above suggested that the inhibitory effect of pyrazole was relatively specific for liver ADH. However, before pyrazole could be reasonably employed in the present studies as a tool to determine directly the role of ADH in ethanol and methanol oxidation in vivo, its influence on the hepatic catalase-peroxidative system had to be determined. Pyrazole was tested 7 ways for interaction with hepatic catalase: (1) As a direct inhibitor of crystalline beef liver catalase activity (Figure 1); (2) As an inhibitor of crystalline beef liver catalase in the presence of hydrogen peroxide (Figure 1); (3) As a substrate for catalase-peroxide complex I (Figure 1); (4) As an inhibitor in vitro of the catalase-peroxidative oxidation of methanol to formaldehyde (Table 2); (5) As a substrate which protects against the aminotriazole-inhibition of catalase-peroxidative oxidation of methanol to formaldehyde in vitro (Table 2); (6) As an inhibitor of hepatic catalase in vivo (Table 1); and, (7) As a substrate for catalase-peroxide complex I in vivo (Table 1). Aminotriazole inhibits catalase activity by reacting competitively and irreversibly with substrates for catalase-peroxide complex I, such as methanol and ethanol (50,153). Therefore,

if pyrazole interacted directly with catalase or with catalase-peroxide complex I, an inhibition of catalase activity would be expected. If, however, pyrazole served as a substrate for catalase-peroxide complex I, this would be reflected in a protection from aminotriazole-inhibition of catalase activity.

The effect of pyrazole on crystalline beef liver catalase activity is illustrated in Figure 1. Pyrazole did not inhibit catalase activity in the absence of a peroxide-generating system, indicating that pyrazole does not interact directly with hepatic catalase. Also, pyrazole did not significantly interact with catalase-peroxide complex I to produce an inhibition. As expected, aminotriazole exerted an inhibition of catalase activity in this system. This inhibition should have been reversed by pyrazole if pyrazole were a substrate for catalase-peroxide complex I. Figure 1 illustrates that reversal of aminotriazole-inhibition of catalase was not effected by pyrazole. The data in Table 2 show that pyrazole has no inhibitory effect on the peroxidative oxidation of methanol in a purified system containing beef liver catalase and the peroxide generating system, glucose oxidase and glucose. Table 2 also shows the expected aminotriazole-inhibition of methanol oxidation, and the inability of pyrazole to reverse this inhibition, further substantiating that pyrazole is not a substrate for catalase-peroxide complex I.

Aminotriazole produces profound inhibition of catalase

in vivo (151). However, the data presented in Table 1 show that pyrazole (2.94 mmoles/kg) has no inhibitory effect on the activity of hepatic catalase *in vivo* under these conditions. Furthermore, pyrazole exerted no significant reversal of the aminotriazole-inhibition of catalase activity *in vivo*, suggesting again that pyrazole fails to serve as a substrate for catalase-peroxide complex I. The conditions used in these experiments were the same as those used in later studies where the effects of pyrazole on ethanol and methanol metabolism were studied.

Although pyrazole in the doses employed does not influence catalase activity *in vivo* over the relatively short time periods in which the effects of pyrazole were examined, it is, of course, possible that pyrazole might be subject to metabolic alteration, thereby forming at later time periods a metabolite, or metabolites, which could interact with a number of biological systems. Indeed, preliminary studies on the urinary excretion of pyrazole in the rat showed that less than 20 percent of the orally administered dose was excreted either as free or conjugated pyrazole (156). Although Lieber *et al.* (241) have reported decreased hepatic catalase activity 24 hours after administration of an acute dose of pyrazole to rats, this finding has little relevancy to the present work for two reasons: (1) measurements in the present experiments were taken very soon after administration of pyrazole, prior to any significant metabolism of pyrazole; (2) if the metabolites formed from pyrazole were

capable of an aminotriazole-type inhibition of hepatic catalase activity, the presence of ethanol or methanol employed in the present studies would protect catalase from such inhibition, until either alcohol was completely metabolized, thereby delaying the inhibitory effects which may have been observed in the absence of ethanol or methanol.

Once it became apparent that pyrazole would not affect the hepatic catalase-peroxidative system under acute conditions, studies were begun to examine the effect of pyrazole on ethanol and methanol oxidation in the intact rat and monkey. Recent reports on inhibition of ethanol metabolism in the rat in vivo (156,170) were confirmed in the present study, as illustrated in Figure 2. A dose of 2.94 mmoles/kg of pyrazole inhibited ethanol oxidation in the rat by about 80 percent. These results further substantiate the view that ethanol is oxidized in the rat through the ADH pathway.

Methanol oxidation in the intact rat was less affected by pyrazole than was ethanol oxidation (Figure 3). This was to be expected, since others (149) showed that the catalase-peroxidative route is largely responsible for the oxidation of methanol in the rat. In light of the dramatic inhibition of methanol oxidation in the rat associated with aminotriazole administration, it was generally assumed that the catalase-peroxidative route was the sole available metabolic route for this alcohol. Not until the discovery of a relatively selective inhibitor of hepatic ADH, was an effective means available by which to determine the role,

if any, of the ADH pathway in methanol oxidation in the rat. It can be seen in Figure 3 that, during a time period when catalase activity is unaffected by 2.94 mmoles/kg of pyrazole, methanol oxidation was inhibited about 45 percent. Although this is a modest inhibition relative to the effect of pyrazole on ethanol oxidation *in vivo*, it is nevertheless significant, and demonstrates for the first time a participation of the ADH pathway in methanol oxidation in the rat.

A number of compounds are known which exhibit toxicities not due to the parent compound *per se*, but rather as a result of biochemical alteration to a toxic metabolite. Examples of compounds considered to undergo oxidation through the ADH pathway to toxic metabolites are methanol, ethylene glycol, and 2-fluoroethanol. Studies in the rat indicated that pyrazole exerts a strong inhibition of ADH *in vivo*. This prompted the suggestion that such an inhibitor might possess a clinical potential in delaying or preventing toxicities arising in patients which had received dangerous amounts of compounds such as methanol, ethylene glycol and 2-fluoroethanol. Therefore, preliminary studies were performed in rats to investigate further some of the characteristics of pyrazole which might have bearing on possible future clinical applications.

The classical treatment for methanol poisoning in man consists of repetitive administration of ethanol. This is based on the assumption that ADH is primarily responsible for methanol oxidation in man. Ethanol is preferentially

oxidized by the ADH system and it occupies ADH while methanol is excreted in the expired air and urine; thereby preventing the accumulation of metabolites of methanol which are believed responsible for "methanol toxicity". Implicit in this treatment of methanol oxidation is the requirement of high blood ethanol levels due to its rapid metabolism.

In the intact rat, doses of pyrazole (2.94 mmoles/kg) which had produced dramatic inhibition of ethanol oxidation exhibited no apparent toxicities when administered alone, or in combination with ethanol (21.7 mmoles/kg) or methanol (31.3 mmoles/kg). Sequential conditions similar to those ordinarily encountered in the treatment of methanol poisoning were examined (Figures 4 and 5) in which pyrazole (2.94 mmoles/kg) was administered hours after administration of ethanol or methanol. Even when this sequence of substrate-inhibitor administration was employed (Figure 5), pyrazole inhibited oxidation in vivo to about the same extent as observed in experiments when the inhibitor was administered prior to the alcohol (Figures 2 and 3). This was not surprising in light of the proposed mechanism by which pyrazole effects its inhibition of hepatic ADH (155). The formation of the inactive ternary complex (ADH-NAD-Pyrazole) has a "compulsory" order in which pyrazole interacts with the enzyme only after the coenzyme has bound. If one assumes for the moment a specific gravity of 1.0 for the mammal, and that pyrazole is evenly distributed throughout body water, then 2.94 mmoles/kg of pyrazole would be equivalent

to 2.94 mM pyrazole at the site of hepatic ADH. The Ki for pyrazole in rat hepatic ADH-mediated ethanol oxidation is about 4 μM (20). Therefore, under these hypothetical conditions the pyrazole concentration in the animal exceeds the Ki concentration by a factor of about 700. However, it is realized that pyrazole distributes unevenly in body water, and in fact may concentrate in the liver.

