THE ENZYMATIC ASPECTS OF ETHANOL METABOLISM

by

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INTRODUCTION

1. History of the Study of Alcohol Metabolism.

The earliest research on the biochemical fate of ethanol attempted to determine whether alcohol was utilized or whether it left the body unchanged. The French researcher Lallemand advocated that ethanol was not used by the body (21). This belief was accepted for fourteen years despite excellent conflicting evidence, primarily because of its endorsement by the then influential teetotalers political party. It was not discarded until two London physicians, Dupre (20) and Anstie (1), demonstrated conclusively that only a small fraction of ingested alcohol could be accounted for in the urine, breath, perspiration and feces.

Much discussion was published near the turn of the century concerning whether ethanol was a food or a poison. Dr. W. Hall pointed out in the Journal of the American Medical Association (35) that: 1) alcohol was an excretion of yeast, 2) organisms excrete only those things that are toxic to them, 3) excretions which are toxic to the organism that excretes them are toxic to all organisms, 4) alcohol is toxic to all organisms. Despite Dr. Hall's logic, it was gradually realized that most of the pathology attributed to alcohol was not due directly to the alcohol but to the inadequacy of the typical alcoholic's diet.

Strassman (88) first demonstrated that prolonged ethanol ingestion resulted in a build-up of adipose tissue.

Atwater (2), using direct calorimetry, demonstrated that alcohol is a metabolic source of energy capable of replacing isocaloric quantities of carbohydrate or fat. He also demonstrated that alcohol was able to spare the protein and fat of the body from metabolism. Mellanby, in a classic paper, demonstrated that the rate of blood ethanol elimination is constant and independent of the concentration of ethanol (58). Nicloux demonstrated that within a few minutes after injection, ethanol could be detected in almost every organ in the body (71).

It was early realized that liver and kidney were able to metabolize alcohol. That liver in particular makes the major contribution to alcohol metabolism has now been amply demonstrated by a number of researchers who found that the perfused liver was able to metabolize alcohol at a rate about half that of the intact animal (26,51,69). Clark demonstrated a 75% decrease in the rate of ethanol metabolism after hepatectomy (13).

The ethanol ---> acetaldehyde ---> acetate pathway was first suggested by Battelli and Stern (5) who found an accumulation of acetaldehyde and acetate in rat liver minces. Reichel and Kohle (77) first studied liver alcohol dehydrogenase in 1935 and C. Lutwak-Mann (53) demonstrated the dependence of the activity of the enzyme on the coenzyme nicotinamide adenine diphosphonucleotide (NAD^+). Early workers were able to achieve a rough purification of the enzyme but it was not until 1948 that the liver enzyme was crystallized in fairly pure form (7). Yeast alcohol dehydrogenase, on the other hand, was crystallized by Negelein and Wolff

in 1937 (61). Subsequent research with liver slices with and without radioisotopes has confirmed that alcohol metabolism is primarily initiated by the alcohol dehydrogenase enzyme of liver.

2. Pathways of Ethanol Metabolism.

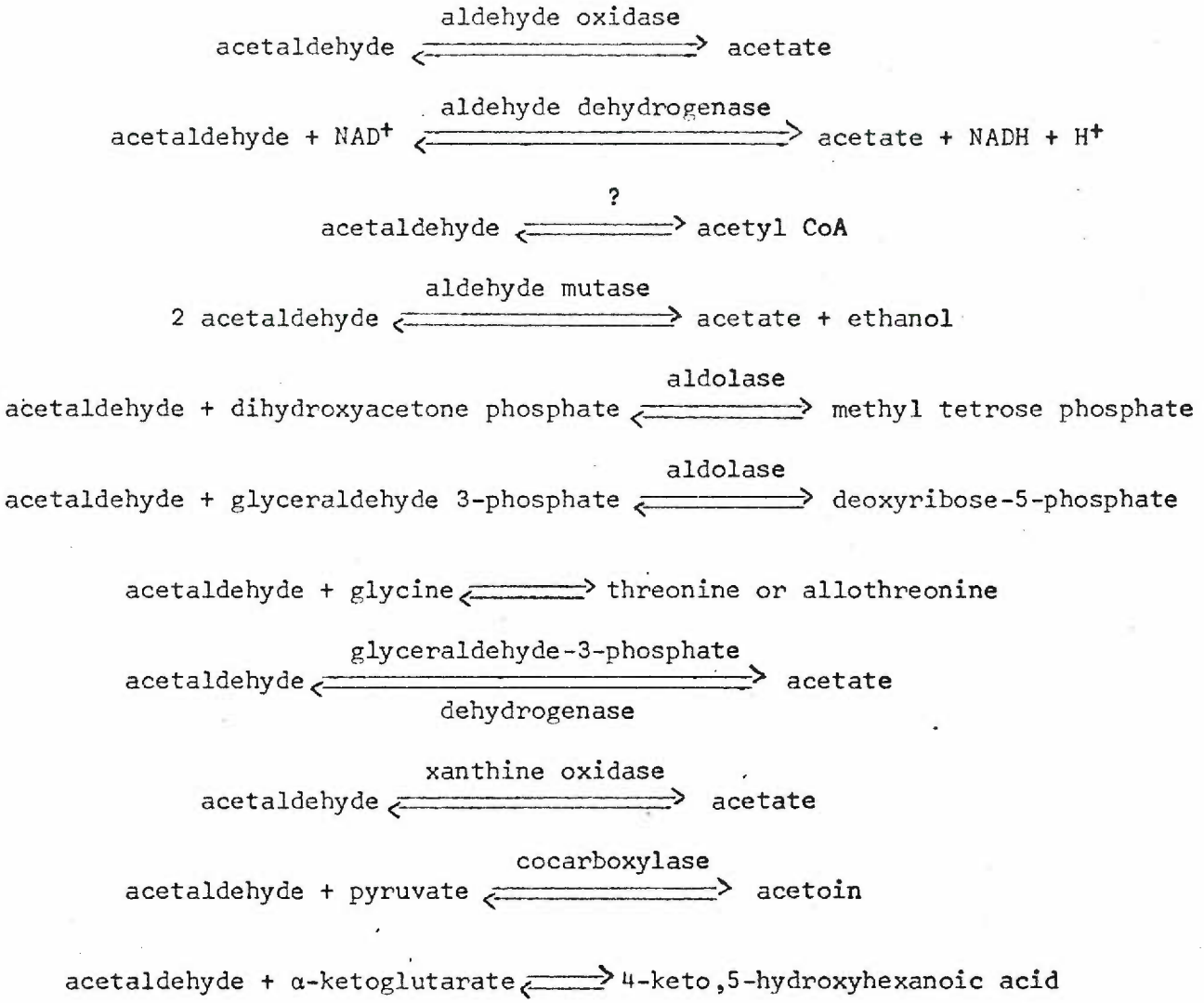
There is general agreement that the first step of alcohol metabolism is ethanol ---> acetaldehyde. Alcohol dehydrogenase is certainly the most important enzyme catalyzing this reaction, although it may not be the only enzyme involved. Other enzymes possibly participating in this first step are xanthine oxidase (31,32) and catalase (50). In the case of catalase, however, Lundquist notes that the endogenous concentration of hydrogen peroxide is probably too low to account for a significant part of the ethanol metabolism. Ethanol can also be eliminated as the conjugation products ethyl glucuronide (39) and ethyl sulfate (9), although their quantitative significance is much less than the oxidation by alcohol dehydrogenase to acetaldehyde. Liver alcohol dehydrogenase may also catalyze the following transhydrogenation reactions (76):

ethanol + n-propionaldehyde $\rightleftharpoons$ acetaldehyde + n-propanol

ethanol + dl-glyceraldehyde $\rightleftharpoons$ acetaldehyde + glycerol.

Both reactions are freely reversible. Although their quantitative significance is unknown, they are of interest biochemically because their equilibrium is independent of either NAD^+ concentration or the $\frac{NAD^+}{NADH}$ ratio.

The metabolic fate of acetaldehyde is much more complex. A number of biochemical reactions have been ascribed to acetaldehyde:



The quantitative significance of these numerous reactions is not known. However, it is usually assumed that acetaldehyde is converted to acetate either by the action of aldehyde oxidase or aldehyde mutase. Acetaldehyde metabolism has not been a popular research topic, and all too little is known of its metabolic fate. Lundquist (49) was able to account for

almost all of a dose of acetaldehyde in an anaerobic rat liver suspension as acetate and ethanol. This would indicate that condensation reactions in that system do not have great quantitative importance. Aldehyde oxidase and xanthine oxidase are both molybdenum containing enzymes. By feeding a tungstate diet to deplete molybdenum from the livers of rats, Richert and Westerfeld (78) demonstrated that the liver enzyme aldehyde dehydrogenase constituted 80-85% of the acetaldehyde oxidizing capability of rat liver homogenate.

Acetaldehyde metabolism, like that of alcohol, seems to be predominately by the liver. Hald, Jacobsen and Larsen (33) have found that perfused livers metabolize acetaldehyde at a rate from 75% - 80% of that of the intact animal. Other tissues are able to metabolize acetaldehyde, but not nearly so rapidly (38). The ability of the body to metabolize acetaldehyde is greater than its ability to metabolize ethanol. Elimination rates of 400-600 mg/kg/hr (47) in cats and 180 mg/kg/hr in rabbits (34) have been measured. These rates exceed the 100-150 mg/kg/hr of ethanol metabolized. Acetaldehyde rarely accumulates to a concentration of greater than 1 mg/100 ml even in acute intoxication (38). Thus it appears to be rapidly eliminated as soon as it is formed. Antabuse is a drug which inhibits xanthine oxidase, aldehyde oxidase and aldehyde dehydrogenase, allowing acetaldehyde to accumulate. Because acetaldehyde is toxic and causes headache and nausea, it has been used to treat alcoholism by associating drinking with the symptoms of acetaldehyde toxicity (19).

For a number of years a controversy has existed as to whether acetaldehyde was transformed to acetate and then acetyl coenzyme A or whether it was directly converted to acetyl coenzyme A. Support for an independent pathway in the metabolism of alcohol has been based upon the observations that labelled alcohol is a better precursor than acetate for the synthesis of cholesterol, fatty acids, phospholipids, and the acetyl group of an acetylated amine (81,82,84,103). However, it has been suggested that this difference between ethanol and acetate metabolism might arise from compartmentation of the acetyl CoA formed from the ethanol within the soluble cytoplasm of the cell (86). Also, Russell and Van Bruggen (80) have shown that ratios of the label incorporated from acetate-1-^{14}C, acetate-2-^{14}C, ethanol-1-^{14}C, and ethanol-2-^{14}C into $^{14}CO_2$, digitonin-precipitable sterols and fatty acids in the intact animal indicate that acetyl CoA is labelled without distinction by acetate-^{14}C and ethanol-^{14}C.

Lundsgaard (52) was first to notice the accumulation of acid, presumably acetic acid, during the metabolism of ethanol. Since then it has been confirmed by a number of investigators (28,47,48,50). Lundquist (50) reports that the level of blood acetate increased 10 to 25 times during ethanol metabolism due to release of acetate by the liver. He reports that acetate can be metabolized at a rate of 0.3 moles hr^{-1}, a rate which is 50% to 100% greater than that of the rate of ethanol metabolism. However, during ethanol metabolism the rate of formation

of acetate is greater than the liver can accommodate, and therefore it is released into the blood stream. Thus the rate limiting step in alcohol metabolism, at least in the liver, is the removal of acetate to a site where it may be utilized.

Lundquist (50) demonstrated that the ethanol that disappears from a rat liver homogenate is nearly quantitatively recovered as free acetate. The weight of evidence favors the ethanol-acetaldehyde-acetate pathway as the primary fate of alcohol. For the complete metabolism of ethanol then, one must consider the metabolic fate of acetate.

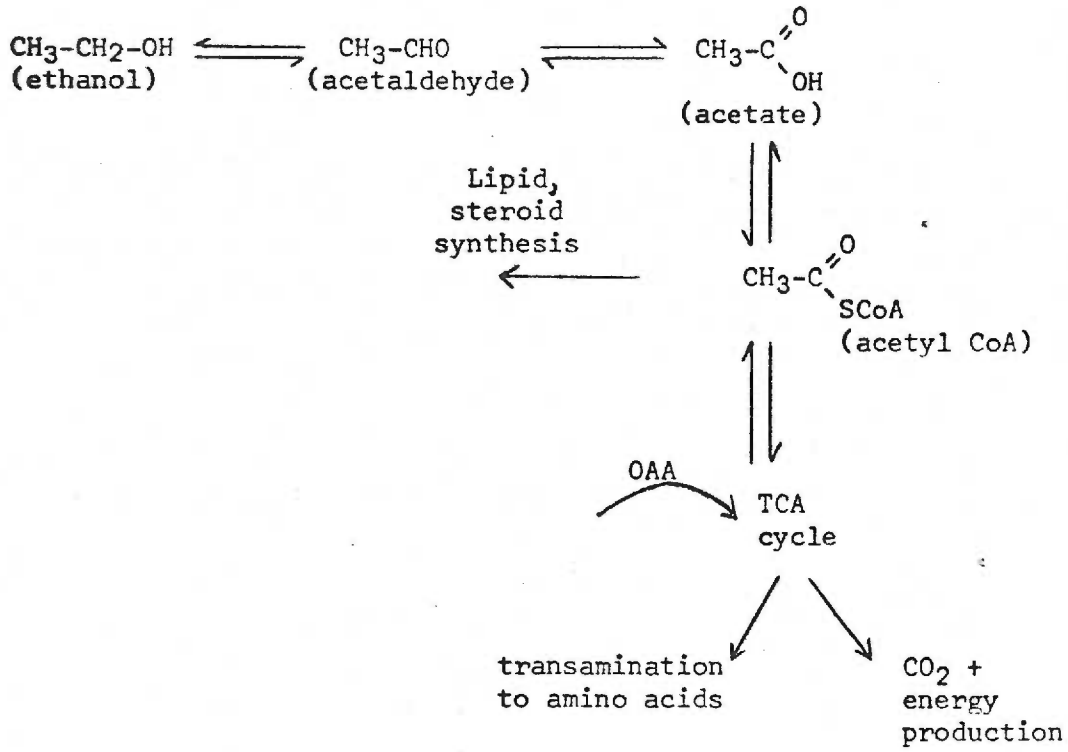
Acetate requires activation prior to being metabolized. Activation may occur by the following series of reactions:

$$\text{acetate} + \text{ATP} \rightleftharpoons \text{acetyl adenylate} + \text{PP}$$

$$\text{acetyl adenylate} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{AMP}$$

After activation many metabolic fates are available to the acetate. It may condense with oxalacetate and enter the Krebs cycle, where it may end up as CO_2, glycogen or other carbohydrates, or it may be incorporated in amino acids via transamination reactions. Acetate may also be incorporated into fatty acids, cholesterol and other steroids.

The primary pathway for alcohol metabolism thus appears to be:



3. Kinetics of Alcohol Dehydrogenase.

As stated before, the first step in the oxidation of ethanol to acetaldehyde is believed to be primarily the result of the catalytic action of alcohol dehydrogenase. The enzyme from both liver and yeast catalyzes the same reaction:

$$R\text{-}CH_2\text{-}OH + NAD^+ \rightleftharpoons R\text{-}CHO + NADH + H^+$$

Despite their similarity of function the enzymes are physically quite different. The yeast enzyme has a molecular weight of 150,000, binds four coenzyme molecules at four active sites (36) and contains 4-5 zinc atoms per molecule (95). When the Zn is removed the enzyme dissociates

into four inactive subunits (85).