Two disadvantages are inherent in the present treatment of methanol poisoning in man. First, it is not desirable to impose the depressant effects of ethanol upon the depressant effects of methanol. Secondly, the suggested repetitive treatment with ethanol for prolonged time periods increases the risk that at some point the inhibition of ADH may not be adequate to prevent methanol oxidation. With the latter disadvantage in mind, experiments were performed to investigate the duration of inhibitory action of pyrazole in vivo (Figure 6). Ethanol oxidation in the rat was maximally inhibited (65 percent) 48 hours after pyrazole administration (Figure 6). Others (156) have reported that under similar conditions, pyrazole inhibition of ethanol oxidation in the rat is detectable as late as 6 days after pyrazole administration. Thus, these studies further supported the sugtestion that an inhibitor of hepatic ADH, such as pyrazole might be employed to advantage in preventing or delaying toxicities from the types of compounds described above.

After the inhibitory effect of pyrazole on ethanol and methanol oxidation was established in the rat, attention

was turned to the oxidation of these alcohols in the monkey. Previous reports had indicated that ethanol is primarily oxidized through the ADH pathway in both the rat (149) and monkey (150). Indeed, pyrazole (2.94 mmoles/kg) inhibited ethanol oxidation in the monkey to about the same extent observed in the rat.

Although the catalase-peroxidative system was shown in the present and earlier studies (149) to comprise the major route for methanol oxidation in the rat, others (150) indicated that its role in the monkey was minimal. The current studies substantiate directly the role of ADH as the primary catalyst for methanol oxidation in the monkey. Figure 7 illustrates the dramatic (about 80 percent) inhibitory effects of pyrazole (2.94 mmoles/kg) on the oxidation of 31.3 mmoles/kg of methanol in the monkey. Although administration of 2.94 mmoles/kg of pyrazole to rats was associated with no apparent toxicities, monkeys receiving this dose of pyrazole with either methanol (31.3 mmoles/kg) or ethanol (21.7 mmoles/kg) exhibited a number of toxic symptoms which often progressed to death. Attempts to characterize the toxicity associated with pyrazole were performed in rats and monkeys and included several standard biochemical and histopathologic procedures. Pronounced hypoglycemia was associated with extremely high doses (14.7 mmoles/kg) of pyrazole in the rat. However, this was not observed in rats or monkeys receiving the doses of pyrazole routinely employed in studies of ADH inhibition <u>in vivo</u>

(0.74-2.94 mmoles/kg). None of the tests employed in the present study were satisfactory indicators of the toxic mechanism of pyrazole. This was consistent with the results of earlier experiments (133) in which pyrazole administered to rats in doses greater than 14.7 mmoles/kg led to coma and death within 12 to 36 hours, however no liver cell alterations were observed microscopically in these animals. In the present studies, the rat and monkey exhibited similar signs and symptoms in response to pyrazole, although the minimal dose necessary to elicit adverse responses in the monkey was about one-fifth of that required in the rat.

A dose of pyrazole lower than that associated with toxicities in monkeys was examined for its capacity to inhibit methanol oxidation in the monkey. It was hoped that a dose of pyrazole could be found which would prove useful in inhibiting methanol oxidation without producing its associated toxic effects. Pyrazole in a dose of 0.74 mmoles/kg (one-fourth the dose associated with toxicity) was found to inhibit methanol oxidation (31.3 mmoles/kg) 50 percent in the monkey (Figure 8). When, during the same experiment, the effect of a low dose of ethanol (5.4 mmoles/kg) was imposed on the existing pyrazole inhibition, methanol oxidation was further inhibited about 80 percent, relative to control methanol oxidation rates. In contrast to the prolonged inhibitory effects of pyrazole, the inhibition of methanol oxidation by a single dose of ethanol was terminated about 3 hours after administration (Figure 8), due to

its rapid metabolism (150 mg/kg/hr). To compare the inhibitory effect on methanol oxidation of the pyrazole-ethanol combination with the effect of ethanol alone, monkeys were administered 5.43 mmoles/kg of ethanol 4 hours after 31.3 mmoles/kg of methanol (Table 3). Methanol oxidation was inhibited about 50 percent during the first hour after ethanol administration similar to the degree of inhibition attributable to ethanol in the ethanol-pyrazole combination (Figure 8). Monkeys administered methanol (31.3 mmoles/kg) + ethanol (5.43 mmoles/kg) + pyrazole (0.74 mmoles/kg) did not display any observable toxicity, in that they behaved normally for at least 1 month, and presented no indication of the toxic manifestations associated with the 2.94 mmoles/kg dose of pyrazole.

An important point was established in the present studies--there is no apparent relationship between the acute inhibitory effects of pyrazole on ADH in vivo and the toxicity associated with high doses of pyrazole. Other derivatives, such as 4-methylpyrazole, appear to possess a more favorable therapeutic index than the parent compound (171). In fact, studies have recently been performed in man using low doses of 4-methylpyrazole and achieving 64 percent inhibition of ethanol-1-^{14}C oxidation with no reported adverse responses to the inhibitor (171).

As would be expected, there appears to be a considerable difference between the adverse effects associated with relatively high acute doses of pyrazole and the frank toxicities

associated with chronic administration of pyrazole alone, or in combination with alcohols. Lelbach (133) has examined liver histology in rats following prolonged ethanol intake and pyrazole administration. A definite hepatotoxic effect due to the pyrazole-ethanol combination was reported, leading to the hypothesis that pyrazole-induced inhibition of ethanol metabolism increased ethanol toxicity. However, this hypothesis was not supported by the findings of Morgan and DiLuzio (237) in which there was an absence of fatty infiltration in acute ethanol-treated rats which had received pyrazole. These latter authors suggested that, in chronic experiments, ethanol may be potentiating the hepatotoxic effects observed when pyrazole is administered in high doses.

During the progress of the present studies, reports appeared in which a "microsomal ethanol oxidizing system" examined *in vitro* was suggested to participate significantly in alcohol oxidation *in vivo* (45,46,210). Considerable debate and attention to mechanistic detail were devoted to this system *in vitro*. However, no detailed consideration nor quantitative determination of the role *in vivo* of this system had been reported. Velocities for ethanol oxidation reactions in this system *in vitro* have been reported by Lieber and DeCarli (45) to be 7.70 nmoles/mg microsomal protein/minute in the untreated male rat, while microsomes prepared from ethanol-pretreated rats oxidized ethanol at a rate of 9.95 nmoles/mg microsomal protein/minute. Microsomes prepared during the course of the present investigation routinely

yielded about 20 mg of microsomal protein per gram (wet weight) of liver. Therefore, approximately 200 nmole of ethanol should be oxidized per gram of liver per minute, or 12,000 nmoles per gram of liver per hour. If one assumes that the liver comprises about 4 percent of the body weight of the rat, and converts to micromoles, one may account for 480 μmoles, or 22 mg/kg/hour. From the foregoing considerations, it would appear that, at most, alcohol oxidation in this microsomal system could theoretically account for only about 7 percent of the total ethanol oxidation rate of the rat. This percentage is based on the knowledge that the normal rate of ethanol oxidation in this species is about 300 mg/kg/hour during periods of linear oxidation rates (156). It was shown in Figure 9 that SKF 525-A does not affect the oxidation *in vivo* of ethanol-1-^{14}C and methanol-^{14}C in the male rat. It should be pointed out that the dose of SKF 525-A employed in these experiments (130 umoles/kg) is sufficient to predictably affect hexobarbital sleeping times and hexobarbital disappearance from the blood (49). SKF 525-A exerts a rather broad range of inhibitory actions on microsomal reactions (238), leading one to expect that if ethanol or methanol were indeed oxidized by a microsomal mixed-function oxidase reaction *in vivo* to any significant extent, treatment by SKF 525-A would have exerted a measurable inhibitory effect.

In Figure 10 it can be seen that treatment of rats with phenobarbital sodium (40 mg/kg) has no effect on the subsequent oxidation of ethanol-1-^{14}C *in vivo*. The N-dealkylation of ethylmorphine in microsomes from rats also treated

with phenobarbital in this manner was increased three-fold beyond that measured in control animals. If a microsomal, mixed-function oxidase reaction were involved in ethanol oxidation *in vivo* to a significant extent, the oxidation of ethanol observed following phenobarbital treatment would have predictably increased. As related earlier, no such increase in ethanol oxidation was observed.

From this series of studies it may be concluded that if ethanol is oxidized by a mixed-function oxidase reaction similar to that participating in the metabolism of many drugs, this system does not play a significant role in alcohol oxidation *in vivo*. This conclusion is based on the conspicuous lack of effect of phenobarbital treatment which usually enhances, and of SKF 525-A treatment which usually inhibits drug oxidation by hepatic microsomes. The possibility exists that microsomal alcohol oxidation may proceed by a reaction mechanism different than that described for drugs. While the main objective of the present experiment was to determine the portion of total alcohol oxidation *in vivo* attributable to the "microsomal ethanol oxidizing system", it was also of interest to consider possible mechanisms which could explain NADPH- and O_2-dependent microsomal ethanol oxidation observed *in vitro*. One such mechanism was based on the above observations and the catalase-peroxidative mechanism described previously. It appears that the microsomal alcohol oxidizing system is composed of a peroxidative reaction where hydrogen peroxide generated from some microsomal oxidase, such as NADPH-

oxidase (239), is supplied to small amounts of residual catalase present in microsomal preparations. If the activity of this system is limited by the rate of peroxide generation, chronic treatment of rats with ethanol or phenobarbital may increase the amount of peroxide generated by the NADPH oxidase system, and could account for observed microsomal alcohol oxidation *in vitro*. That is, enhancement of ethanol oxidation may represent corresponding enhancement of peroxide generation. Experimental support for this view has recently been presented by Roach *et al.* (48), who observed that aminotriazole inhibited microsomal ethanol oxidation, and further, that NADPH can be replaced by a peroxide-generating system. More recently Isselbacher and Carter (51) have reported that microsomes prepared by the usual differential centrifugation techniques contain NADPH oxidase, catalase, and alcohol dehydrogenase. Catalase and ADH were considered contaminants, the activity of which would be decreased by washing procedures. These authors found no evidence for the presence of a distinct microsomal ethanol oxidase, and concluded that observed NADPH- and O_2-dependent microsomal ethanol oxidation results from coupled and combined activities of NADPH oxidase, catalase and alcohol dehydrogenase. It should be reemphasized that the present experiments gave no reason to conclude a significant role in ethanol and methanol oxidation *in vivo* for the "microsomal ethanol oxidizing system".

A number of compounds other than pyrazole and certain derivatives have been reported to serve as effective inhibitors of hepatic ADH, including various amides (1,168,240) and

oximes (1,174). Koe and Tenen (174) have recently reported that n-butyraldoxime effectively inhibits mouse hepatic ADH activity *in vivo* and *in vitro*. In addition, they reported that n-butyraldoxime exerted an inhibitory effect on aldehyde dehydrogenase activity *in vivo*, but not *in vitro*. Lester and Benson (172) have considered the results of Koe and Tenen (174) as supporting the view that n-butyraldoxime and pyrazole inhibit liver ADH by different mechanisms. In the current investigation the potent inhibitory effect of butyraldoxime was confirmed on liver ADH activity *in vitro*, and on ethanol and methanol oxidation in the intact rat and monkey. Further, butyraldoxime (1 mM or less) did not inhibit acetaldehyde oxidation by partially purified rat liver AldDH. This confirms the findings of Koe and Tenen (174). However, it was noted in the course of these experiments that n-butyraldoxime serves as a substrate for hepatic and retinal AldDH. This finding could offer a tentative explanation for some of the characteristics observed with n-butyraldoxime administration *in vivo*. Butyraldoxime probably produces its "Antabuse reaction" (adverse reaction due to increased levels of blood acetaldehyde formed through liver ADH following ethanol administration *in vivo*) at least in part by competing with acetaldehyde for the active site of AldDH, thereby resulting in acetaldehyde accumulation. Further, the inhibitory effect of butyraldoxime on alcohol oxidation *in vivo* has been reported by others (172), and confirmed in the present study, to be of much shorter duration than pyrazole. This

could be accounted for by the possibility that a means of metabolism of this inhibitor of ADH lies in the adjacent enzyme of the ADH pathway.

The studies described in the first portion of this dissertation show directly that alcohol dehydrogenase plays an important role in the oxidation of ethanol and methanol, particularly in the primate. The proposal was made in the present study that certain inhibitors of ADH possess a clinical potential as agents capable of preventing the production of toxic metabolites enzymatically formed through oxidation of a relatively nontoxic parent compound by the ADH-pathway, such as methanol, ethylene glycol or 2-fluoroethanol. In addition, other human applications for inhibitors of ADH have been proposed (156). These compounds may serve as useful conjuctive therapy in the treatment of serious toxicities arising due to a dangerous elevation in acetaldehyde levels following the treatment of alcoholics with disulfiram (Antabuse) or calcium cyanamide. ADH-inhibitors may be of value in the treatment of delerium tremens, by virtue of their capacity to provide a prolonged persistence of the action of alcohol. Many of the metabolic derangements associated with alcoholism have been attributed to alterations in the normal hepatic NAD/NADH ratio. If this is in fact the primary metabolic lesion, it is possible that the use of an ADH inhibitor to control ethanol metabolism may be of partial value in treating the broader disease of alcoholism.

The studies described in this dissertation combined with

earlier work provide a groundwork for reflecting on the relative utilities of pyrazole and n-butyraldoxime. Both compounds inhibit the oxidation *in vivo* of methanol, ethanol, and possibly ethylene glycol and 2-fluoroethanol. Both compounds have been shown to exert an inhibition of hepatic ADH. While pyrazole does not inhibit liver AldDH, butyraldoxime does appear to possess this additional inhibitory effect *in vivo*, possibly by virtue of its ability to function as a substrate. The duration of inhibition by pyrazole far exceeds that of butyraldoxime. Clinical treatment of toxicities exemplified by methanol poisoning would probably indicate the use of long-acting inhibitors of the pyrazole-type. On the other hand, clinical situations may arise which would indicate an inhibition of liver ADH over which more control could be exercised. In this latter case a drug of the butyraldoxime-type would be more appropriate. Neither pyrazole nor butyraldoxime is free of toxic potential in man. At the present time it would be predicted from experiments in primates that pyrazole possesses a higher therapeutic ratio (LD_{50}/ED_{50}) than butyraldoxime. However, this is not meant to indicate that the same situation holds when either of these compounds is administered in combination with alcohols. Indeed, following ethanol administration, butyraldoxime results in an elevation of blood acetaldehyde concentration which may cause a variety of toxic manifestations. Because pyrazole does not inhibit AldDH, no such effect has been associated with this inhibitor during ethanol oxidation *in vivo*. Anal-

agous toxicities should be anticipated when the metabolism of other alcohols is modified by the actions of butyraldoxime-type compounds. The above considerations are of importance in attempting to develop compounds which are both safe and effective in man. Although pyrazole and n-butyraldoxime have proved to be extremely useful experimental tools, it is felt at this time that neither compound should be administered in man. However, certain derivatives of pyrazole and butyraldoxime appear to exert a greater inhibitory effect on hepatic ADH, and possess a lower mammalian toxicity than the parent compounds. These derivatives would be the likely candidates for any future clinical applications of inhibitors of liver ADH.

The latter portion of this dissertation was devoted to describing a series of studies which were prompted by the contemporary and growing use of inhibitors of ADH in man.(171). The decision was made to investigate the effect of these inhibitors on retinal alcohol dehydrogenase activity in order to determine whether they posed a toxic potential by virtue of a capacity to inhibit retinal ADH activity. Until a few years ago it was generally accepted that retinol-retinaldehyde interconversion was mediated by alcohol dehydrogenase (E.C. 1.1.1.1). This concept was based and sustained primarily on observations made using either purified hepatic ADH (82, 84,20) or crude retinal preparations (83,86,87,88). However, evidence has been presented which suggests that the "hepatic type" ADH is absent from the retina, and that retinol is oxidized in the retina and liver by specific and distinct

enzymes (85). The concern in the present investigation was the following: if retinal ADH participates in visual pigment metabolism, and if the retinal enzyme is similar to hepatic ADH, inhibitors of hepatic ADH could interrupt normal visual pigment generation.

Meaningful interpretation of earlier studies dealing with retinal ADH activity was hindered by either the use of hepatic ADH to describe retinal phenomena, or the crude nature of the retinal enzyme preparations employed in those studies. Therefore, it was decided that attempts would be made at purifying retinal ADH activity and examining some of its properties, including its susceptability to inhibitors of hepatic ADH. It was also decided that, if present, retinal AldDH activity should be examined for its possible role in retinol-retinaldehyde metabolism, and because it may interfere in the assay for retinal ADH activity. No reports on purification procedures for retinal ADH or AldDH had been published prior to these studies, therefore necessitating certain analyses or procedures not ordinarily required in the purification of better documented enzymes.

Initial attempts at purification of retinal ADH and AldDH activities were performed on rat retinas. Retinal NAD-dependent oxidation of ethanol (RADH) and NAD-dependent oxidation of acetaldehyde (RAldDH) were found exclusively in the supernatant obtained after centrifugation of the retinal homogenate at 105,000 x g for 1 hour (cytosol). In this respect the soluble nature of RADH and RAldDH were similar to the hepatic

ADH. RADH and RAldDH activities were precipitated with ammonium sulfate (Table 4) and further purified by DEAE-chromatography (Figure 11, Tables 4 and 5), obtaining final purification of 7.4- and 7.3-fold, respectively, relative to the original cytosol preparation.