The liver enzyme has a molecular weight of 84,000 (24), binds two coenzyme molecules at two active sites and contains two zinc atoms per molecule. It does not dissociate into subunits. The enzyme, depending on the source, exhibits substrate inhibition at alcohol concentrations in the range of 2-20 mM. In man, the figure is 20 mM (100), corresponding to a blood alcohol concentration of 90 mg/100 ml. Since a blood alcohol concentration of 400-600 mg/100 ml may cause death, it is possible the body may become increasingly unable to metabolize alcohol at progressively higher blood alcohol concentrations, thus increasing the chance of a fatality occurring.

Liver alcohol dehydrogenase has an unusually wide specificity. It has been reported to catalyze the oxidation of many primary, secondary and cyclic alcohols and several aldehydes and ketones (16). Activity increases with chain length of primary alcohols (106) and it is perhaps surprising that ethanol is such a good substrate.

The kinetics of liver alcohol dehydrogenase have been perhaps more widely studied than any other NAD-dependent dehydrogenase enzyme in hopes of using it as a model system for pyridine nucleotide linked hydrogen transfer. The reaction below is characterized by the following constants (56).

$$CH_3\text{-}CHO + NADH^+ + H^+ \rightleftharpoons CH_3\text{-}CH_2\text{-}OH + NAD^+$$

$Keq = 8.01 \times 10^{-2}$ M

$\Delta F = 14.89$ Kcal mole^{-1} at 20 C

$\Delta S = -26.5$ Kcal mole^{-1}

$\Delta H^\circ = 7130 \pm 200$ cal mole^{-1}

In 1951 Theorell and Chance (90) first proposed the kinetic mechanism of action that bears their names.

$$E + NADH \underset{K_2}{\overset{K_1}{\rightleftharpoons}} E\text{-}NADH \overset{K_3}{\rightleftharpoons} (E\text{-}NADH\text{-}aldehyde{=}E\text{-}NAD\text{-}alcohol) \overset{K_3'}{\Longrightarrow} E\text{-}NAD \underset{K_1}{\overset{K_2'}{\rightleftharpoons}} E + NAD$$

The characteristics of this mechanism include:

1) The only kinetically significant binary compounds are formed between the enzyme and the coenzyme. The enzyme-alcohol and enzyme-aldehyde compounds either do not form or have a sufficiently unfavorable equilibrium such that they do not affect the kinetics.

2) The ternary complexes E-NADH-ald and E-NAD-alc are in rapid equilibrium with each other and at no time do they become rate limiting. Therefore they, too, do not affect the kinetics.

3) The aldehyde and alcohol dissociate from the ternary complex much more rapidly than does the coenzyme.

4) The rate limiting steps are K_3' for the forward and K_3 for the reverse reaction.

The mechanism was first supported (91), then questioned (15), then finally confirmed (92,93,94). The question of whether enzyme-alcohol and

enzyme-acetaldehyde binary complexes may exist without influencing the kinetics is still unanswered, but the current consensus (16) is that at least the alcohol-enzyme complex does exist.

Liver alcohol dehydrogenase possesses a strong substrate inhibition by alcohol at concentrations between 2 mM and 20 mM, depending on the source of the enzyme. The cause of this inhibition has not been determined, but possibilities include formation of an abortive alcohol-NADH-enzyme complex (89) and binding of ethanol by the enzyme at other than the active site (107).

4. Enzyme Levels.

Comparison of enzyme levels of alcohol dehydrogenase published by different researchers is complicated by the variety of assay conditions used. Another difficulty is that the most commonly used assay procedure, that published by Bonnischen and Brink in Methods in Enzymology (8), is not the best procedure available. The Bonnischen and Brink procedure uses far too great an alcohol concentration causing a strong substrate inhibition that results in a low sensitivity. Also, the procedure reports enzyme levels in arbitrary "units of enzyme" rather than the more desirable measure of moles substrate utilized per minute per weight of tissue or protein. Consequently comparison of enzyme levels cited in the literature is at best confusing.

A list of liver enzyme levels and the experimental conditions by which they were obtained are given on the following page.

	Experimenter	Temperature	Assay pH	Ethanol	NAD^+	Animal	Activity
(75)	Raiha Koskinen	room	8.7	0.51 M	0.46 mM	rats	0.96 I.U./g
(79)	Rogers & McClearn	35°	9.8-9.9	0.017 M	1 mM	mice	3.5-7.4 I.U./g tissue
(55)	McClearn	35°	9.8-9.9	0.017 M	1 mM	mice	2.0-3.2 I.U./g tissue
(87)	Spencer	room	9.6	0.47 M	0.46 mM	rats	3.7 B+B units/mg protein
(83)	Smith & Newman	room	9.6	0.51 M	0.46 mM	rats	7.8 units/g tissue
(97)	Von Wartburg	room	9.6	0.51 M	0.46 mM	rats	14.0ΔA/10min/g tissue
(12)	Cherrick & Leevy	room	9.6	0.51 M	0.46 mM	rats	0.64 I.U./g tissue
(59)	Mikata	38°	7.4	0.66 M	2 mM	rats	0.60 I.U./g tissue

I.U. = International Enzyme Units = $\frac{\mu\text{mole substrate utilized}}{\text{min.}}$ at defined conditions.

B.B. units = units as reported by Bonnischen and Brink in Methods in Enzymology, vol. I, 1955, p. 496.

Although the experimental results are quite variable due to the wide variety of experimental conditions employed, by varying the experimental conditions it has been possible to demonstrate that the values obtained by the various experimenters are quite consistent with each other and also with the experimental results obtained by us (see Results section). We were not able to ascertain the meaning of the units described by Smith and Newman. Thus enzyme levels in rat and mice liver have been fairly well established and seem to be consistent. Among different animals there are, however, substantial differences in reported enzyme levels. Nyberg (72) found that horse liver contained more than 10 times as much enzyme as does rat liver, and that the amount of enzyme in guinea pig liver was far less than that of rat liver. Approximately 80% of the enzyme activity is located in the soluble fraction, the rest distributed in the microsomes, mitochondria and nuclear fractions(72,75). A NADP dependent alcohol dehydrogenase has been found in rat, rabbit and pig liver microsomes (73). Liver enzyme levels decrease in alcohol induced cirrhosis in both man and rats (27,59) but may increase slightly at first by means of enzyme induction (14).

Much less has been published on alcohol dehydrogenase levels in other organs. Cherrick and Leevy (12) were unable to find any alcohol dehydrogenase in kidney, but this is not a surprising result in view of their experimental conditions. Dajani and Orton (14) were able to detect alcohol dehydrogenase in human and rat serum, whereas Figueroa and Klotz

(27) were not able to do so. Spencer (87) was able to demonstrate extremely high enzyme levels in rat stomach; indeed, up to eight times as much as found in liver. He also found very high enzyme levels in intestinal tissue, indicating that possibly the digestive tract could make a considerable contribution to ethanol metabolism. Heart muscle was found to be devoid of enzyme (12). It has recently been realized that alcohol dehydrogenase, like so many other enzymes, is not homogenous but rather consists of isozymes. Von Wartburg (100) has recently demonstrated three isozymes of alcohol dehydrogenase from the rhesus monkey and found enzyme activity in liver, stomach and intestine, kidney, bladder, lung and spleen.

5. Enzyme Levels vs. Rate of Metabolism.

There is little doubt that the liver is the principle site of ethanol metabolisms. Perfusion studies, blood alcohol curves after hepatectomy, and studies with liver poisoning have all demonstrated that liver is responsible for at least half, and possibly as much as ninety per cent of *in vivo* ethanol metabolism. *In vitro* studies with tissue slices demonstrate the capability of liver to metabolize ethanol. Also, tracer work (81,4,100,98,60) has confirmed the ability of liver to convert the isotopic carbon of ethanol into $^{14}CO_2$.

Liver tissue contains considerable alcohol dehydrogenase. Indeed, it is the recommended tissue for isolation of the enzyme. When this enzyme is assayed at physiological pH and optimal substrate concentrations,

an average human liver weighing 1500 g contains 2700 I.U.'s of enzyme. This corresponds to a metabolic capacity of 6-7 grams of ethanol per hour (100,6,99). By comparing this to information obtained from blood alcohol curves, it can be seen that the human liver has the capacity to metabolize 60-95% of a lightly intoxicating dose of ethanol. It is very important to note that to do this the enzyme must be working at a near maximal rate; substrate concentrations must be near optimal, the effect of inhibitors must be negligible.

The role of the kidneys in alcohol metabolism is much more confusing. It must be kept in mind that the total weight of kidney tissue is approximately one-fifth that of liver. The alcohol dehydrogenase content of kidney is sufficiently low as to be undetectable under the assay conditions of Bonnischen and Brink (12); yet it is present (100). Leloir and Munoz found kidney slices to be able to slowly metabolize ethanol (46). Recent tracer work has caused much confusion with respect to the role of the kidney in ethanol metabolism. Bartlett and Barnet (4) found kidney to be able to oxidize ethanol to $^{14}CO_2$ at a rate of 2.1 μMoles of ethanol 100 mg tissue^{-1} hour^{-1}. Von Wartburg found a rate of 5.7 μMoles of ethanol 100 mg tissue^{-1} hour^{-1} (96). Both of these values represent quite significant ethanol oxidation rates. Moir (60) has also demonstrated high rates of metabolism. Indeed, both Bartlett and Barnet and Von Wartburg found kidney to convert tracer ethanol into $^{14}CO_2$ at a rate of approximately three times that of liver. However, as has been previously noted, the liver is unable to metabolize all of the acetate

formed from the ethanol and thus the rate of breakdown of ethanol here may be much higher than the $^{14}CO_2$ values indicate.

Although high alcohol dehydrogenase levels have been found in rat stomach (87), no studies have been done either *in vivo* or *in vitro* to examine whether or not the stomach can metabolize ethanol. It is known that evisceration causes a decline in the rate of elimination of ethanol from the blood, indicating that the intestinal tract may participate in alcohol metabolism. One questionable study found intestine, testis, diaphram and spleen unable to metabolize ethanol (46). Bartlett and Barnet found both diaphram and heart capable of producing small amounts of $^{14}CO_2$ from tracer ethanol (4), but no one has attempted to determine enzyme levels in those tissues using a sensitive assay procedure. Dewan (19) claimed to have found alcohol dehydrogenase activity in beef brain, but no one has been able to either confirm the result or measure $^{14}CO_2$ activity from tracer ethanol in nerve tissue.

6. Rate of Ethanol Metabolism.

The disappearance rate of ethanol from the blood and from the whole animal has been extensively studied in many animals. A summary of representative data is listed below.

Experimenter	Animal	Rate of elimination mg kg^{-1} hr^{-1}
Larsen (42,43)	cats	103
Newman & Lehman (66)	cats	142
Eggleton (22)	cats	114-150
Heim (37)	cats	126
Nelson & Kinard (64)	dogs	155
Newman (65)	dogs	186
Newman, Newman & Wilson (69)	man	155
Widmark (104)	man	10
Aull, Roberts & Kinard (3)	rats	293
Mikata (59)	rats	309
Kinard, Aull & Ulmer (41)	rats	249
Nelson & Abbenhaus (62)	baby rats	493
Nelson, Kinard & Hays (63)	mice	600

The shape of the blood alcohol elimination curve has been a source of controversy for over thirty years, and the question has still not been entirely resolved. Mellanby first proposed that the curve was linear, indicating that the rate of elimination was independent of concentration (58). However, other evidence, including a re-evaluation of Mellanby's original data, seemed to indicate that there was some decline in rate with decreasing concentration. The argument may well be pointless and it is perhaps

best to summarize, as Westerfeld did (101), that the blood ethanol disappearance rate is much more nearly rectilinear then exponential, except at very low blood ethanol concentrations where it very rapidly declines (54,42,43). This is entirely reasonable in terms of enzyme kinetics. One might expect a slight decrease in the metabolic rate of ethanol until the point was reached where the enzyme was no longer saturated, whereupon a more rapid decline in rate would result (96). Also, it must be remembered that the losses based on diffusion through respiration, perspiration and in the urine will be concentration dependent and will be greater at higher ethanol concentrations.

Much more confusing is the "conditioning" effect described by Newman, Lehman & Cutting (67). They found that when a dog was given a large dose of ethanol, and when the concentration of blood alcohol had almost reached zero, the dog was given a second but much smaller dose of ethanol, the rate of disappearance of the smaller dose was the same as the larger dose. The rate of disappearance of the smaller dose when given alone, however, was significantly less. Thus it was postulated that the initial maximal metabolic rate was able to effect, i.e., "condition" the subsequent rate of metabolism. However, no satisfactory explanation for this phenomenon has been offered.

The effect of carbohydrate metabolism on the metabolism of alcohol has been studied intensively. Leloir and Munoz first demonstrated that

pyruvate added to liver slices caused an increase in the rate of ethanol metabolism (46). Westerfeld, Stotz and Berg confirmed the effect in intact dogs (102). Starvation has the opposite effect; it slows down ethanol metabolism (44,45). Tissue slices from starved rats are able to metabolize alcohol only about one-half as fast as those from fed rats (46,83). Some researchers have been able to show an acceleration of ethanol metabolism by glucose (11), insulin (70,74,101), glucose plus insulin (70), fructose (74) and alanine (83,46,23). It is not hard to envisualize how pyruvate and alanine, a direct precursor of pyruvate, could accelerate ethanol metabolism. Lactic dehydrogenase will convert pyruvate to lactate and in so doing reoxidize the NADH formed by the oxidation of alcohol. This would result in cycling the coenzyme and could theoretically increase the rate of the alcohol dehydrogenase step. Presumably, then, the effect of carbohydrates on alcohol metabolism is mediated through the control of the NAD^+ concentration. Westerfeld (101) notes that the carbohydrate effect is present only when the initial rate of alcohol metabolism is less than maximal. He states that the maximal rate of ethanol metabolism is limited to the amount of alcohol dehydrogenase present, but that the maximal rate is not achieved by the enzyme alone. The demands of alcohol dehydrogenase and aldehyde dehydrogenase during ethanol metabolism for the available NAD^+ may be so great that the usual mechanisms for reoxidation of NADH may be inadequate; a coupling substrate such as pyruvate may be necessary.

Since carbohydrates, alanine and insulin will all increase the amount of pyruvate, they will all increase alcohol metabolism. Newman and Smith (83) note that during alcohol metabolism the $NAD^+/NADH$ ratio falls, and that the fall is greater in starved than fed animals. This could explain the effect of starvation on the rate of alcohol metabolism. Evidence that in the normal well-fed animal NAD^+ is not normally a rate-limiting factor was demonstrated when rats were fed a diet designed to increase NAD^+ levels in rat liver by a factor of 10 (10). These rats were able to metabolize ethanol no faster than controls. This is in accord with published levels of NAD^+ of about 0.7 mM (12,40) which is sufficient to saturate the enzyme *in vitro*.