Separation and purification of monkey RADH and RAldDH activities proceeded similarly to the purification of the rat retinal enzymes (Tables 6 and 7). RADH and RAldDH activities were obtained free of each other in both the rat and monkey.

Once the desired enzymes were prepared in partially purified forms, studies were performed on RADH in which a variety of characteristics were examined, including substrate specificity, and the effect of inhibitors of hepatic ADH. These characteristics were compared with hepatic ADH and are summarized in Table 10. Only enzymes from the rat were incorporated in this table, for, partially purified RADH from the rat and monkey were found to possess no notable differences. Both liver ADH and RADH (Figure 12) were found to possess pH optima of approximately 10.0 under the conditions of the standard assay. Liver ADH appeared to function most effectively as a catalyst of aldehyde reduction, whereas RADH catalyzed alcohol oxidation with greater effectiveness. Both enzymes catalyzed ethanol-oxidation more effectively with NAD than with NADP as a co-substrate. About the only similarity which could be drawn between the reactivities of liver ADH and RADH with a series of straight-chain aliphatic alcohols was that both were progressively more reactive with

increasing carbon-chain length. Three notable differences were found: (1) RADH was unreactive with methanol, whereas liver ADH was reactive with this substrate; (2) with alcohol substrates other than methanol, the Michaelis constants with RADH were between 100 and 1000 times greater than those obtained and reported for the hepatic enzyme; and, (3) RADH exhibited no activity with retinol as substrate, whereas liver ADH did accept retinol as a substrate.

Retinol was recently shown to serve as a substrate for purified rat liver ADH (20), as was confirmed during the present study (Km = 3 μM). Contrary to expectations, all assay procedures employed in the present study indicated that retinol does not interact with RADH as a substrate or as an inhibitor of ethanol oxidation. Initially, these results appeared to be at total variance with the results of Koen and Shaw (85). These authors applied extracts of rat liver and rat retinas to starch gels, and prepared zymograms of alcohol dehydrogenase activity (E.C. 1.1.1.1) and "retinol dehydrogenase" activity (retinol:NAD oxidoreductase). The extracts employed in these studies were prepared by homogenizing in distilled water, repetitive freezing and thawing, and centrifuging at either 10,000 x g (retinas) or 13,000 x g (liver). Under the conditions of these reported experiments, the liver extract was resolved into five dehydrogenase bands, four of which were presumed to be isozymes of hepatic ADH capable of oxidizing ethanol and retinol, and a fifth band ("retinol dehydrogenase 3") oxidized only retinol. Under

the same conditions, electrophoresis of retinal extracts results in the appearance of 4 dehydrogenase bands, only one of which corresponded to the nonspecific hepatic ADH isozyme. Two of these 4 isozymes ("retinol dehydrogenases 1 and 2") were specific for retinol, and the fourth was specific for ethanol. The authors concluded that the oxidation of vitamin A (retinol) proceeds in the retina and liver by specific and different enzymes. In light of the results obtained in the current study, a review of Koen and Shaw's (85) procedures revealed a number of points which could account for the apparent discrepancies between their work and the findings of the present study. First, it must be remembered that prior to these studies, no one had discerned whether the retinal capacity to oxidize ethanol or retinol was mediated by a "soluble" or particulate enzyme. The procedure of centrifuging retinal homogenates at 10,000 x g (for a duration not specified by the authors) is usually not sufficient to yield a supernatant which is free of insoluble homogenate components. Further, application of the resulting suspension to a porous starch gel matrix would not impede the electrophoretic migration of particulate enzymes. Indeed, a review of published zymograms (85) of ADH (substrate: ethanol) and "retinol dehydrogenase" (substrate: retinol) activities show that hepatic "retinol dehydrogenase" (a soluble enzyme) and retinal ADH (a soluble enzyme) both migrate a substantial distance from the origin toward the anode. However, "retinol dehydrogenase" activity from the retina migrated a relatively

short distance from the origin toward the anode. This could be indicative of the particulate state of this enzyme, in that one would not expect the migratory distances of soluble and particulate enzymes to be similar. An additional point should be made that the procedure of submitting relatively crude tissue preparations to electrophoresis, and attempting to define specific dehydrogenases by means of a dehydrogenase activity stain may be hazardous. Many substances in addition to the enzyme under study are included in crude tissue preparations. Occasionally these substances are capable of reducing the dye system intended to define a particular dehydrogenase, resulting in a "false" enzyme activity stain. This interpretation may be avoided by preparing a suitable control in which all reaction components except the substrate for the particular dehydrogenase under study are combined on the gel. "Non-specific" and specific activities can then be differentiated from one another. Such controls were not reported in the report of Koen and Shaw (85), and would have aided in evaluating the significance of the reported dehydrogenase activities. Therefore, the foregoing considerations would not suggest that the report of Koen and Shaw (85) is invalid, but rather that their procedures did not prove the soluble nature of their "retinol dehydrogenase", nor did they disprove the insoluble nature of their "retinol dehydrogenase". The present studies show that the locus of RADH activity is the cytosol fraction, and that the NAD- or NADP-dependent capacity to oxidize retinol to retinaldehyde does not appear in the cytosol fraction.

Rat RADH in the presence of NADH or NADPH was unreactive with formaldehyde and retinaldehyde, and only slightly reactive with acetaldehyde (Table 10). On the other hand, partially purified rat liver ADH under the same conditions (NADH only) was reactive with formaldehyde, acetaldehyde and retinaldehyde.

Three compounds known to inhibit hepatic ADH were examined for their effects on RADH-mediated ethanol oxidation (Table 10). The Ki values for pyrazole and butyraldoxime with liver ADH, and for butyraldoxime with RADH were 4 μM, 5 μM and 2 μM, respectively. This would suggest that butyraldoxime exerts an inhibition of RADH similar to that observed with liver ADH. However, the Ki for pyrazole with RADH was 0.5 mM--more than 100 times the coresponding Ki observed with hepatic ADH. This could indicate an inhibitory mechanism for pyrazole different than the classical pyrazole-Hepatic ADH mechanism discussed by Theorell and Yonetani (155), and further that pyrazole and butyraldoxime may effect inhibition of RADH by different mechanisms. The observation that 1,10-phenanthroline possessed no inhibitory effect on RADH activity until extremely high inhibitor concentrations were employed would suggest one of the following possible explanations: (a) RADH may contain no zinc; (b) RADH may contain zinc which does not participate in the reactions under study; and, (c) RADH may contain zinc which participates in the catalytic oxidation of ethanol, but is somehow protected from the chelating capacity of 1,10-phenanthroline. A separ-

ate study devoted to determining the presence and role of zinc in the catalytic function of RADH would be required to determine which of these possibilities apply. However, the observations that, relative to liver ADH, RADH activity proceeds rather unfavorably; and that over 100 times the concentration of pyrazole required to inhibit liver ADH is necessary to inhibit RADH, are suggestive of either possibility (a) or (b) presented above.

Analagous to the observations on RADH, partially purified retinal aldehyde dehydrogenase (RAldDH) was unreactive with the one-carbon substrate (formaldehyde) and was reactive with acetaldehyde (Figure 17), although this reactivity was much lower than that routinely observed with hepatic AldDH.

Two observations primary to the theme of the present studies were made concerning rat and monkey RADH: (1) RADH is unreactive with methanol; and, (2) RADH is unreactive with retinol. These observations suggested that RADH is not involved in producing toxic metabolites proposed to be responsible for retinopathies associated with methanol poisoning. In addition, although inhibitors of liver ADH were observed to possess an inhibitory effect on RADH, the consequences were obviously of little significance because RADH was apparently not a participant in the metabolism of retinol or retinaldehyde. Therefore, it was decided to investigate rat and bovine retinal homogenates for their capcity to engage in the oxidation-reduction of retinol or retinaldehyde.

These homogenates were effective catalysts in mediating the oxidation of retinol (Table 9). Experiments employing

standard centrifugation and sucrose gradient techniques (236) showed that the retinal pyridine nucleotide-dependent capacity to oxidize retinol is located in a particulate subcellular organelle, probably in the outer segments. The results of these studies indicate that the terminologoy of others (85) applied to this activity ("retinol dehydrogenase" = retinol: NAD oxidoreductase) would be more correct if changed to retinol dehydrogenase = retinol:NADP oxidoreductase. That is, NADP was found to function about twice as effectively as NAD in serving as the co-substrate for this activity.