7. Statement of Purpose.

It is the intention to increase the understanding of ethanol metabolism by examining the *in vitro* metabolism of ethanol ^{14}C in the light of the amount of alcohol dehydrogenase present in various tissues. Proper evaluation of tissue levels of alcohol dehydrogenase has been lacking, and as a consequence, published enzyme levels in various tissues are numerically different and difficult to compare because of the wide variety of assay conditions used by the researchers.

In vitro studies of ethanol, in some cases with the use of tracer and in some cases without, have examined the ability of several tissues to metabolize alcohol. However, we know of no study in which the same

researcher, using the same experimental procedures, has examined the ability of a number of tissues to metabolize ethanol. This approach is necessary to evaluate quantitatively the contribution of these tissues to total body ethanol metabolism.

Some attempt to relate the levels of alcohol dehydrogenase of various tissues to the metabolic capability of these tissues to utilize ethanol should be made. But even with accurate estimates of both enzyme levels and ethanol metabolizing capabilities in the tissues, there is no assurance that there will be any correlation between the two. Enzymes other than alcohol dehydrogenase may prove to be sufficiently important that they will override or mask its action. But even if other enzymes are not responsible for a significant contribution to alcohol metabolism, there may still be no correspondence between the amount of enzyme and its activity in the cell. It must be considered that the activity of an enzyme in the intact cell and in a test tube may be substantially different. The intracellular activity may be significantly elevated or inhibited by various metabolic cell constituents.

Specific goals for this research are therefore:

1. To develop a procedure that will enable us to accurately determine the amount of alcohol dehydrogenase in a given tissue. This technique must be sufficiently sensitive to detect enzyme in all tissues which make a contribution to total body alcohol metabolism due to the presence of the enzyme.

2. To obtain quantitative, easily reproducible determinations of enzyme activity in all tissues in which alcohol dehydrogenase is known to be present.
3. To determine, using ^{14}C ethanol as a substrate, the ability of various tissues to metabolize tracer ethanol. Also, by using a set of experimental procedures kept as constant as possible for these various tissues, to obtain a rough estimate of their quantitative contribution to total body ethanol metabolism.
4. To determine, by examining a number of tissue fractions, the eventual fate of the tracer ethanol carbon. By comparing the fate of the tracer ethanol in several tissues it may be possible to find qualitative differences in ethanol metabolism in these tissues.
5. To examine the possibility that there may be a correspondence between the amount of alcohol dehydrogenase present in a tissue and the ability of that tissue to metabolize ethanol.

METHODS AND MATERIALS

1. Materials.

Radioactive tracers used in this study were ethanol-1-^{14}C, acetic acid-1-^{14}C, palmitic acid-2-^{14}C, and cholesterol-4-^{14}C. These tracers were obtained from New England Nuclear Corporation.

The tracer ethanol was stored at -16°C in water solution and was routinely distilled from NaOH to remove CO_2 and acetic acid formed by the breakdown of the tracer ethanol. The concentration of the ethanol was 2.5 μMoles EtoH per ml. The other tracers were used without further purification.

All of the tracer work and most of the enzyme assay work was done with 200-240 g Sprague-Dawley rats obtained from Berkeley, California. A few enzyme assays were done with rats obtained from Pacord Research Animals in Beaverton, Oregon. The enzyme levels of these animals from the two sources were not significantly different.

Prior to their use in the tracer studies, all animals were starved 12 to 15 hours but were given free access to water.

Crystalline horse liver alcohol dehydrogenase was obtained from Sigma Chemical Company.

2. Alcohol Dehydrogenase Assay Procedure.

The problem of developing an accurate and sensitive method for determination of tissue levels of alcohol dehydrogenase was encountered.

The standard procedure for assay, published by Bonnischen and Brink in Methods in Enzymology (8) was found to be lacking in sensitivity, subsequently found to be due to the excess of alcohol used in the assay and the lack of sufficient NAD^+. It has also the added disadvantage that results are calculated as micrograms enzyme rather than the more conventional micromoles product formed min^{-1} g $tissue^{-1}$ at optimal conditions, a measure recommended by the Commission on Enzymes of the International Union of Biochemistry (1961).

A. Theory of the Method

The NAD^+-enzyme complex oxidizes ethanol to acetaldehyde and NADH-enzyme. The NADH is measured spectrophotometrically at its absorption maximum of 340 mμ. The reaction is:

$$CH_3\text{-}CH_2\text{-}OH + NAD^+ \rightleftharpoons CH_3\text{-}CHO + NADH + H^+$$

which has the equilibrium constant of: (pH 7-10, ionic strength of 0.1)

$$K_{eq.} = \frac{(\text{Acetaldehyde})\ (\text{NADH})\ (H^+)}{(\text{Ethanol})\ (NAD^+)} = 0.801 \times 10^{-11}$$

Since the reaction is H^+ dependent, a high pH will favor acetaldehyde formation. The pH of 9.6, recommended by Bonnischen and Brink, has been adopted. At a given pH the equilibrium may be conveniently expressed:

$$K_{eq.}^{H^+} = \frac{(\text{Acetaldehyde})\ (\text{NADH})}{(\text{Ethanol})\ \ (\text{NAD}^+)}$$

for pH = 9.6, the constant will be

$$K = \frac{(\text{Acetaldehyde})\ \ (\text{NADH})}{(\text{Ethanol})\ \ (\text{NAD}^+)} = 3.2 \times 10^{-2}$$

Although the equilibrium favors ethanol formation, the high initial concentrations of ethanol, NAD^+ and the high pH result in first order kinetics with respect to enzyme until a change in absorbance of 0.225 or greater is attained.

B. Assay Procedure for Alcohol Dehydrogenase

The tissue is lightly chopped and one gram is homogenized with 9 ml of ice cold distilled water. The homogenate is then centrifuged at 8000 x g for 60 minutes at 0°C. A Beckman spectrophotometer is used to measure the increase in absorbance at 340 mμ. for a three-minute period. Each cuvette contains 0.1 ml NAD^+ solution (70 mg NAD^+/ml), 0.1 ml of tissue homogenate, 2.78 ml of 0.1 N glycine - NaOH buffer, pH = 9.6, and 0.02 ml of 5% ethanol. In the blank cuvette the ethanol volume is replaced by additional buffer to a final buffer volume of 2.8 ml. The addition of ethanol in the test cuvette initiates the reaction and the change in absorbance in three minutes is recorded.

In the modified procedure below allowance is made for endogenous reduction of NAD^+ and internal correction is made for initial differences

between the blank and the sample cuvettes. The homogenate and the buffer are added to the control and test cuvettes then at the indicated times, perform the manipulated cited:

Time	Duty
0 sec.	Add NAD^+ to control cuvette.
15 sec.	Add NAD^+ to experimental cuvette.
2 min. 30 sec.	Add ethanol to experimental cuvette.
2 min. 45 sec.	Set spectrophotometer on zero density with the control cuvette in place.
3 min.	Take the first reading on the test cuvette.
5 min. 45 sec.	Reset spectrophotometer on zero for blank cuvette.
6 min.	Take final reading of test cuvette.

Subtract the initial reading from the final reading to give the absorption change for the three minute period. Endogenous reduction of NAD^+ has been procedurally subtracted.

C. Calculations

The extinction coefficient for NADH is $6.22 \times 10^3\ M^{-1}\ cm^{-1}$; therefore,

$$\mu\text{moles NADH formed min}^{-1}\text{ g tissue}^{-1} = \frac{(\Delta A340)\ (3\ ml)\ (10^3)}{(6.22 \times 10^3)\ (3\ min)\ (.01\ g\ tissue)}$$

$$\mu\text{moles NADH formed min}^{-1}\text{ g tissue}^{-1} = \frac{\Delta A340}{0.0622}$$

D. Preparation of the Homogenate

It was demonstrated that rat liver, when frozen immediately after

decapitation of the animal and removal of the tissue, could be stored for up to five days with no loss of activity. Raiha and Koskinen (75) found that freezing and thawing the tissue may enhance the alcohol dehydrogenase activity of the rat liver.

The liver homogenate prepared for use is much less stable. One sample stored at 8°C for twenty-four hours lost 40% of its activity, while another sample stored under similar conditions lost 30% of its activity in seventy-two hours. However, we have found no loss of activity for at least three and a half hours after centrifugation when the homogenate is stored on ice.

Both distilled water and 0.25 M sucrose were considered as possible extraction media. The resultant alcohol dehydrogenase activity was approximately the same with either.

Extraction with Triton X-100, a non-ionic surface acting agent, has been reported to result in a 40% increase in rat liver alcohol dehydrogenase activity (75). We have confirmed this effect in both rat liver and rat kidney. Part of this increase has been demonstrated to be due to the release of alcohol dehydrogenase from the nuclear and mitochondrial fractions. However, the Triton X-100 has an unexplained activating effect on the supernatant enzyme activity.

The centrifugation rate seems to make a slight difference in activity, apparently due to removal of less active cell debris. A centrifugation rate of 8000 g is about 5% more active than one of 800 g

and also yields a preparation that is more homogeneous and easier to work with.

E. The Concentration of NAD^+ and the Reaction Rate

The rate of the reaction is dependent upon the concentration of NAD^+. We found that for rat liver the activity of the enzyme rises rapidly with increasing NAD^+ until about 7 mg of coenzyme has been added, corresponding to a concentration of about 3.25 mM. At this point the activity is very near a maximum. It is clear that sufficient attention has not previously been paid to the concentration of coenzyme. The adjustment of coenzyme concentration yields twice the sensitivity of the old method. A plot of (NAD^+) vs. activity is shown in Fig. I. A Lineweaver-Burke plot of the same data yields a straight line.

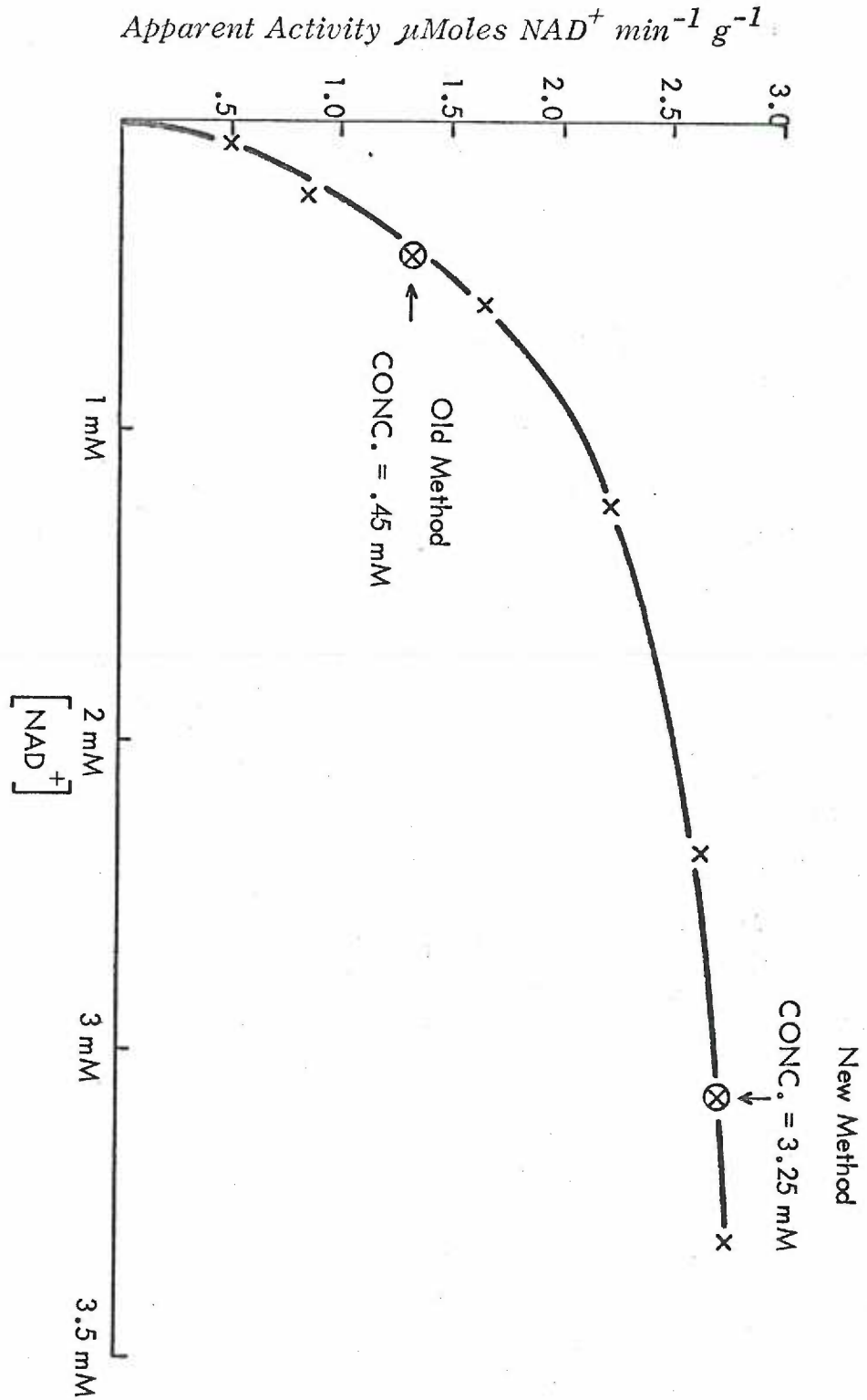
F. Ethanol Concentration and Reaction Rate

Liver alcohol dehydrogenase shows substrate inhibition at high ethanol concentrations, and thus the ethanol concentration is a very critical variable in the assay. The optimal ethanol concentration varies slightly from tissue to tissue. We have found that for rat liver a concentration of 5.4 mM (1 µl/3 ml) yields maximal activity, for horse liver it is about twice that, for rabbit liver, 3-4 times as much. For unknown tissues preliminary determination of the optimal ethanol concentration is recommended.

We have found that alcohol dehydrogenase from rat stomach and intestine does not exhibit substrate inhibition. We therefore have used

Fig. I

Activity of Rat Liver Homogenate with Varying NAD^+ Concentration. The Recommended Concentration is Contrasted with the Concentration used in Bonnischen and Brink Procedure.


Apparent Activity μMoles NAD+ min-1 g-1
.5
1.0
1.5
2.0
2.5
3.0
1 mM
2 mM
3 mM
3.5 mM
[NAD+]
Old Method
CONC. = .45 mM
New Method
CONC. = 3.25 mM

an ethanol concentration of 0.54 M (100 µl of 95% ethanol/3 ml) to assay enzyme from this tissue. This gives a very sensitive assay.

The activity of rat liver enzyme is shown over a wide range of ethanol concentrations (Fig. II).