Based on the results of these studies, a number of comparisons may be drawn between the particulate retinol dehydrogenase activity, RADH and LADH (Table 10). The pH optimum of retinol dehydrogenase (approximately pH 7.5) was significantly different from the pH optima of RADH and hepatic ADH (approximately pH 10). Retinol dehydrogenase, in the presence of NAD or NADP, was unreactive with methanol, ethanol, 1-propanol and 1-butanol. This was evidenced by the fact that none of these alcohols were observed to undergo oxidation under the conditions employed, nor did ethanol or methanol significantly influence retinol oxidation under these conditions (Table 9). Furthermore, in the presence of NADH or NADPH, retinol dehydrogenase was unreactive with formaldehyde or acetaldehyde. Whereas RADH was unreactive with retinol (+ NAD) or retinaldehyde (+ NADH), retinol dehydrogenase was relatively specific in oxidizing and reducing these two forms of vitamin A, and was not influenced by the

presence of high concentrations of pyrazole (Table 9).

It seemed reasonable that, of the two retinal enzyme activities under investigation, RADH and retinol dehydrogenase, the latter was most probably of physiologic importance by virtue of its apparent participation in retinol-retinaldehyde metabolism. The possibility existed that RADH may merely represent an artifactual phenomenon resulting from manipulation in vitro of the retina. It was reasoned that if this were indeed the case, then varying degrees of tissue trauma should yield varying cytosol concentrations of RADH. Experiments were performed to measure the yield of RADH from whole retinas or retinal particulate fractions which had been purposely homogenized for different time durations with manual or mechanical tissue homogenizers. The effects of repetitive freezing and thawing, and sonication were also evaluated. Even the most severe of these treatments failed to result in a significant change in the levels of RADH. Furthermore, the treatments described did not measurably release retinol dehydrogenase into the buffered media. These experiments strongly suggest that RADH exists in the normal retinal cell as an enzyme distinctly different from the particulate retinol dehydrogenase.

These kinetic studies show that in the retina, there is a soluble NAD-dependent alcohol dehydrogenase which does not participate in the oxidation of retinol or methanol, but does catalyze the enzymatic oxidation of ethanol, 1-propanol, and 1-butanol, and is subject to inhibition by compounds known to inhibit hepatic ADH. Also, there is in the retina,

a particulate dehydrogenase, retinol dehydrogenase, which participates in NADP-dependent retinol-retinaldehyde metabolism and is not influenced by the presence of high concentrations of methanol, ethanol, formaldehyde, acetaldehyde or pyrazole.

On the basis of these studies it would appear unlikely that administration of inhibitors of hepatic ADH would influence visual pigment generation by virtue of inhibiting the dehydrogenase responsible for retinol-retinaldehyde interconversion. Furthermore, studies presented in the earlier portion of this dissertation showed directly that ADH is of substantial significance in the oxidation of methanol and ethanol in the rodent and particularly the primate. Once inhibitors of hepatic ADH are found which exhibit relatively low toxic potential, these compounds may be of use in treating or examining metabolic toxicities associated with a wide variety of exogenous or endogenous alcohols.

SUMMARY

This dissertation shows the participation of alcohol dehydrogenase (ADH) in the oxidation of methanol in the rat and monkey. The activity of this enzyme was studied *in vivo* by measuring the influence of well-defined inhibitors of purified hepatic ADH, such as pyrazole, on the rate of methanol and ethanol metabolism in intact animals. Pyrazole did not influence other known enzymic systems concerned with alcohol oxidation *in vivo* during the time intervals investigated.

In the monkey, pyrazole dramatically inhibited the oxidation of ethanol and methanol. In the rat, pyrazole exerted a profound inhibition of ethanol oxidation and a modest, yet significant inhibition of methanol oxidation. These results support the concept that hepatic ADH is the major catalyst in ethanol and methanol oxidation in the monkey, and ethanol oxidation in the rat. Although the catalase-peroxidative system may still be considered the major oxidative route for methanol in the rat, these studies show that the hepatic ADH system participates in this process.

Pyrazole in doses which exhibited no apparent toxicity in rats was seriously toxic in monkeys. Standard tests for toxicity did not define the mechanism of pyrazole toxicity in the rat or monkey. No apparent relationship exists between the acute inhibitory effects of pyrazole on ADH *in vivo* and the toxicity associated with high doses of pyrazole. A combination of ethanol and pyrazole at doses which exhibited no apparent toxicity to the monkey served to inhibit methanol oxidation to a degree observed with either compound employed alone in

higher doses, suggesting possible clinical applications in the treatment of methanol poisoning in man. Also of clinical significance were the observations *in vivo* that pyrazole is an effective inhibitor of alcohol oxidation when administered after the alcohol; and, that pyrazole exhibits prolonged inhibitory effects.

Studies were conducted to determine whether a "microsomal ethanol oxidizing system" observed *in vitro* plays a significant role in the oxidation of alcohols *in vivo*. No role for this system could be demonstrated in the rat, as shown by experiments *in vivo* in which treatment with SKF 525-A which inhibits, and phenobarbital induction, which enhances microsomal hydroxylations did not alter alcohol oxidation.

The second part of this dissertation was devoted to studies on the role of retinal ADH (RADH) in the metabolism of alcohols and vitamin A. Studies were performed to assess the effect of inhibitors of hepatic ADH on RADH activity and vitamin A metabolism. It was determined that RADH does not participate in the interconversion of retinol and retinaldehyde. Such interconversion is, however, mediated by an enzyme separate and distinct from RADH and hepatic ADH, and which is located in the particulate fraction of retinal homogenates.

Studies showed that RADH resembles hepatic ADH (E.C. 1.1.1.1) in that both enzymes are located in cytosol, are NAD-dependent, exhibit similar pH optima, are reactive with ethanol, 1-propanol and 1-butanol, and are inhibited by pyrazole and n-butyraldoxime. Despite these qualitative similarities between RADH and liver ADH activities, Michaelis

constants and inhibitor constants were many-fold greater for RADH than liver ADH. Also, RADH differed from hepatic ADH in that RADH is unreactive with retinol, retinaldehyde and methanol; is only slightly reactive with acetaldehyde; and is not inhibited by concentrations of 1,10-phenanthroline which inhibit hepatic ADH.

Retinol dehydrogenase activity located in the particulate fraction of retinal homogenates was shown to exhibit a pH optimum different from RADH or hepatic ADH; was more reactive with NADP than NAD; and was unreactive with methanol, ethanol, 1-propanol, 1-butanol, formaldehyde and acetaldehyde. Furthermore, this enzyme was reactive with retinol (oxidation) and retinaldehyde (reduction) and neither of these activities were inhibited by high concentrations of pyrazole, ethanol or methanol.

Therefore, neither RADH nor retinol dehydrogenase would be expected to participate in the retinal oxidation of methanol. Furthermore, it is unlikely that administration of inhibitors of hepatic ADH would exert an effect on retinol-retinaldehyde interconversion.

Although a well-defined toxicologic role of hepatic alcohol dehydrogenase has emerged, no definitive statement can be made at this time for alcohol dehydrogenase activity in other organs.

BIBLIOGRAPHY

1. Sund, H. and Theorell, H., in Boyer, P. D., Lardey, A. A., and Myrbäck, K. (eds.) The Enzymes, 7:25 (1963), New York, Academic Press.