3. Tissue Slice Preparations for Metabolism Studies.

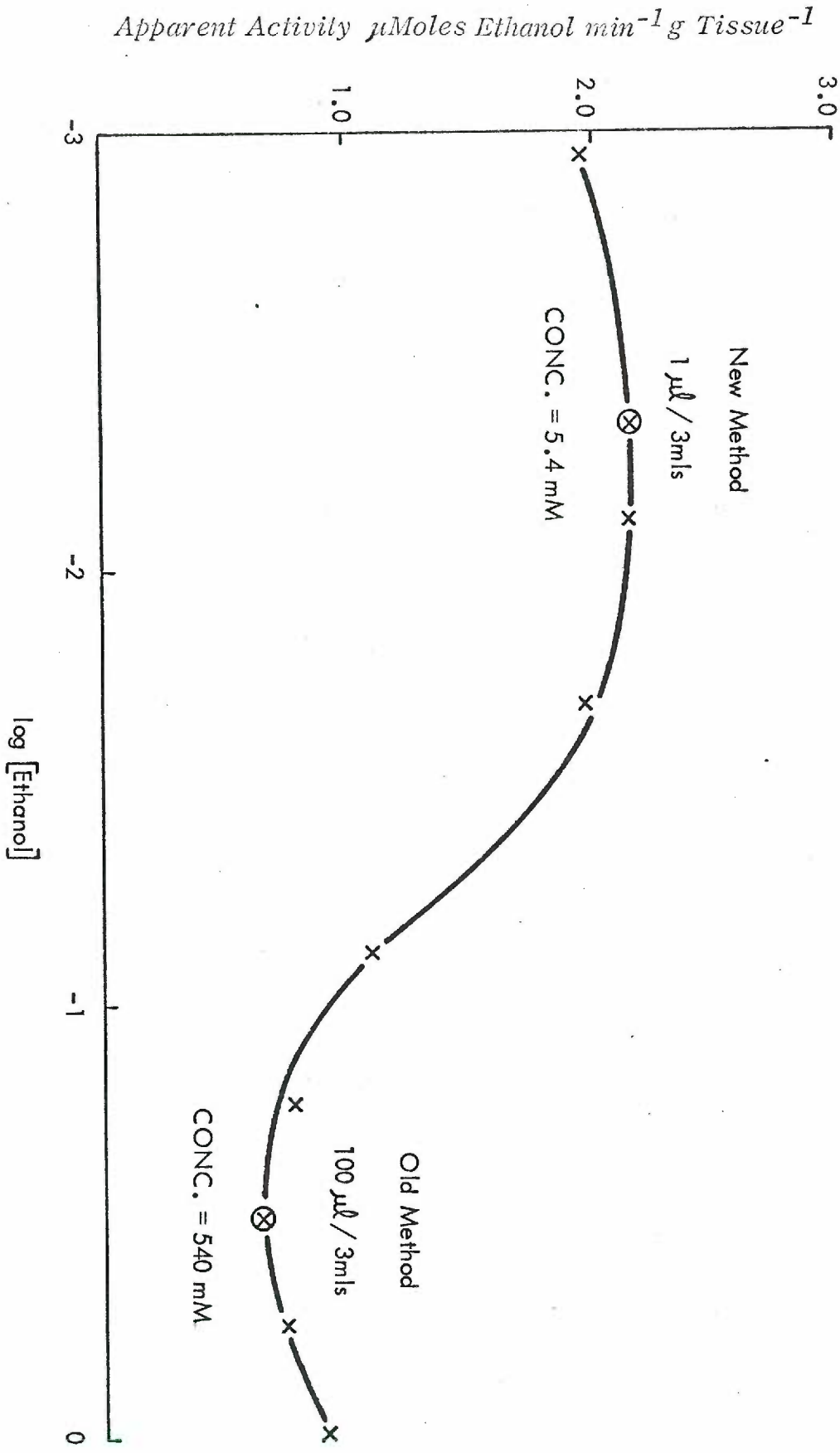
The animals were killed by decapitation, the tissue immediately removed and immersed in ice cold Krebs phosphate buffer, pH = 7.4.

Liver slices were cut to an approximate thickness of 0.5 mm using a Stadie-Riggs microtome in the cold room. Other tissues were cut to a thickness of 0.5 mm using a Mickle tissue chopper while the tissues were kept cold.

Approximately 200 mg of tissue slices were placed in preweighed, chilled Warburg flasks containing 2.7 ml of Kreb's phosphate buffer, pH = 7.4, and with 0.2 ml of 12N H_2SO_4 in one side arm. The flasks were then weighed and the increase in weight used as the tissue weight. Just prior to equilibration small CO_2 collection tubes that fit into the center well of the Warburg flask were put in place. These tubes contained 0.2 ml of 6N carbon dioxide free NaOH absorbed on a small folded-square of filter paper. The substrate ^{14}C ethanol was then added to one of the side arms, the flasks were flushed with 100% O_2 at a flow rate of 1-1.5 L/min for one minute, immediately connected to their manometers and inserted in the Warburg bath. The flasks were equilibrated for 10 minutes

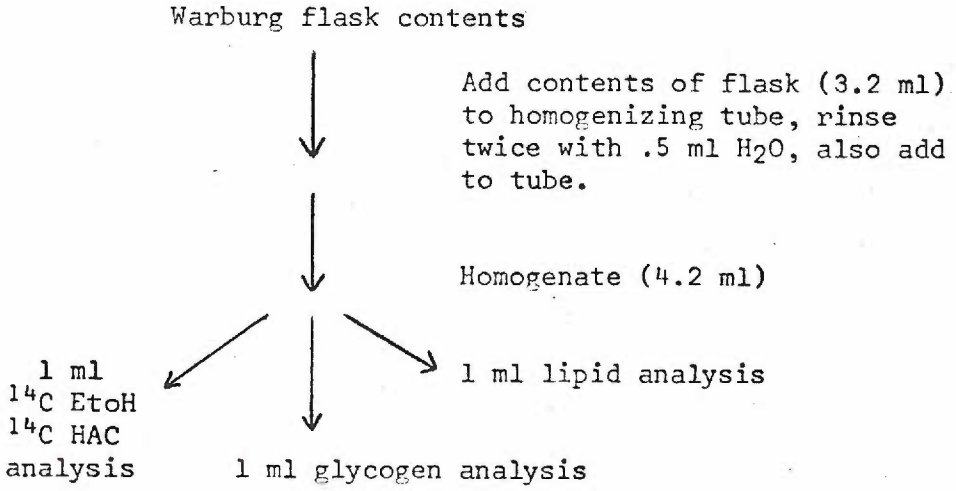
Fig. II

Activity of Rat Liver Homogenate with Varying Ethanol Concentration. The Recommended Concentration is Contrasted with the Concentration used in the Bonnischen and Brink Procedure.


Apparent Activity μMoles Ethanol min⁻¹ g Tissue⁻¹
3.0
2.0
1.0
-3
-2
-1
0
log [Ethanol]
New Method
1 μl / 3mls
CONC. = 5.4 mM
Old Method
100 μl / 3mls
CONC. = 540 mM

at 37°C and the substrate is then added. After a one-hour incubation period the tissue was "killed" by dumping H_2SO_4 from the side arm into the contents of the flask. Forty-five minutes later the flasks were removed from the bath, covered, and placed on ice. The contents of the flask were then placed in chilled glass homogenization tubes. The flasks were rinsed with cold distilled water and this, too, added to the homogenization tubes. The removal of the flask contents was done as quickly as possible to minimize the evaporation of remaining ethanol tracer. The tissues were then homogenized with a teflon plunger at 0°C. The homogenate was then frozen at -16°C until it was used for fraction analysis. Immediately after the flasks were removed from the Warburg bath, the center well CO_2 collection tubes were removed and placed in a 9.8 ml of distilled water for subsequent $^{14}CO_2$ radioassay.

A flow chart for fraction analysis is shown below.



4. Collection of ^{14}C Acetate and ^{14}C Ethanol.

A modified method based on a procedure for blood alcohol determinations was used (17). To a 1 ml sample of Warburg homogenate the following were added: 100 µl of 95% ethanol, 100 µl of acetic acid, and 1.8 ml of dioxane.

The sample was thoroughly mixed for one minute and then centrifuged in the cold room for ten minutes. The precipitate was discarded. The sample was then placed on ice and analyzed immediately.

Ethanol and acetic acid fractions were separated by gas chromatography using an F & M Model 720 gas chromatograph and a preparative column eight feet in length and 5/8" in outside diameter containing 45-50 mesh chromosorb W and coated with 20% by weight silicone rubber. The conditions and separation for a typical run are shown in Table I. The ethanol fraction was collected with a U-tube immersed in dry ice-acetone. Loss of vapor was avoided by the use of a 1/4" rubber gasket. The acetic acid fraction was collected by bubbling the exit port gas through a small test tube immersed in an ice-water bath containing 7 ml of 0.3N NaOH. The tube was then rinsed with 2 ml of NaOH and this was added to the solution. Recoveries of tracer ethanol and acetate are shown on Table II.

The time course studies of elution of radioactivity from the gas chromatography column was done by bubbling the exit port gas through tubes of ethanol or toluene-ethanol scintillation fluid. The first ten tubes were immersed in dry ice-acetone and the subsequent tubes in ice water.

TABLE I

Ethanol and Acetic Acid fractions superimposed on a typical chromatograph tracing.

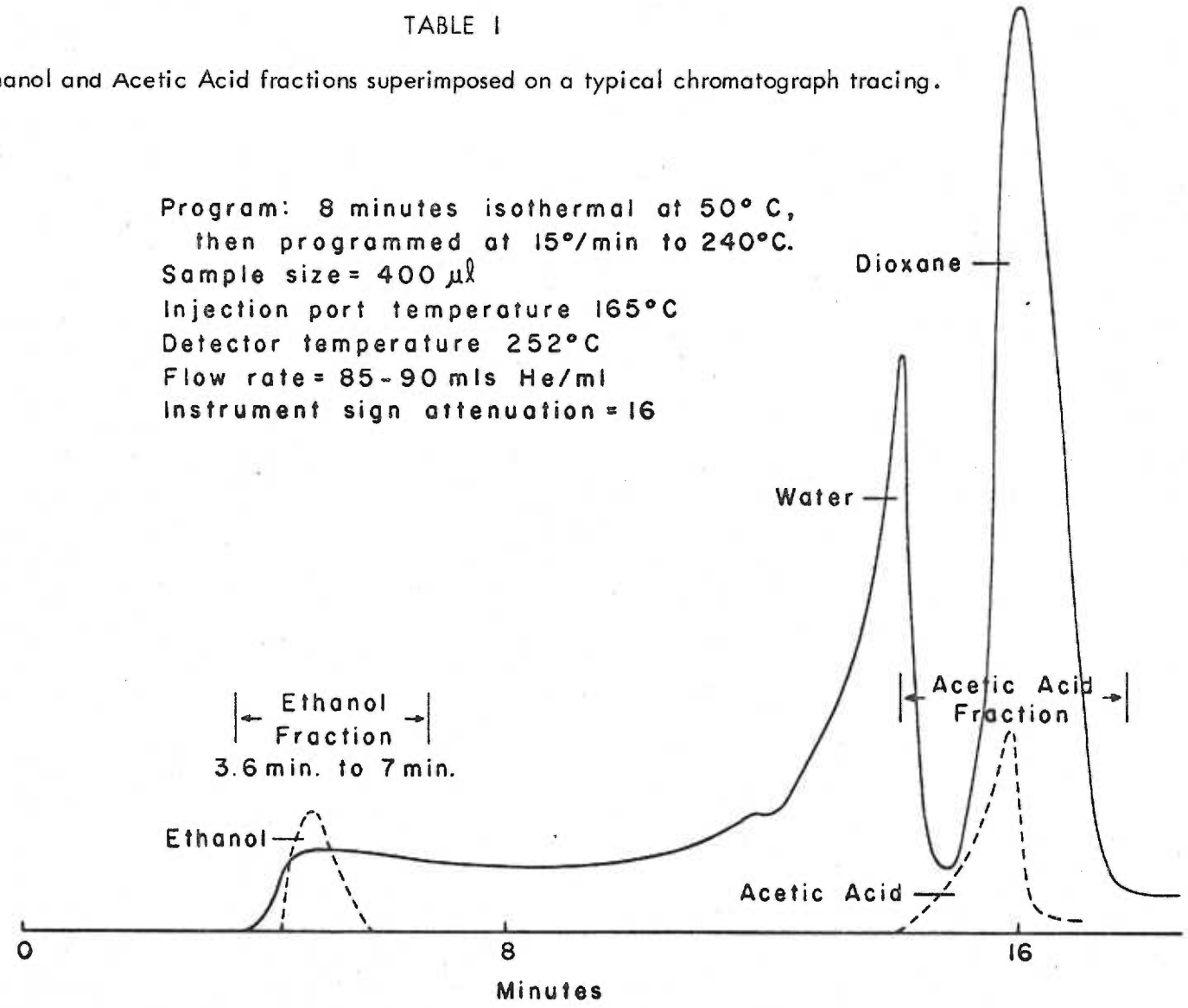


TABLE II

Recovery of Ethanol-1-^{14}C and Acetic Acid-1-^{14}C

by Gas Chromatography

Ethanol Recovery

from	cpm added	cpm recovered	% recovery
liver extract	135,086	131,500	97.4
" "	27,057	28,208	104.2
water	7,185	7,592	95.0
"	1,996	2,036	102.0

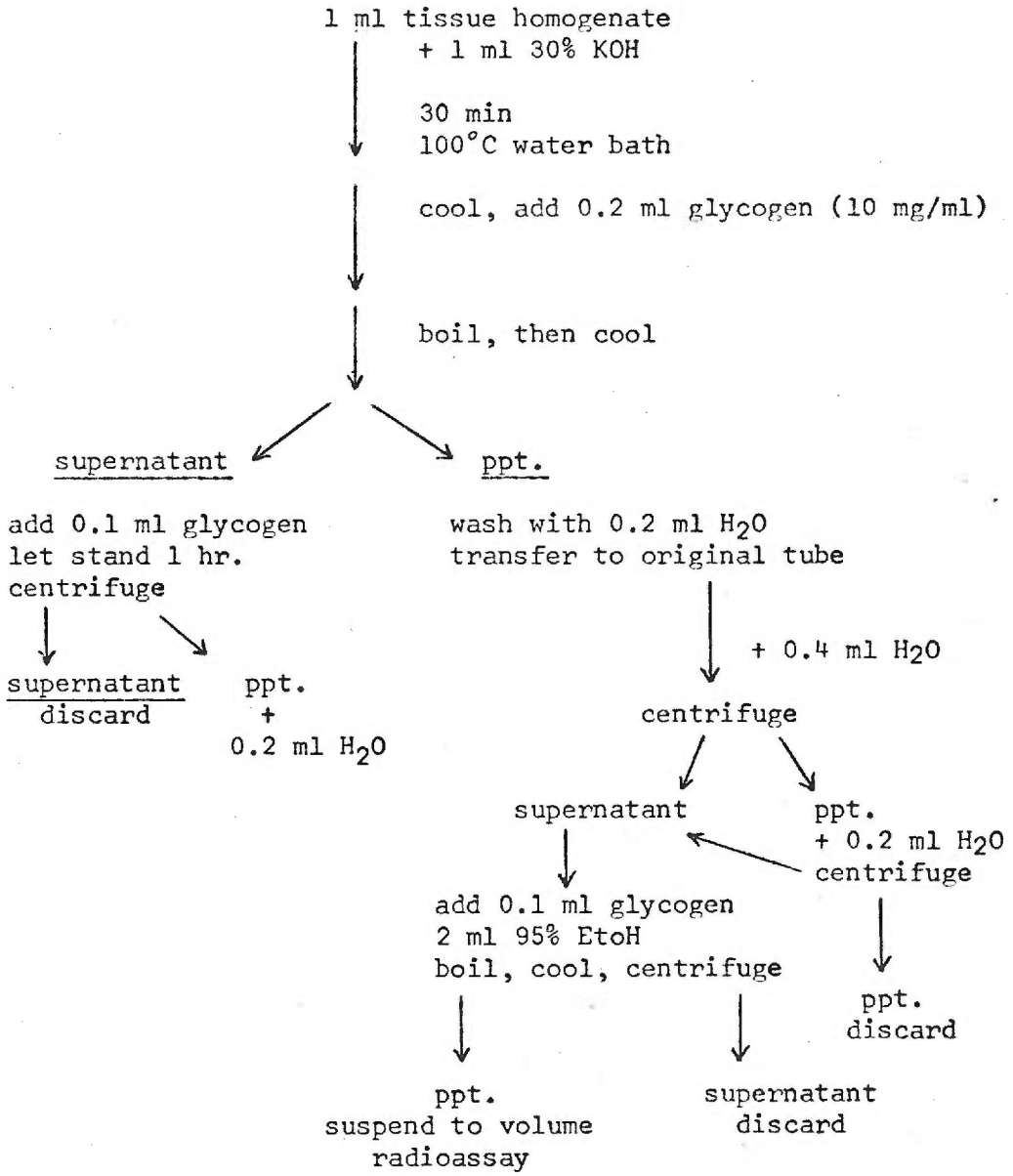
Acetic Acid Recovery

from	cpm added	cpm recovered	% recovery
liver extract	462,724	408,300	88.2
" "	462,724	421,836	91.1
" "	462,724	427,736	92.4
" "	462,724	417,704	90.3

The fractions were collected at one-minute intervals.