2. Raskin, N. H., and Sokoloff, L., Science, 162: 131 (1968).

3. Von Wartburg, J. P., Papenberg, J., and Aebi, H., Can. J. Biochem., 43: 889 (1965).

4. Mistilis, S. P., and Birchall, A., Nature, 223: 199 (1969).

5. Schmiedeberg, O., Arch. Exptl. Pathol. Pharmakol. Naunyn-Schmiedeberg's, 14: 288 (1881).

6. Jaquet, A., Arch. Exptl. Pathol. Pharmakol. Naunyn-Schmiedeberg's, 29: 386 (1892).

7. Pohl, J., Arch. Exptl. Pathol. Pharmakol. Naunyn-Schmiedeberg's, 31: 281 (1893).

8. Battelli, F., and Stern, L., Compt. rend. soc. biol., 67: 419 (1909).

9. Battelli, F., and Stern, L., Biochem. Z., 28: 145 (1910).

10. Anderson, B., Z. physiol. Chem., 217: 186 (1933).

11. Müller, D., Biochem. Z., 262: 239 (1933).

12. Von Euler, H., Adler, E., and Hellström, H., Z. physiol. Chem., 241: 239 (1936).

13. Negelein, E., and Wulff, H. J., Biochem. Z., 289: 436; 290: 445; 293: 351 (1937).

14. Lutwak-Mann, C., Biochem. J., 32: 1364 (1938).

15. Theorell, H., The Harvey Lectures, 61: 17 (1967).

16. Bonnichsen, R. K., and Wassen, A. M., Arch. Biochem. Biophys., 18: 361 (1948).

17. Dalziel, K., Acta. Chem Scand., 12: 459 (1958).

18. Von Wartburg, J. P., Bethune, J. L., and Vallee, B. L., Biochemistry, 3: 1775 (1964).

19. Makar, A. B., and Mannering, G. J., Mol. Pharmacol., 4: 484 (1968).

20. Reynier, M., Acta Chem. Scand., 23: 1119 (1969).

21. Backlin, K.-I., Acta Chem. Scand., 12: 1279 (1958).

22. Lundquist, F., in Radouco-Thomas, C. (ed.) International Encyclopedia of Pharmacology and Therapeutics, Sec. 20: 1: 95 (1970), New York, Pergamon Press.

23. Lester, D., Quart. J. Stud. Alc., 22: 554 (1961).

24. Shore, J. D., and Theorell, H., Arch. Biochem. Biophys., 117: 375 (1966).

25. Ungar, F., Acta Endocrinol. Suppl., 51: 727 (1960).

26. Ungar, F., Goldstein, M., and Kao, C. M., Steroids, Suppl. 1: 131 (1965).

27. Graves, J. M. H., Clark, A., and Ringold, H. J., Biochemistry, 4: 2655 (1965).

28. Waller, G. R., Theorell, H., and Sjövall, J., Archs. Biochem. Biophys., 111: 671 (1965).

29. Reynier, M., Theorell, H., and Sjövall, J., Acta Chem. Scand., 23: 1130 (1969).

30. Pietruszko, R., Clark, A., Graves, J. M. H., and Ringold, H.J., Biochem. Biophys. Res. Commun., 23: 526 (1966).

31. Theorell, H., Taniguchi, S., Åkeson, Å., and Skursky, L., Biochem. Biophys. Res. Commun., 24: 603 (1966).

32. Treble, D. H., Biochem. J., 82: 129 (1962).

33. Theorell, H., and Bonnichsen, R. K., Acta Chem Scand., 5: 1105 (1951).

34. Merritt, A. D., and Tomkins, G. M., J. Biol. Chem., 234: 2778 (1959).

35. Kini, M. M., and Cooper, J. R., Biochem. Pharmacol., 8: 207 (1961).

36. Theorell, H., and Chance, B., Acta Chem Scand., 5: 1127 (1951).

37. Mourad, N., and Woronick, C. L., Arch. Biochem. Biophys., 121: 431 (1967).

38. Blair, A. H., and Vallee, B. L., Biochemistry, 5: 2026 (1966).

39. Von Wartburg, J. P., and Schürch, P. M., Ann. N. Y. Acad. Sci., 151: 936 (1968).

40. McKinley-McKee, J. S., and Moss, D. W., Biochem. J., 96: 583 (1965).

41. Keilin, D., and Hartree, E. F., Proc. Roy. Soc. B., 119: 141 (1936).

42. Keilin, D., and Hartree, E. F., Biochem. J., 39: 293 (1945).

43. Chance, B., Acta Chem. Scand., 1: 236 (1947).

44. Orme-Johnson, W. H., and Ziegler, D. M., Biochem. Biophys. Res. Commun., 21: 78 (1965).

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

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

47. Roach, M. K., Reese, W. N., Jr., and Creaven, P. J., Fed. Proc., 28: 546 (1969).

48. Roach, M. K., Reese, W. N., Jr., and Creaven, P. J., Biochem. Biophys. Res. Commun., 36: 596 (1969).

49. Mannering, G. J., in Tedeschi, D. H., and Tedeschi, R. E. (eds.) Importance of Fundamental Principles In Drug Evaluation, 105 (1968), New York, Raven Press.

50. Margoliash, E., Novogrodsky, A., and Schejter, A., Biochem. J., 74: 339 (1960).

51. Isselbacher, K. J., and Carter, E. A., Biochem. Biophys. Res. Commun., 39: 539 (1970).

52. Holzer, H., and Schneider, S., Klin. Wachschr., 33: 1006 (1955).

53. Holzer, H., Schneider, S., and Lange, K., Angew. Chem., 67: 276 (1955).

54. Dorfman, R. I., and Ungar, F., Metabolism of Steroid Hormones, (1965), New York, Academic Press.

55. Grangaud, R., Nicol, M., Jovan, P., and LeGall, J. Y., Compt. Rend. Soc. Biol., 157: 2173 (1963).

56. Grangaud, R., and Nicol, M., Compt. Rend. Acad. Sci. Paris, 262: 2640 (1966).

57. Juneja, H. S., Murthy, S. K., and Ganguly, J., Biochem. J., 99: 138 (1966).

58. Maxwell, E. S., and Topper, Y. J., J. Biol. Chem., 236: 1032 (1961).

59. Maxwell, E. S., J. Biol. Chem., 237: 1699 (1962).

60. Elder, T. D., and Topper, Y. J., Biochem. Biophys. Acta, 64: 430 (1962).

61. Christophe, J., and Popják, G., J. Lipid Res., 2: 244 (1961).

62. Waller, G. R., Nature, Lond., 207: 1389 (1965).

63. Gustafsson, J.-Å., Sjövall, J., and Theorell, H., (unpublished results).

64. Gillette, J. R., J. Biol. Chem., 234: 139 (1959).

65. Moir, N. J., Miller, W. L., and Gaylor, J. L., Biochem. Biophys. Res. Commun., 33: 916 (1968).

66. Snyder, F., and Blank, M. L., Arch. Biochem. Biophys., 130: 101 (1969).

67. Gershbein, L. L., and Singh, E. J., J. Amer. Oil Chem. Soc., 46: 34 (1969).

68. Blank, M. L., and Snyder, F., Lipids, 5: 337 (1970).

69. Gilbertson, J. R., Ferrell, W. J., and Gelman, R. A., J. Lipid Res., 8: 38 (1967).

70. Ferrell, W. J., Radloff, J. F., and Jackiw, A. B., Lipids, 4: 278 (1968).

71. Goldfine, H., in Boyer, P. D. (ed.) Annual Review of Biochemistry, 37: 303 (1968), Palo Alto, Annual Reviews, Inc.

72. Radloff, J. F., and Ferrell, W. J., Physiol. Chem. And Physics, 2: 105 (1970).

73. Ferrell, W. J., and Gilbertson, J. R., Proc. W. Va. Acad. Sci., 39: 263 (1967).

74. Snyder, F., Malone, B., and Nykle, R. L., Biochem. Biophys. Res. Commun., 34: 40 (1969).

75. Snyder, F., and Pfleger, R. C., Lipids, 1: 328 (1966).

76. Pfleger, R. C., Piantadosi, C., and Snyder, F., Biochem. Biophys. Acta, 144: 633 (1967).

77. Tietz, A., Lindberg, M., and Kennedy, E.P., J. Biol. Chem., 239: 4081 (1964).

78. Ferrell, W. J., and Radloff, J. F., Bioscience, 19: 243 (1969).

79. Ellingboe, J., and Karnovsky, M. L., J. Biol. Chem., 242: 5693 (1967).

80. Ball S., Glover, J., Goodwin, T., and Morton, R., Biochem. J., 41: XXIV (1947).

81. Bliss, A. F., Biol. Bull., 97: 221 (1949).

82. Bliss, A. F., Arch. Biochem. Biophys., 31: 197 (1951).

83. Wald, G., and Hubbard, R., J. Gen. Physiol., 32: 367 (1949).

84. Zachman R. D., and Olson, J. A., J. Biol. Chem., 236: 2309 (1961).

85. Koen, A. L., and Shaw, C. R., Biochem. Biophys. Acta, 128: 48 (1966).

86. Wald, G., and Hubbard, R., in Boyer, P. D., Lardy, H., and Myrbäck, K. (eds.) The Enzymes, 3: 369 (1960), New York, Academic Press.