5. ^{14}C Glycogen Recovery.

A method of glycogen determination (20) modified by D. T. Wong (108) was used. A flow sheet for the procedure follows.



6. Recovery of ^{14}C Lipid

One ml of tissue homogenate was placed in a 16 mm x 130 mm screw-cap tube containing 5 ml of 11% alcoholic KOH. The tubes were then heated on a steam bath for two hours and the alcohol which had evaporated from the tubes during the saponification was replaced with distilled water. After cooling, the solution was acidified with concentrated HCl. to a pH of less than 3 using pH test paper. The solution was then extracted at least six times with an equal volume of petroleum ether. The petroleum ether was placed directly in Packard scintillation counting vials and taken to dryness in a nitrogen atmosphere. One ml of 95% ethanol was added to dissolve the lipid, the solution heated on a steam bath under nitrogen and the volume reduced to approximately 250 µl. Scintillation fluid was then added to the vial and the sample was counted. Recovery of tracer cholesterol and palmitic acid by this procedure is shown in Table III.

TABLE III

Recovery of ^{14}C Cholesterol and

^{14}C Palmitic Acid from Warburg Homogenates

Standard	cpm added	cpm recovered	% recovery
Cholesterol	434,800	374,980	86.2
	434,800	369,903	83.4
	172,235	168,440	98.0
	171,242	145,009	96.1
Palmitic Acid	449,600	414,071	92.1
	449,600	415,876	92.5
	173,675	149,649	86.3
	172,902	149,405	86.2

RESULTS

1. Development of the Assay Procedure.

In the work on alcohol metabolism the problem of adopting a method for the assay of tissue levels of alcohol dehydrogenase was encountered. A method for this enzyme assay was published by Bonnischen and Brink in Methods in Enzymology in 1955 (8). In use, however, the method was disappointing. In our hands it was possible to find only a trace of activity with rat liver, although rabbit liver showed considerable activity. We were dubious that between species the liver enzyme concentrations could be so different. Also, we knew from other work done here that rat kidney could metabolize ethanol into $^{14}CO_2$ faster than liver, yet no enzyme activity could be found in kidney. Since the theoretical basis for the assay was sound, we began varying conditions in hopes of developing a procedure that would be more efficient. In doing so there were a number of difficulties found representative of those that would be encountered in any assay of an enzyme in a crude tissue homogenate. Since purification almost invariably means denaturation of some of the enzymes, crude tissue preparations are preferred for assays of tissue levels of enzymes. Despite certain limitations of the following procedure, we believe that an assay procedure has been developed that is reasonably accurate and sufficiently sensitive to be useful.

2. Enzyme Kinetics.

In an enzyme assay it is necessary that the reaction must not continue so far that removal of substrates and initiation of the reverse reaction do not cause it to slow down. In the assay of alcohol dehydrogenase the reaction should not be allowed to proceed past a change in absorbance of 0.225. This has been confirmed for enzyme from several sources, as is shown in Fig. III.

Since the purpose of an enzyme assay is to measure the amount of enzyme, the measured activity must be proportional to the amount of enzyme present. This was found to be the case with commercially purified horse liver alcohol dehydrogenase, as can be seen in Fig. IV. However, we were not able to demonstrate such first-order kinetics with respect to enzyme when dilutions of rat liver homogenate were assayed. The greater the concentration of the homogenate, the greater deviation from the expected activity was found (Fig. V). This led us to question whether there were one or more alcohol dehydrogenase inhibitors present in the homogenate. Since dilution of the homogenate would cause dilution of the inhibitors, one would expect greater inhibition at higher homogenate concentrations, as was the case. To verify this, a rat liver homogenate was fortified with varying amounts of purified horse liver alcohol dehydrogenase. First order kinetics were obtained with respect to the added enzyme. Further, the added enzyme was inhibited

FIGURE III

Linearity of Alcohol Dehydrogenase Assay with Time.

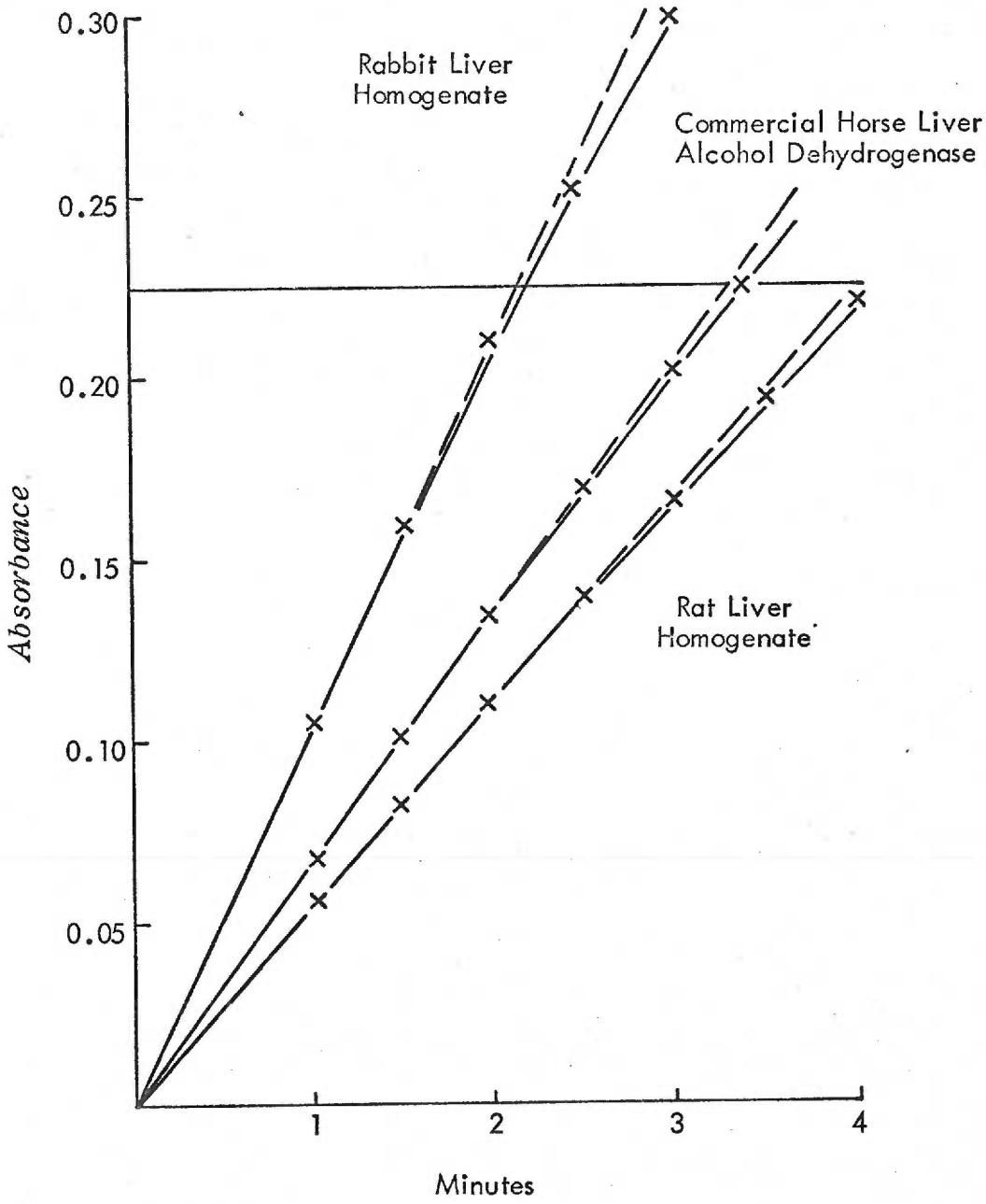

0.30
0.25
0.20
0.15
0.10
0.05
Absorbance
Rabbit Liver
Homogenate
Commercial Horse Liver
Alcohol Dehydrogenase
Rat Liver
Homogenate
1
2
3
4
Minutes

FIGURE IV

Assay of Different Concentrations of Commercial Horse Liver Alcohol Dehydrogenase.

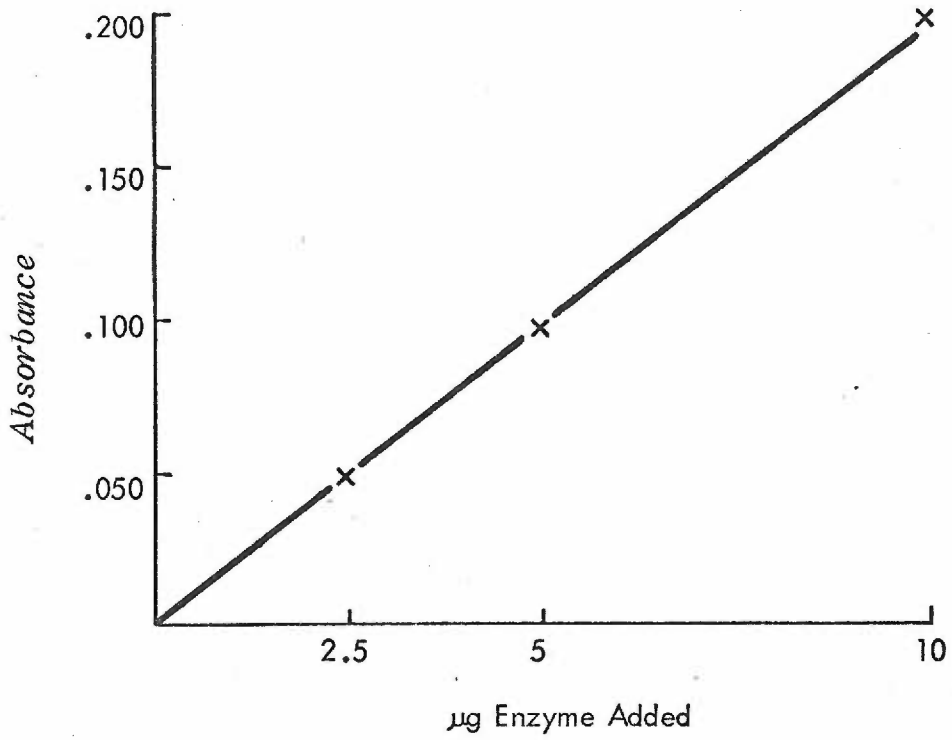
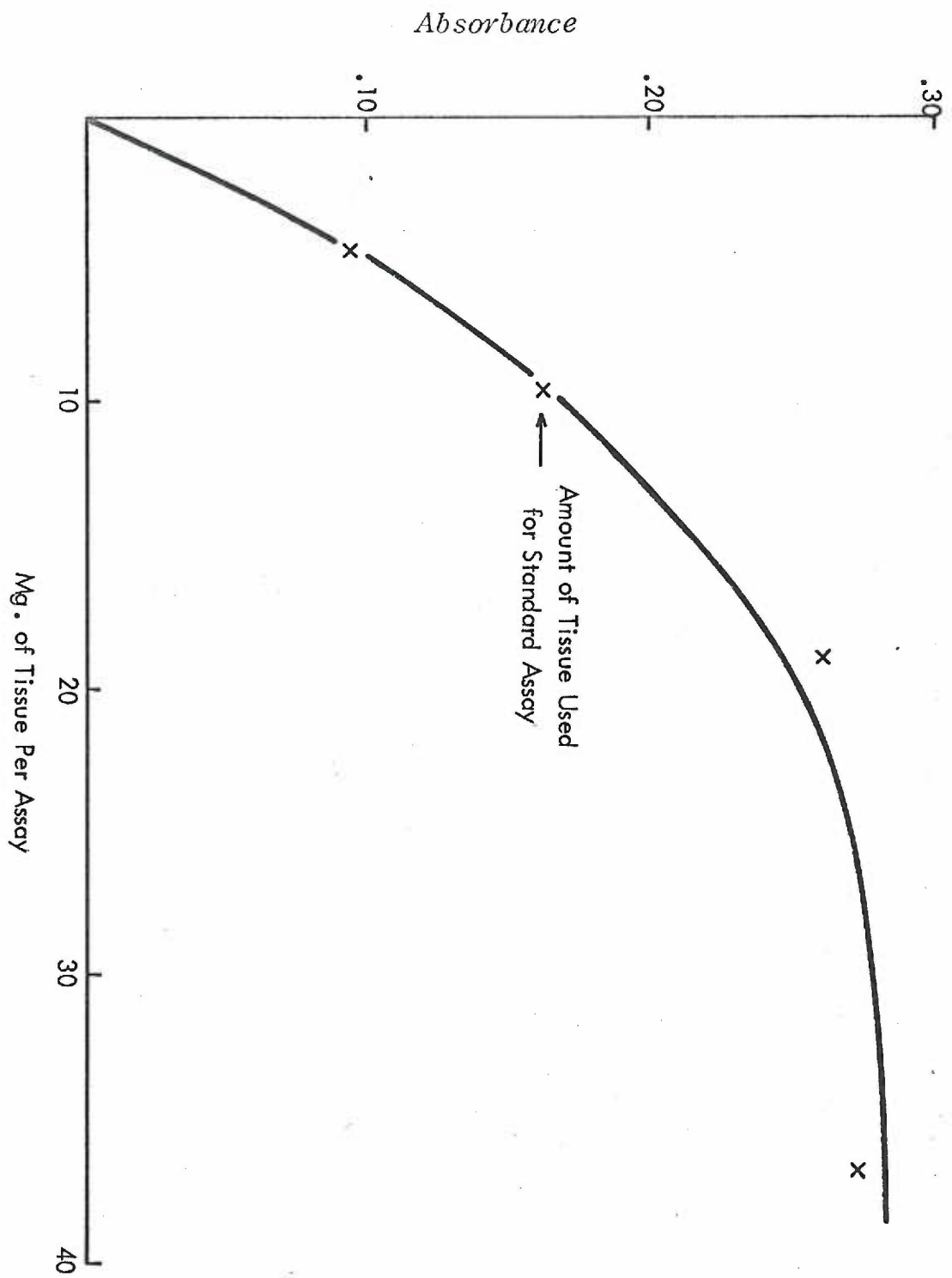

.200
.150
.100
.050
Absorbance
2.5
5
10
µg Enzyme Added

FIGURE V

Activity of Rat Liver Homogenate vs. Amount of Tissue Assayed.


Absorbance
.10
.20
.30
10
20
30
40
Mg. of Tissue Per Assay
Amount of Tissue Used
for Standard Assay

40% by the homogenate, as shown by Fig. VI. When the experiment was repeated using a rabbit liver homogenate, the level of inhibition was below 10%. Thus some metabolic constituent or constituents of a tissue homogenate are able to effect the results of the alcohol dehydrogenase assay. Of course, one can circumvent the problem by using a higher dilution of homogenate. However, this is not practical in any tissue besides stomach and liver, because there is so little activity in such tissues and the amount of enzyme is approaching the limits of sensitivity of the assay procedure. Just what is causing the effect is not known, but many metabolic compounds, such as caproate, imidazole, folic acid, hydroxylamine, acetyl CoA and even chloride ions, have been shown to effect the activity of alcohol dehydrogenase (100, 56).

3. Enzyme Levels.

Enzyme assays were determined on a number of tissues and the activity measured in μmoles min^{-1} g^{-1}. The averages are given in Table IV. As is mentioned in the methods section, the stomach and intestine were assayed with a different amount of ethanol. The use of 100 μl (540 mM) of ethanol rather than 1 μl (5.4 mM) greatly increased the sensitivity of the assay of enzyme from these sources. The stomach and liver showed the greatest enzyme activity, possessing far more activity than the other tissues tested. Both lung and fat tissue contain small amounts of enzyme. In these tissues the assay period was extended

FIGURE VI

Inhibition of Commercial Horse Liver Alcohol Dehydrogenase by Rat Liver Homogenate.

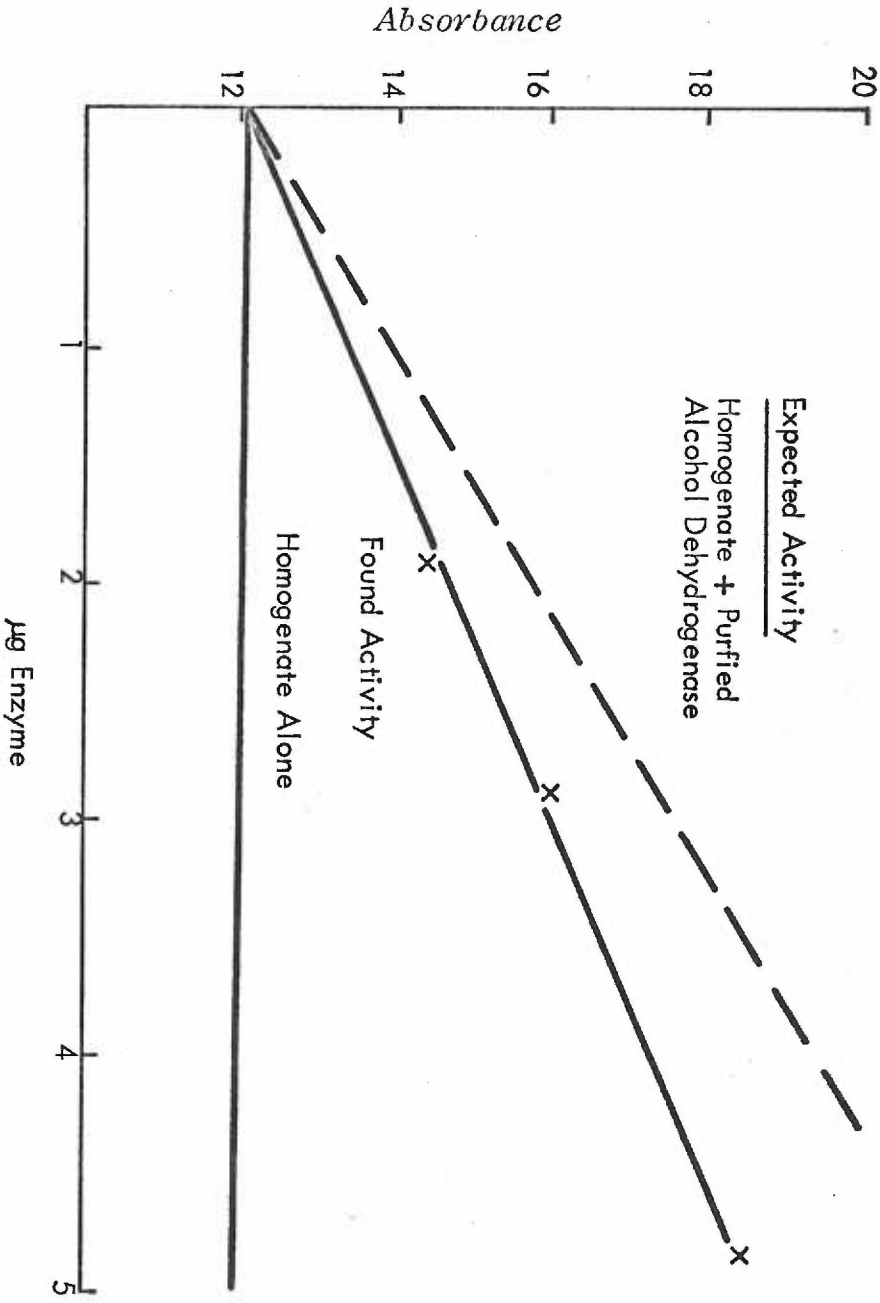

Absorbance
20
18
16
14
12
1
2
3
4
5
μg Enzyme
Expected Activity
Homogenate + Purfied
Alcohol Dehydrogenase
Found Activity
Homogenate Alone

TABLE IV

Alcohol Dehydrogenase Levels in Rat Tissues

Tissue	Animals Tested	Amount of Enzyme µmoles Ethanol min^{-1} g $tissue^{-1}$
Liver	8	2.57
Kidney	4	0.15
Stomach*	3	3.62
Small intestine*	3	0.22
Colon	1	0.30
Lung	1	0.021
Fat	1	0.011
Heart muscle	1	none
Skeletal muscle	1	none

* 540 mM ethanol (100 µl 95% ethanol/3 ml) used in the assay of these tissues.

to fifteen minutes, and a continual and constant reduction of NAD^+ was monitored. Nc activity at all was found in skeletal muscle. Also, two samples of human blood serum were tested and found to be devoid of activity.

The alcohol dehydrogenase activity of liver tissue from several animals other than rat were tested. This is shown in Table V. Rabbit liver contains a very high concentration of alcohol dehydrogenase, much higher than the other species tested.

Kidney tissue of two animals other than rat were also tested for alcohol dehydrogenase (Table VI). Mouse kidney contained approximately the same activity as rat kidney, whereas guinea pig kidney contained considerably less. Human sera contained no detectable amount of enzyme.

4. Rat Stomach Alcohol Dehydrogenase.

During our investigation of rat tissue enzyme levels it came to our attention that the enzyme from the stomach was quite different from that of liver. Most noticeable was its lack of substrate inhibition, as is shown on Fig. VII. The rat stomach enzyme increases in activity with increasing ethanol concentration, while the liver enzyme declines in activity beyond a concentration of 5.4 mM. The graph of the activity of rat stomach homogenate against the ethanol concentration can be plotted on a Lineweaver-Burke plot and gives a straight line. It is

TABLE V

Alcohol Dehydrogenase Levels

in Liver Tissue of Several Animals

Animal	Amount of Enzyme µmoles Ethanol Utilized min^{-1} g^{-1}
Rat	2.57
Mouse	1.67
Dog	1.39
Rabbit	6.61
Guinea pig	1.51

TABLE VI

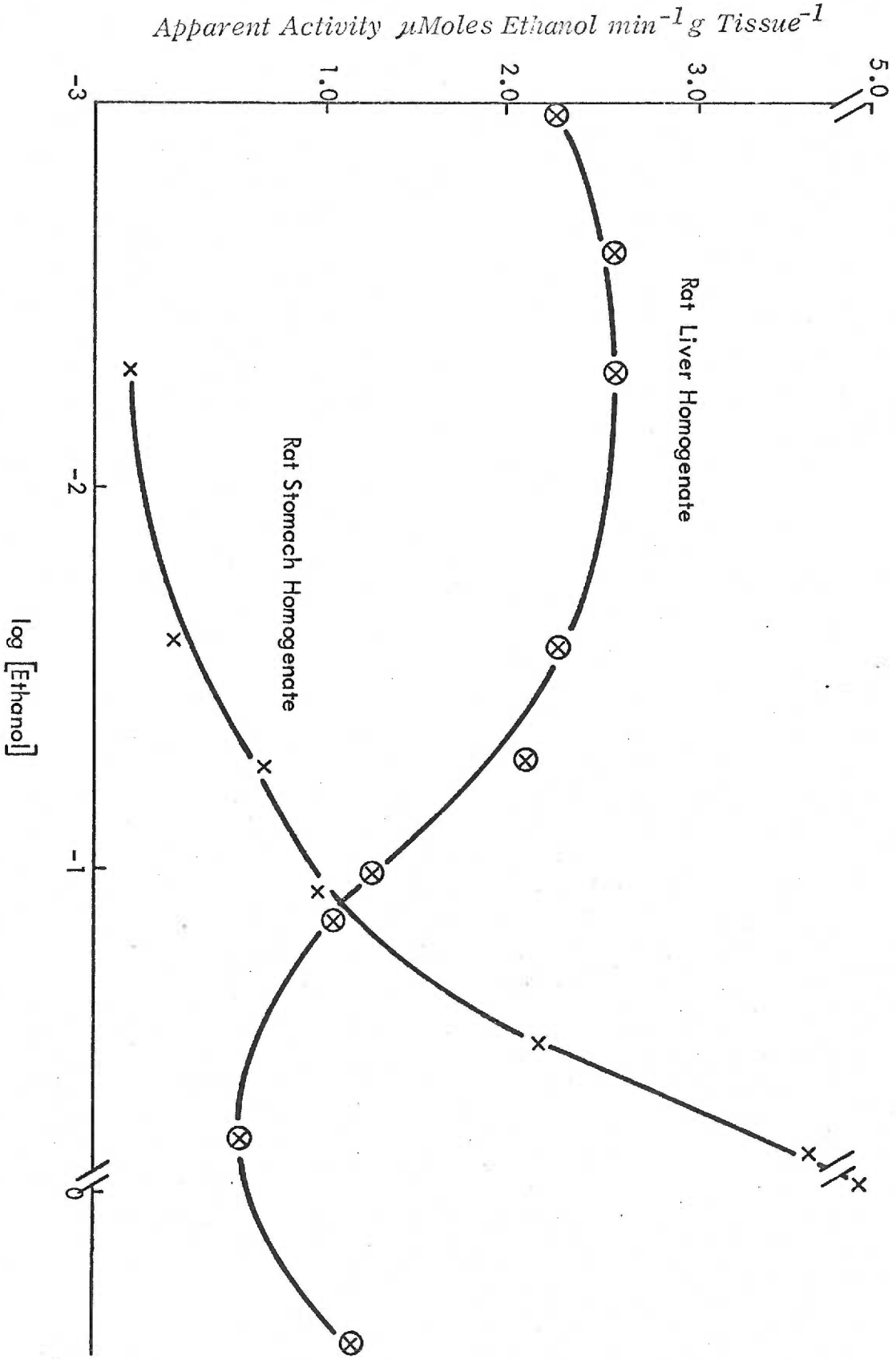
Alcohol Dehydrogenase Levels

in Kidney Tissue of Several Animals

Animal	Amount of Enzyme µmoles Ethanol Utilized min^{-1} g^{-1}
Rat	0.15
Mouse	0.19
Guinea pig	0.016

FIGURE VII

Comparison of Rat Liver and Stomach Homogenates with Varying Ethanol Concentrations.


Apparent Activity μMoles Ethanol min⁻¹ g Tissue⁻¹
1.0
2.0
3.0
5.0
-3
-2
-1
0
log [Ethanol]
Rat Liver Homogenate
Rat Stomach Homogenate

not known why the activity of rat liver homogenate increases when an ethanol concentration greater than 540 mM (100 µl 95% ethanol) is used for the assay. It is possible that some other dehydrogenase enzyme is able to oxidize ethanol at this very high concentration. However, for the present discussion the question is not relevant because of the unphysiological nature of the assay solution.

It was desired to obtain some idea of the alcohol dehydrogenase activity of the tissue homogenates at physiological pH and temperature. Thus these conversions were obtained:

	Activity at pH=7.4 37°, 0.1M Phosphate	Activity at pH=9.6 26°, 0.1N Glycine
Liver and kidney	1.08	1.0
Stomach	0.12	1.0

A Lineweaver-Burke plot was made of stomach homogenate at pH=7.4, 0.1M phosphate, plotting its activity against ethanol concentration. The activity was calculated at an ethanol concentration of 10 mM. It was necessary to do this because there was too little activity to measure directly. The plot is shown in Fig. VIII. A similar plot was made of liver homogenate, Fig. IX.

5. Metabolic Studies.

After having obtained an estimate of the amounts of alcohol dehydrogenase present in the various tissues, it was desired to compare this information with the ability of the tissues to metabolize ethanol. We

FIGURE VIII

Lineweaver-Burke plot of Rat Stomach Alcohol Dehydrogenase.

pH=7.4, 0.1M Sodium Phosphate Buffer.