87. Reading, H. W., and Sorsby, A., Biochem. J., 99: 3c (1966).

88. Muirhead, J. F., Invest. Ophthal., 6: 635 (1967).

89. Futterman, S., J. Biol. Chem., 237: 677 (1963).

90. Glover, J., Goodwin, T. W., and Morton, R. A., Biochem. J., 43: 109 (1948).

91. Fidge, N. H., and Goodman, D. S., Fed. Proc., 26: 849.

92. Culp, H. W., and McMahon, R. E., J. Biol. Chem., 243: 848 (1968).

93. Ganguly, J., J. Sci. Ind. Res., 26: 110 (1967).

94. Grangaud, R., and Nicol, M., Compt. Rend. Acad. Sci. Paris, 263: 446 (1966).

95. Grangaud, R., Nicol, M., and Delauney, J., Compt. Rend. Acad. Sci. Paris, 260: 2339 (1965).

96. Grangaud, R., Nicol, M., Legall, J. Y., and Delauney, J., Arch. Sci. Physiol., 20: 123 (1966).

97. Olson, J. A., Pharm. Rev., 19: 559 (1967).

98. Wolf, G., and Varandani, P., Biochem. Biophys. Acta, 43: 501 (1960).

99. Rogers, W. E., Jr., Fed. Proc., 26: 636 (1967).

100. Johnson, B. C., Abs. Amer. Chem. Soc. 152nd, Sept. 1966, New York, C-277.

101. Yonemoto, T., and Johnson, B. C., Fed. Proc., 26: 635 (1967).

102. Subba Rao, K., and Olson, R. E., Fed. Proc., 26: 635 (1967).

103. Wiss, O., and Gloor, U., Vitam. and Horm., 18: 485 (1960).

104. Rogers, W. E., Jr., Amer. J. Clin. Nutr., 22: 1003 (1969).

105. Kean, E. L., J. Lipid Res., 11: 248 (1970).

106. McManus. I. R., Contag. A. O.. and Olson, R. E., Science, 131: 102 (1960).

107. McManus, I. R., Contag, A. O., and Olson, R. E., J. Biol. Chem., 241: 349 (1966).

108. Krebs, H. A., and Perkins, J. R., Biochem. J., 118: 635 (1970).

109. Lester, D., Quart. J. Stud. Alc., 22: 554 (1961).

110. Lundquist, F., and Wolthers, H., Acta Pharmacol. et Toxicol., 14: 265 (1958).

111. Juni, E., and Heym, G. A., J. Biol. Chem., 218: 365 (1956).

112. Pihl, A., and Fritzson, P., J. Biol. Chem., 215: 345 (1955).

113. Braunstein, A. E., and Vilenkina, G. Y., Dokl. Akad. Nauk. SSS R, 66: 243 (1949).

114. Racker, E., J. Biol. Chem., 196: 347 (1952).

115. Ritchie, J. M., in Goodman, L. S., and Gilman, A. (eds.) The Pharmacological Basis of Therapeutics, IV Edition: 135 (1970), New York, Macmillan.

116. Raaflaub, J., Experientia, 22: 258 (1966).

117. McDaniel, H. G., Podgainy, H., and Bressler, R., J. Pharmacol. Exp. Ther., 167: 91 (1969).

118. Friedman, P. J., and Cooper, J. R., J. Pharmacol. Exp. Therap., 129: 373 (1960).

119. Mackay, F. J., and Cooper, J. R., J. Pharmacol. Exp. Therap., 135: 271 (1962).

120. Cooper, J. R., and Friedman, P. J., Biochem. Pharmacol., 1: 76 (1958).

121. Blake, D. A., Rozman, R. S., Cascorbi, H. F., and Krantz, J. C., Jr., Biochem. Pharmacol., 16: 1237 (1967).

122. Van Dyke, R. A., and Chenoweth, M. B., Anesthesiology, 26: 348 (1965).

123. Parke, D. V., The Biochemistry of Foreign Compounds, 212 (1968), New York, Pergamon Press.

124. Goldstein, A., Aronow, L., and Kalman, S. M., Principles of Drug Action, (1969), New York, Hoeber Medical Division, Harper and Row.

125. Isselbacher, K. J., and Greenberger, N. J., New Eng. J. Med., 270: 351,402 (1964).

126. Isselbacher, K. J., and Krane, S. M., J. Biol. Chem., 236: 2394 (1961).

127. Forsander, O. A., Räihä, N., Salaspuro, M., and Mäenpää, P., Biochem. J., 94: 259 (1965).

128. Lindros, K. O., and Aro, H., Ann. Med. exp. Fenn., 47: 39 (1969).

129. Forsander, O. A., Biochem. J., 98: 244 (1966).

130. Forsander, O. A., in Radouco-Thomas, C. (ed.) International Encyclopedia of Pharmacology and Therapeutics, Sec. 20: 1: 117 (1970), New York, Pergamon Press.

131. Lieber, C. S., Leevy, C. M., Stein, S. W., George, W. S., Cherrick, G. R., Abelmann, W. H., and Davidson, C. S., J. Lab. Clin. Med., 59: 826 (1962).

132. Bustos, O. G., Kalant, H., Khanna, J. M., and Loth, J., Science, 168: 1598 (1970).

133. Lelbach, W. K., Experientia, 25: 16 (1969).

134. Madison, L. L., Lochner, A., and Wulff, J., Diabetes, 16: 252 (1967).

135. Freinkel, N., Cohen, A. K., Arky, R. A., and Foster, A. E., J. Clin. Endocrinol. Metab., 25: 76 (1965).

136. Krebs, H. A., Freedland, R. A., Hems, R., and Stubbs, M., Biochem. J., 112: 117 (1969).

137. Williamson, J. R., Scholz, R., Browning, E. T., Thurman, R. G., and Fukami, M. M., J. Biol. Chem., 244: 5044 (1969).

138. Lumeng, L., and Davis, E. J., J. Biol. Chem., 245: 3179 (1970).

139. Frieden, C., J. Biol. Chem., 234: 809 (1959).

140. Häkkinen, H.-M., Kulonen, E., and Wallgren, H., Biochem. J., 98: 488 (1963).

141. Häkkinen, H.-M., and Kulonen, E., Biochem. J., 78: 588 (1961).

142. Flock, E. V., Tyce, G. M., and Owen, C. A., Jr., Proc. Soc. Exp. Biol. Med., 131: 214 (1969).

143. Feldstein, A., Hoagland, H., Freeman, H., and Williamson, D., Life Sci., 6: 53 (1967).

144. Davis, V., Brown, H., Huff, J., and Cashaw, J., J. Lab. Clin. Med., 69: 132 (1967).

145. Cronholm, T., and Sjövall, J., Eur. J. Biochem., 13: 124 (1970).

146. Lundquist, F., Tygstrup, N., Winkler, K., Mellemgaard, K., and Munck-Petersen, S., J. Clin. Invest., 41: 955 (1962).

147. Hunt, R., Bull. Johns Hopkins Hosp., 13: 213 (1902).

148. Gilger, A. P., and Potts, A. M., Am. J. Ophthal., 39: 63 (1955).

149. Tephly, T. R., Parks, R. E., Jr., and Mannering, G. J., J. Pharmacol. Exp. Therap., 143: 292 (1964).

150. Makar, A. B., Tephly, T. R., and Mannering, G. J., Mol. Pharmacol., 4: 471 (1968).

151. Heim, W. G., Appleman, D., and Pyfrom, H. T., Science, 122: 693 (1955).

152. Mannering, G. J., and Parks, R. E., Jr., Science, 126: 1241 (1957).

153. Tephly, T. R., Mannering, G. J., and Parks, R. E., Jr., J. Pharmacol. Exp. Therap., 134: 77 (1961).

154. Mannering, G. J., Van Harken, D. R., Makar, A. B., Tephly, T. R., Watkins, W. D., and Goodman, J. I., Ann. N. Y. Acad. Sci., 168: 265 (1969).

155. Theorell, H., and Yonetani, T., Biochem. Z., 338: 537 (1963).

156. Lester, D., Keokosky, W. Z., and Felzenberg, F., Quart. J. Stud. Alc., 29: 449 (1968).

157. Gessner, P. K., Parke, D. V., and Williams, R. T., Biochem. J., 79: 482 (1961).

158. Holzer, H., Goedde, H. W., and Schneider, S., Biochem. Z., 327: 245 (1955).

159. Peterson, D. I., Peterson, J. E., Hardinge, M. G., and Wacker, W. E. C., J. Am. Med. Assoc., 186: 955 (1963).

160. Van Harken, D. R., Role of the Hepatic Catalase Peroxidative System in the Metabolism of Methanol by the Rat, Ph.D. Thesis, Dept. Pharmacol., Univ. Minnesota, 1965.