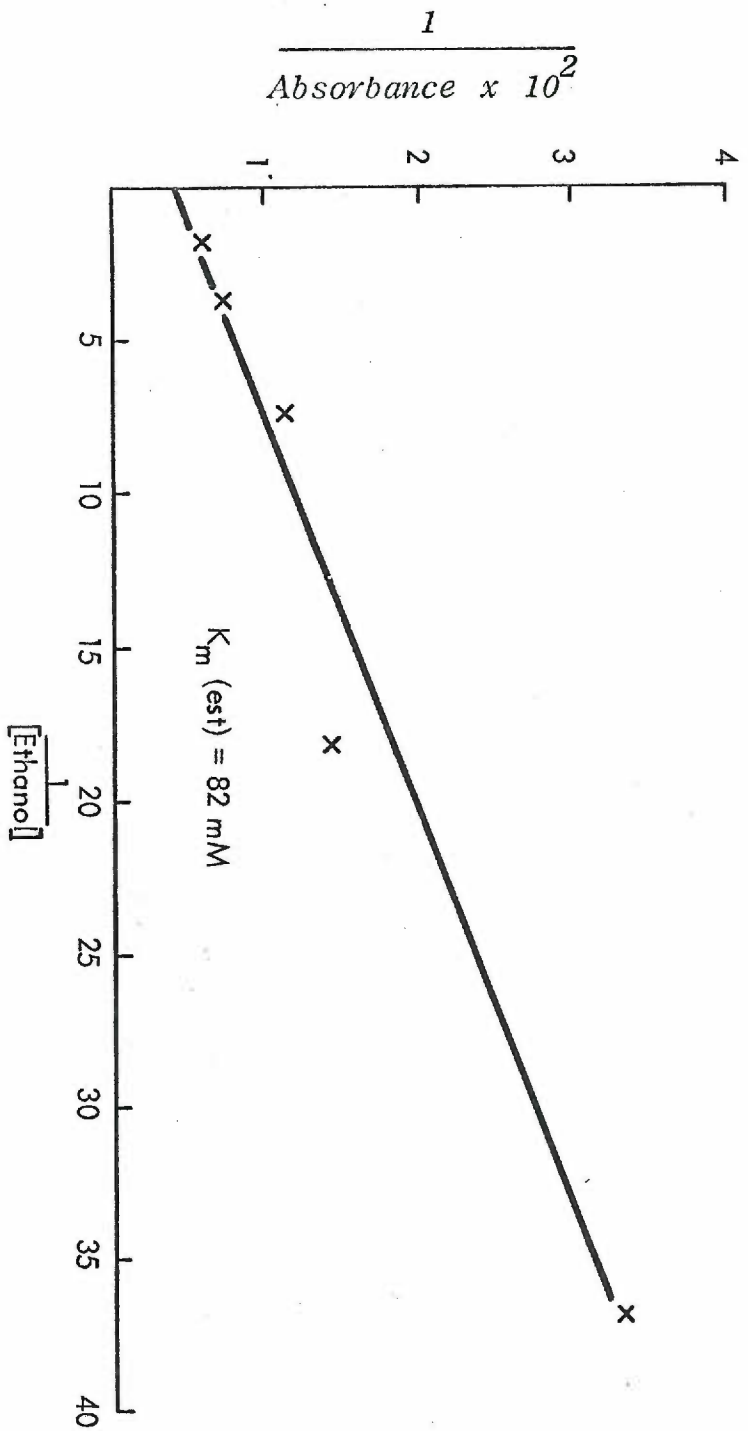
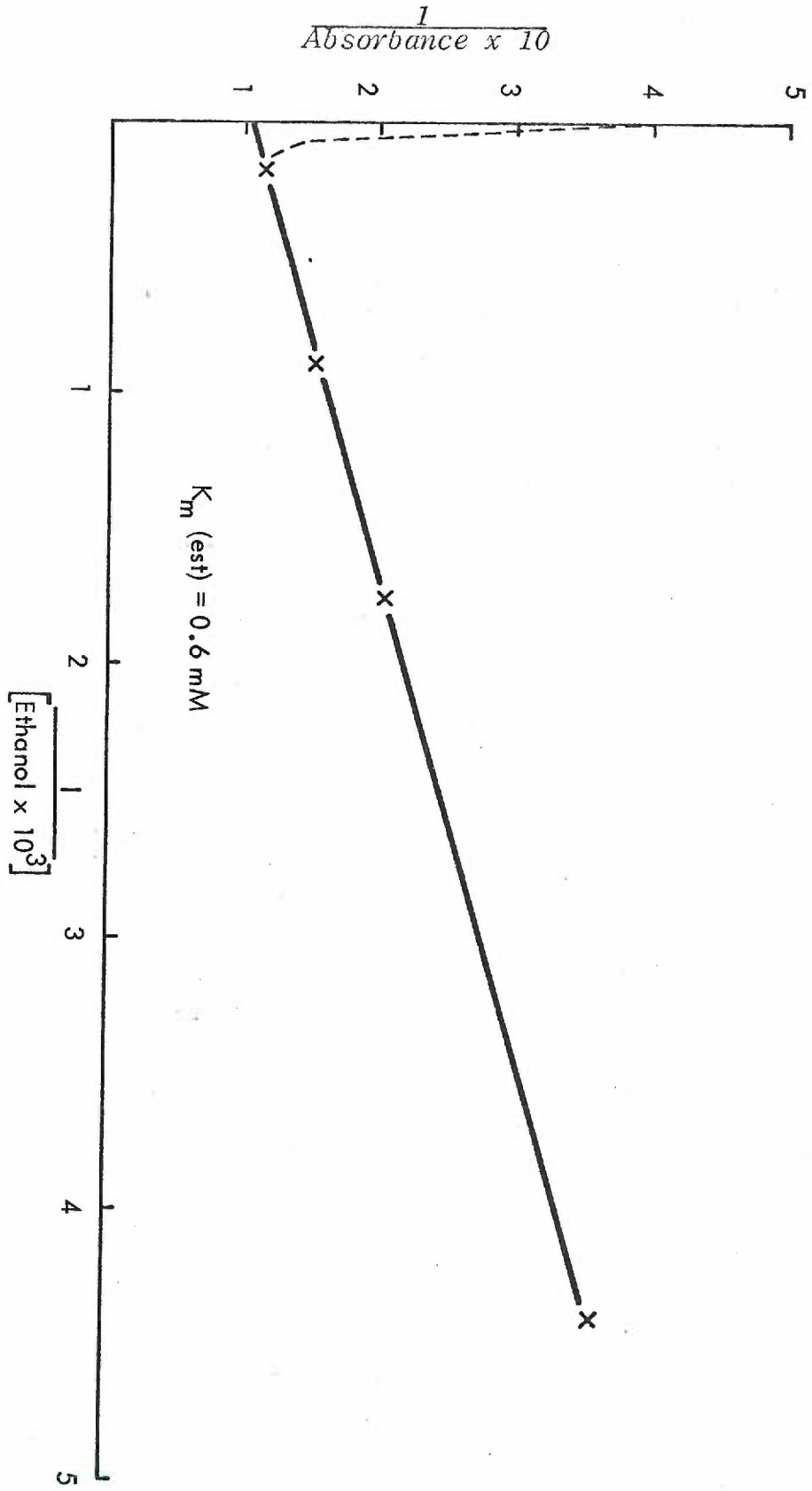

1 / Absorbance x 10²
1
2
3
4
5
10
15
20
25
30
35
40
1 / [Ethanol]
Km (est) = 82 mM

FIGURE IX

Lineweaver-Burke plot of Rat Liver Homogenate.

pH=7.4, 0.1M Sodium Phosphate Buffer.


1 / Absorbance x 10
1
2
3
4
5
K_m (est) = 0.6 mM
1 / [Ethanol x 10^3]
1
2
3
4
5

hoped to get an estimate of the metabolic capability of various tissues to oxidize ethanol by incubating slices in a Warburg bath in the presence of ethanol-1-^{14}C. In addition, it was desired to obtain some idea of what compounds into which the isotope label would be incorporated.

The total amount of ethanol metabolized was measured by collecting the remaining ethanol tracer after the incubation, by gas chromatography and comparing it with the amount of ethanol tracer originally introduced into the flask. There was some loss of the unmetabolized ethanol in several steps, e.g., in the gas phase of the flask, during the homogenization of the Warburg tissue, in the preparation of the solution to be chromatographed, and also during the chromatographic collection. This unrecovered ethanol would be falsely included as part of the ethanol metabolism. In tissues with a low ethanol oxidizing capacity the amount of tracer lost was greater than the amount metabolized. Consequently, a Warburg run was made without tissue, and the solution from the flask treated as though it was a flask containing tissue. The solution was chromatographed and the recoveries determined to average 90.3%. Subsequent correction of ethanol recovery data was made on the basis of the control of 90% recovery.

A precaution must be taken in the interpretation of the ethanol

utilization studies. A relationship but no direct proportionality should be inferred between the percentage of ethanol oxidized by the tissues and their actual capacity to metabolize ethanol. This is a very important point and is due to three factors.

1. A high percentage of the ethanol label is used by most tissues tested. In the case of liver, for example, the 200 mg of tissue consumed 85% of the original label. As the amount of ethanol decreased, the reaction rate decreased also. Thus the rates measured are not initial rates; they are instead average rates. Since those tissues that have the slowest rates of ethanol utilization most nearly approach initial rate kinetics, while the tissues that oxidize ethanol readily show the greatest deviation, the values obtained by the more active tissues will be more effected. Thus the more active tissues may be able to metabolize far more ethanol in proportion to the less capable tissues than the relative per cent label hr^{-1} g^{-1} figures indicate.

2. In an enzyme assay ample substrate is provided, and there is far less enzyme than substrate. Under these conditions the rate of the reaction is proportional to the amount of enzyme present. These conditions did not exist during the Warburg tissue slice experiments. Since the reaction is taking place in the tissues and not in the surrounding medium, tissue concentrations of substrates and enzyme are those that must be evaluated. A comparison of the tissue concentrations

of substrate and enzyme with those employed in the tissue enzyme assay procedure is given:

		Added in assay procedure	Warburg tissue slice concentrations
Ethanol		5.4 mM	≈ 0.15 mM
NAD^+		3.25 mM	0.7 mM
Enzyme	(liver)	0.0086 Units/ml	2.5 Units/g
	(kidney)	0.0005 Units/ml	0.15 Units/g

As can be seen, the concentrations in the Warburg tissue slice experiments are far from ideal. The ethanol concentration is about 1/35 the optimal concentration, while the amount of enzyme in liver and kidney is too large. Consequently the conditions of the assay are more conducive to an assay of the amount of ethanol than the amount of alcohol dehydrogenase. Experimental evidence that this is the case comes from comparison of the data on kidney ethanol metabolism reported in this paper and that reported by Moir (71). The tissues were prepared in the same way and their ability to consume oxygen was the same as found in our study. Approximately the same per cent of the label was incorporated into $^{14}CO_2$, despite the fact that the tracer ethanol concentration used by Moir was approximately four times as high. Also, one experiment was done by us in which the amount of tracer was cut in half without changing the per cent label utilization. Consequently the rate of the reaction in the tissue slice preparations

is more dependent upon the ethanol concentration than upon the amount of enzyme present.

3. It is possible that in some tissues an enzyme in addition to alcohol dehydrogenase contributes to alcohol metabolism. If this is the case and the enzymes have different Michealis constants for ethanol then the contribution to ethanol metabolism of these tissues will change in comparison to other tissues depending on the concentration of ethanol.

The oxygen consumption values for various tissues are given in Table VII. As can be seen, kidney is quite a metabolically active organ, consuming more oxygen than the other tissues.

Table VIII shows the metabolism of ethanol by liver slices. The acetate fraction contained the greatest amount of the isotope label. A surprisingly small percentage is fully oxidized to $^{14}CO_2$. The glycogen and lipid fractions contained small amounts of the label. It had been expected that a greater percentage of the label would be found in the lipid fraction. However, the rats were starved at least 10 hours, and this has been demonstrated to significantly reduce lipid synthesis from acetate (109). A fairly large percentage of the label has not been identified, and is probably largely incorporated into Kreb's cycle intermediates.

Table IX shows the metabolism of ethanol by kidney slices. Kidney

TABLE VII

O_2 Consumption in Tissue Slice Preparations

Tissue	Number of Flasks	O_2 Consumption in μmoles hr^{-1} 100 mg^{-1}
Liver	27	3.9 ± 0.4
Kidney	35	11.5 ± 1.2
Stomach	10	2.1 ± 0.5
Intestine	19	1.92 ± 0.71
Muscle	12	1.12 ± 0.23

Mean ± standard deviation

TABLE VIII

Fate of Ethanol-1-^{14}C in Liver Slices

Utilization of Ethanol % Label Utilized hr^{-1} 100 mg^{-1}	Recipient Metabolite % Label Recovered hr^{-1} 100 mg^{-1}			
	$^{14}CO_2$	^{14}C Acetate	^{14}C Lipid	^{14}C Glycogen
*43.46 ± 6.60	5.80±	23.28±	0.16±	0.14±
	0.74	4.60	0.06	0.06

* mean ± standard deviation

No. of flasks = 27

TABLE IX

Fate of Ethanol-1-^{14}C in Kidney Slices

Utilization of Ethanol % Label Utilized hr^{-1} 100 mg^{-1}	Recipient Metabolite % Label hr^{-1} 100 mg^{-1}			
	$^{14}CO_2$	^{14}C Acetate	^{14}C Lipid	^{14}C Glycogen
*29.34 ± 3.89	16.44 ± 2.62	1.58 ± 0.75	0.07 ± 0.01	0.06 ± 0.03

* mean ± standard deviation

No. of flasks = 34

was able to readily metabolize ethanol, but in this system it did not have the ethanol-oxidizing capacity of liver. Kidney was much more capable than liver of utilizing acetate, as shown by the much lower percentage of the isotope label as acetate, and the greater label incorporation into $^{14}CO_2$. Only small amounts of label were present in the glycogen and lipid fractions.

Table X shows the metabolism of ethanol by stomach slices. Stomach was able to metabolize ethanol, but not extremely well. Significant amounts of the metabolized ethanol were located as ethanol and acetate.

Table XI shows the metabolism of ethanol by intestine. One intestine possessed an extremely high capacity to metabolize ethanol, as high a capacity as was found in kidney. Intestines from four other animals showed a much lower ethanol oxidizing ability. Acetate contains a significant fraction of the label and in the extremely active intestine a great deal of the label was found as acetate.

Muscle tissue was able to metabolize a small amount of ethanol (Table XII). In measuring the amount of ethanol remaining, as was done in this work, those tissues that have low rates of ethanol oxidation will be most subject to experimental error. For example, the difference between 85% and 95% recovery of ethanol does not seem great, yet the lower figure will give an ethanol utilization rate three times greater than the higher. Since the number of flasks used to study muscle ethanol

TABLE X

Fate of Ethanol-1-^{14}C in Stomach Slices

Utilization of Ethanol % Label Utilized hr^{-1} 100 mg^{-1}	Recipient Metabolite % Label Recovered hr^{-1} 100 mg^{-1}			
	$^{14}CO_2$	^{14}C Acetate	^{14}C Lipid	^{14}C Glycogen
*9.41 ± 2.80	1.25 ± 0.19	1.29 ± 0.40	-	0.03 ± 0.003

* mean ± standard deviation

No. of flasks = 10

TABLE XI

Fate of Ethanol-1-^{14}C in Intestine Slices

Utilization of Ethanol % Label Utilized hr^{-1} 100 mg^{-1}	Recipient Metabolite % Label hr^{-1} 100 mg^{-1}			
	$^{14}CO_2$	^{14}C Acetate	^{14}C Lipid	^{14}C Glycogen
*10.98 ± 2.39	2.34 ±	2.61 ±	0.06 ±	0.03 ±
	0.80	2.61	0.02	0.003

* mean ± standard deviation

No. of flasks = 19

TABLE XII

Fate of Ethanol-1-^{14}C in Muscle Slice Preparations

Utilization of Ethanol % Label Utilized hr^{-1} $100\ mg^{-1}$	Utilized as:	
	$^{14}CO_2$	^{14}C Acetate
*4.20 ± 1.93	0.41 ± 0.09	0.42 ± 0.08

* mean ± standard deviation

No. of flasks = 12

metabolism was not great, we therefore do not wish to put a great deal of reliance on the percentage of label incorporation by muscle. However, muscle tissue is definitely able to metabolize ethanol, as is shown by the incorporation of label into the $^{14}CO_2$ and acetate fractions.

A summary of the amount of label utilized by the various tissues is given in Table XIII.

The elution of radioactivity from the gas chromatograph is shown in Figures X, XI, and XII for liver, kidney, and stomach homogenate. In the liver homogenate there was at least one peak eluting from the column after the acetate peak that contained a significant portion of the ethanol label. The identity of the compound or compounds responsible is not known. No analogous peaks were found in kidney and stomach Warburg homogenates. It is possible that this represents the label present in one or more of the Kreb's cycle acids. This is consistent with the results from the Warburg fraction analysis which demonstrated a considerable amount of label present in unidentified fractions.