161. Makar, A. B., Methanol Metabolism in the Monkey, Ph.D. Thesis, Dept. Pharmacol., Univ. Minnesota, 1966.

162. Kun, E. J., J. Biol. Chem., 194: 603 (1952).

163. Kun, E. J., Dechary, J. M., and Pitot, H. C., J. Biol. Chem., 210: 269 (1954).

164. Peters, R. A., Proc. Roy. Soc. B., 139: 143 (1952).

165. Baron, E. S. G., and Levine, S., Arch. Biochem. Biophys., 41: 175 (1952).

166. Blake, D. A., Cascorbi, H. F., Rozman, R. S., Meyer, F. J., Toxic. Appl. Pharmacol., 15: 83 (1969).

167. Theorell, H., Experientia (Basel), 21: 553 (1965).

168. Theorell, H., Yonetani, T., and Sjöberg, B., Acta Chem. Scand., 23: 255 (1969).

169. Li, T., and Theorell, H., Acta Chem. Scand., 23: 892 (1969).

170. Goldberg, L., and Rydberg, U., Biochem. Pharmacol., 18: 1749 (1969).

171. Blomstrand, R., and Theorell, H., Life Sci., 9: 631 (1970).

172. Lester, D., and Benson, G. D., Science, 169: 282 (1970).

173. Lewis, W., and Schwartz, L., Med. Ann. D. C., 25: 485 (1956).

174. Koe, B. K., and Tenen, S. S., J. Pharmacol. Exp. Ther., 174: 434 (1970).

175. Deitrich, R. A., and Hellerman, L., J. Biol. Chem., 238: 1683 (1963).

176. Forsander, O. A., Biochem. Pharmacol., 19: 2131 (1970).

177. Edwards, J. A., and Price, J., Nature, 214: 190 (1967).

178. Gelder, M. G., and Edwards, G., Br. J. Psychiat., 114: 473 (1968).

179. Khouw, L. B., Burbridge, T. N., and Sutherland, V. C., Biochem. Biophys. Acta, 73: 173 (1963).

180. Perlman, R. L., and Wolff, J., Science, 160: 317 (1968).

181. Larsen, J. A., and Madsen, J., Proc. Soc. Exptl Biol. Med., 109: 120 (1962).

182. Fitzgerald, M. G., Gaddie, R., Malins, J. M., and O'Sullivan, D. J., Diabetes, 11: 40 (1962).

183. Flitman, R., and Worth, M. H., Jr., J. Biol. Chem., 241: 669 (1966).

184. Gilleland, M. J., and Shore, J. D., J. Biol. Chem., 244: 5357 (1969).

185. McCarthy, K., and Lovenberg, W., J. Biol. Chem., 244: 3760 (1969).

186. Racker, E., J. Biol. Chem., 177: 883 (1949).

187. Lundquist, F., Svendsen, I., and Petersen, P. H., Biochem. J., 86: 119 (1963).

188. Kraemer, R. J., and Deitrich, R. A., J. Biol. Chem., 243: 6402 (1968).

189. Blair, A. H., and Bodley, F. H., Can. J. Biochem., 47: 265 (1969).

190. Strittmatter, P., and Ball, E. G., J. Biol. Chem., 213: 445 (1951).

191. Albers, R. W., and Koval, G. K., Biochem. Biophys. Acta, 52: 29 (1961).

192. Embree, L. J., and Albers, R. W., Biochem. Pharmacol., 13: 1209 (1964).

193. Deitrich, R. A., Hellerman, L., and Wein, J., J. Biol. Chem., 237: 560 (1962).

194. Robbins, J. H., Arch. Biochem. Biophys., 114: 585 (1966).

195. Weissbach, H., Redfield, B. G., and Udenfriend, S., J. Biol. Chem., 229: 953 (1957).

196. Weissbach, H., Goodwin, F., and Maxwell, E. S., Biochem. Biophys. Acta, 49: 384 (1961).

197. Kapeller-Adler, R., and Fletcher, M., Biochem. Biophys. Acta. 33: 1 (1959).

198. Leeper, L. C., Weissbach, H., and Udenfriend, S., Arch. Biochem. Biophys., 77: 417 (1958).

199. Fellman, J. H., Biochem. Biophys. Acta, 35: 530 (1959).

200. Truitt, E. B., Jr., Bell, F. K., and Krantz, J. C., Jr., Quart. J. Stud. Alc., 17: 594 (1956).

201. Rehak, M. J., and Truitt, E. B., Jr., Quart. J. Stud. Alc., 19: 399 (1958).

202. Beer, C. T., and Quastel, J. H., Canad. J. Biochem., 36: 531 (1958).

203. Christopherson, B. O., Biochem. Biophys. Acta, 86: 14 (1964).

204. Blaschko, H., Pharmacol. Rev., 4: 415 (1952).

205. Carlsson, A., and Hillarp, N. A., Acta Physiol. Scand., 55: 95 (1962).

206. Erwin, V. G., and Deitrich, R. A., J. Biol. Chem., 241: 3533 (1966).

207. Tabakoff, B., and Erwin, V. G., J. Biol. Chem., 245: 3263 (1970).

208. Larsen, V., Acta Pharm. Tox., 4: 321 (1948).

209. Hald, J., Jacobsen, E., and Larsen, V., Acta Pharm. Tox., 8: 329 (1952).

210. Rubin, E., and Lieber, C. S., Science, 162: 690 (1968).

211. Maling, H. M., in Radouco-Thomas, C. (ed.) International Encyclopedia of Pharmacology and Therapeutics, Sec. 20: 1: 277 (1970), New York, Pergamon Press.

212. Leake, C. D. (ed.) Biological Actions of Dimethyl Sulfoxide, Ann. N. Y. Acad. Sci., Art. 1: 1 (1967).

213. Huff, E., and Rudney, H., J. Biol. Chem., 234: 1060 (1959).

214. Mallia, A. K., and Cama, H. R., Anal. Biochem., 30: 86 (1969).

215. Feinstein, R. N., J. Biol. Chem., 180: 1197 (1949).

216. Von Euler, H., and Josephson, K., Ann. Chem. Liebigs, 452: 158 (1927).

217. Goodman, J. I., and Tephly, T. R., Mol. Pharmacol., 4: 492 (1968).

218. Nash, T., Biochem. J., 55: 416 (1953).

219. Horecker, B. L., and Kornberg, A., J. Biol. Chem., 175: 383 (1948).

220. Futterman, S., and Saslow, L. D., J. Biol. Chem., 236: 1652 (1961).

221. Gupta, N. K., and Robinson, W. G., Biochem. Biophys. Acta, 118: 431 (1966).

222. Huff, E., Biochem. Biophys. Acta, 48: 506 (1961).

223. Jones, L. R., and Riddick, J. A., Anal. Chem., 29: 1214 (1957).

224. Dietz, A. A., and Lubrano, T., Anal. Biochem., 20: 246 (1967).

225. Davis, B. J., Ann. N. Y. Acad. Sci., 121: 404 (1964).

226. Chrambach, C., Reisfeld, R. A., Wyckoff, M., and Zaccari, J., Anal. Biochem., 20: 150 (1967).

227. Wilkinson, G. N., Biochem. J., 80: 324 (1961).

228. Cleland, W. W., Nature, 198: 463 (1963).

229. Gornall, A. G., Bardwill, C. S., and David, M. M., J. Biol. Chem., 177: 75 (1949).

230. Warburg, O., and Christian, W., Biochem. Z., 310: 384 (1941).

231. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193: 265 (1951).

232. Gerritsen, G. C., Dulin, W. E., J. Pharmacol. Exp. Ther., 150: 491 (1965).

233. Gerritsen, G. C., Dulin, W. E., Diabetes, 14: 507 (1965).

234. Keston, A. S., Abs., 129th Meeting, ACS, Dallas, Texas, April, 310 (1956).

235. Karmen, A., Wroblewski, F., and LaDue, J. S., J. Clin. Invest., 34: 126 (1955).

236. McConnell, D. G., Ozga, G. W., and Soze, D. A., Biochim. Biophys. Acta, 184: 11 (1969).

237. Morgan, J. C., and DiLuzio, N. R., Proc. Soc. Exp. Biol. Med., 134: 462 (1970).

238. Stitzel, R. E., Anders, M. W., and Mannering, G. J., Mol. Pharmacol., 335: 2 (1966).

239. Gillette, J. R., Brodie, B. B., and LaDu, B. N., J. Pharmacol. Exp. Ther., 119: 532 (1957).

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

241. Lieber, C. S., Ruben, E., DeCarli, L. M., Misra, P., and Gang, H., Lab. Invest., 22: 615 (1970).