No other important peaks other than those formed by ethanol and acetate are found in the homogenates of any of the tissues tested.

TABLE XIII

Utilization of ^{14}C Ethanol in Several Tissues

Tissue	% label utilized hr^{-1} 100 mg $tissue^{-1}$
Liver	43.5* ± 6.6
Kidney	29.3 ± 3.9
Stomach	9.4 ± 2.8
Intestine	11.0 ± 2.4
Muscle	4.2 ± 1.9

* mean ± standard deviation

FIGURE X

Gas Chromatograph of Dioxane Precipitated Liver Warburg Homogenate: Elution of Radioactivity

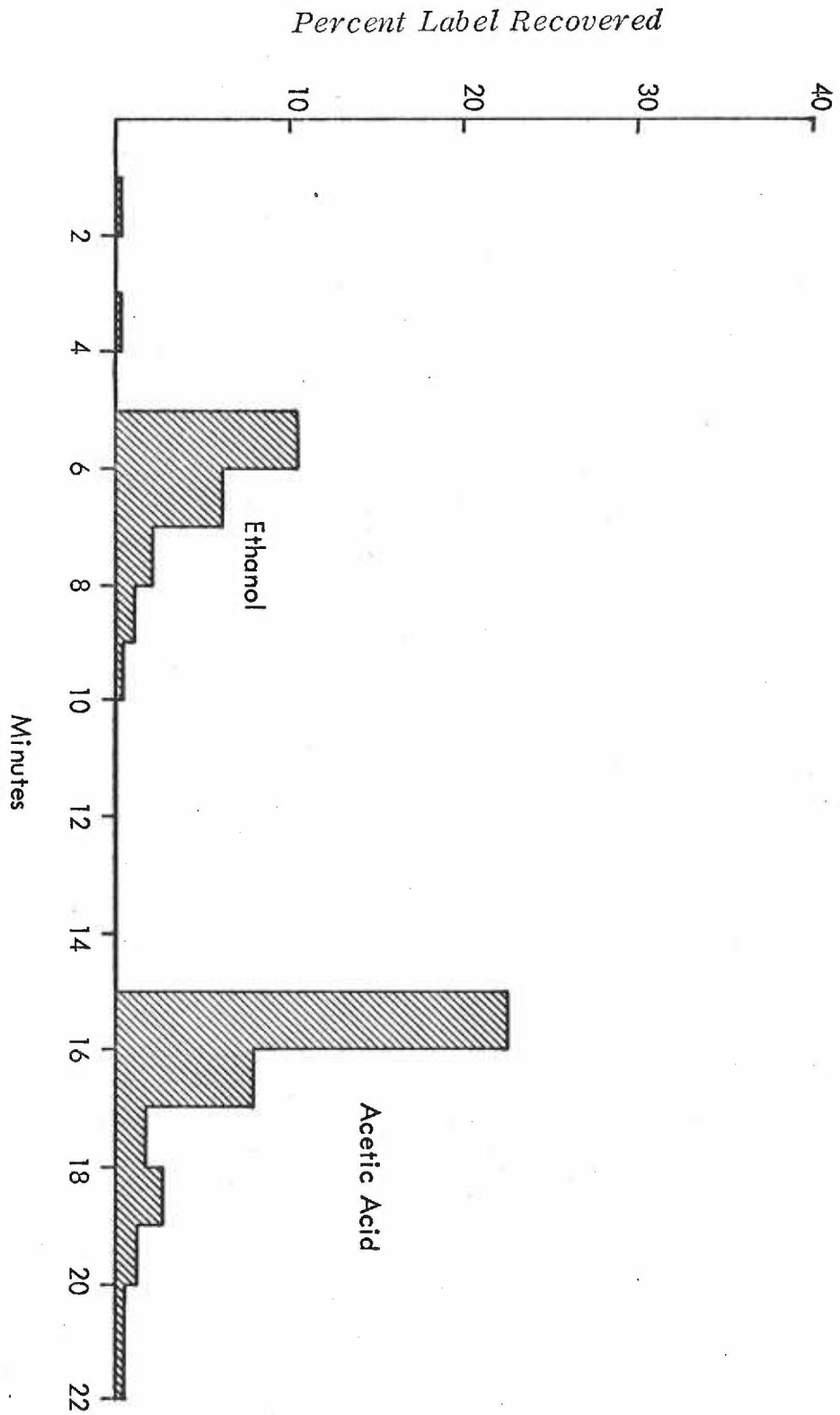

Percent Label Recovered
10
20
30
40
2
4
6
8
10
12
14
16
18
20
22
Minutes
Ethanol
Acetic Acid

FIGURE XI

Gas Chromatograph of Dioxane Precipitated Kidney Homogenate: Elution of Radioactivity

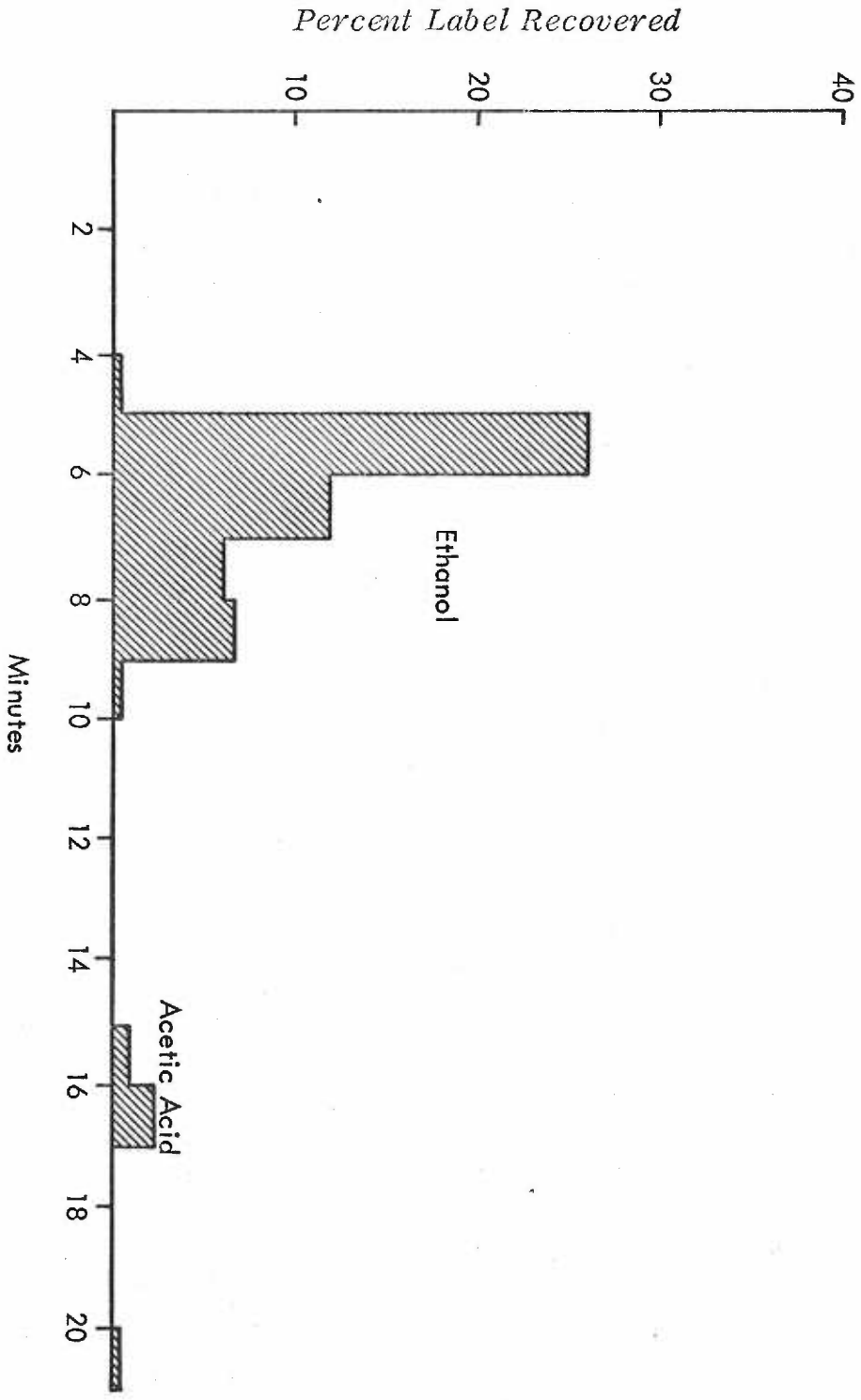
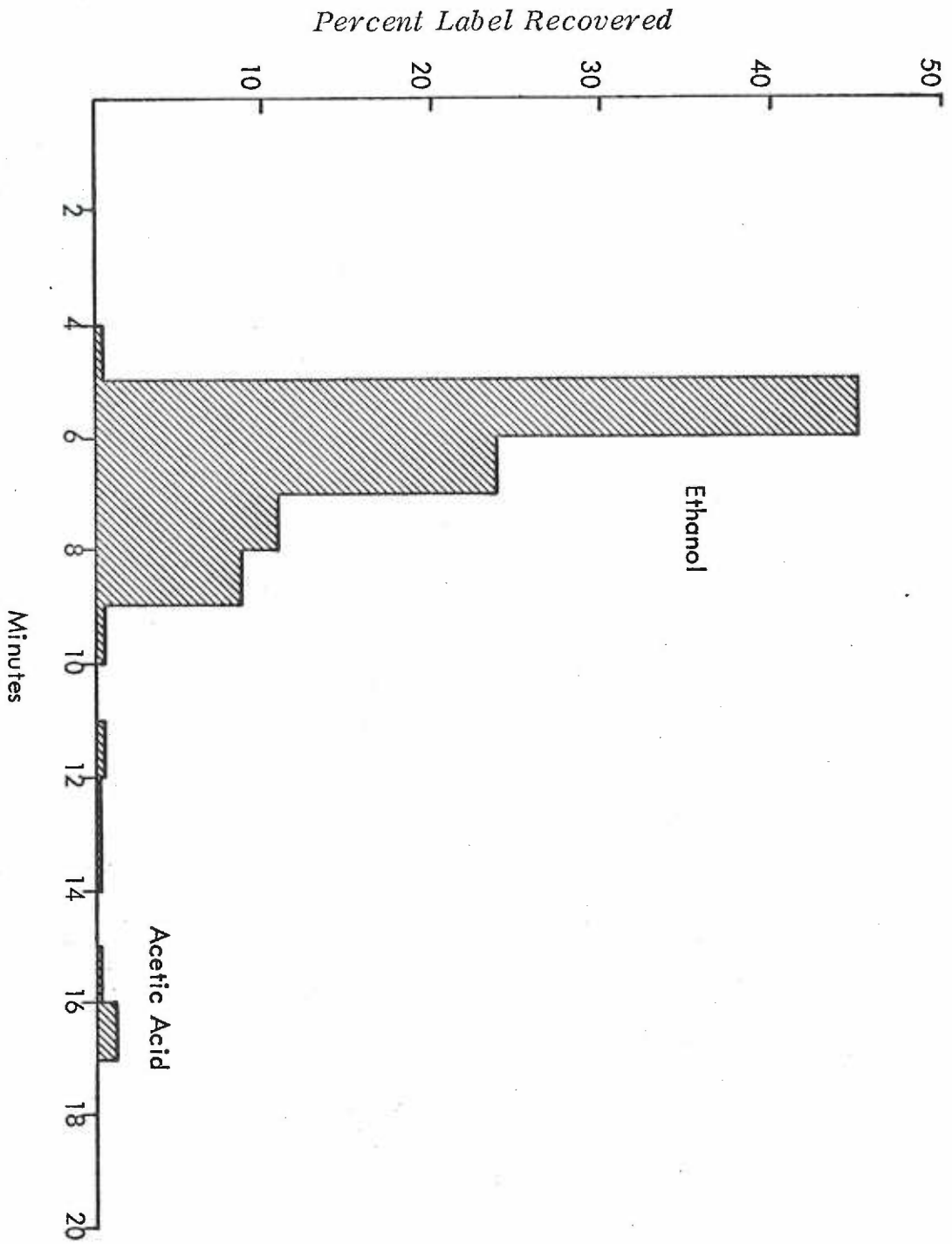

Percent Label Recovered
10
20
30
40
2
4
6
8
10
12
14
16
18
20
Minutes
Ethanol
Acetic Acid

FIGURE XII

Gas Chromatograph of Dioxane Precipitated Stomach Warburg Homogenate: Elution of Radioactivity


Percent Label Recovered
10
20
30
40
50
2
4
6
8
10
12
14
16
18
20
Minutes
Ethanol
Acetic Acid

DISCUSSION

1. The Assay Procedure.

The alcohol dehydrogenase method described in this thesis does provide as good an assay for this enzyme as is possible when working with a crude tissue homogenate. There are, however, several problems still inherent in the method. Inhibitors may profoundly effect the activity of the enzyme and thus yield qualified results. As shown above, we have demonstrated that when known amounts of purified horse liver ADH are added to a rat liver tissue homogenate considerable inhibition of the added enzyme occurs. The failure to obtain first order kinetics when dilutions of a tissue homogenate are assayed is considered a consequence of the presence of inhibitors in the homogenate.

The ADH molecules from the livers of different species are not the same and it appears that isozymes exist in the same species and even in the same individual (100). We have found different maximal ethanol concentrations for rat liver, rabbit liver and purified horse liver enzyme. Thus, the recommended ethanol concentration may not be maximal in every case and should be adjusted by the investigator.

2. The Role of the Liver in Alcohol Metabolism.

There can be little doubt of the importance of the liver in the metabolism of ethanol. It has more enzyme than any other tissue and because of its relatively large mass it is the locus of most of the alcohol dehydrogenase in the body. At pH 7.4, 37°C, at an ethanol

concentration corresponding to a state of light intoxication (a dose of 1-2 grams of ethanol/kg body weight), a liver homogenate has 16 times as much enzymatic activity per weight as kidney and 100-120 times the amount of activity found in the stomach. An average assay of alcohol dehydrogenase at pH 7.4, 37°C in 0.1 M phosphate buffer yields an ethanol oxidizing capacity of 2.5 μmoles ethanol/g tissue/min. Although this number may be a bit low due to inhibition from the homogenate, it probably does not exceed 4.0. This is equivalent to a total body ethanol metabolizing ability of 330 mg/kg/hr. This is slightly higher than the value obtained from blood ethanol elimination studies for the rat (59,3,41). It is possible that the actual metabolic ability of liver ADH is slightly below this value due to the difference in invo NAD^+ concentration (0.7 mM) and that used in the assay (3.25 mM). Still, there is every indication that for a physiological dose of ethanol the rate of alcohol dehydrogenase reaction in the liver is 60-80% of the maximal turnover rate of the isolated enzyme.

The metabolic studies confirm the ability of liver to metabolize ethanol. Our work is in complete agreement with the previous tracer work of Von Wartburg (98) and Bartlett and Barnet (4).

One might think that it would be possible to convert the values obtained from the metabolic studies into a measure of the ethanol oxidizing capacity of the tissue. This is not possible. The reason for this is that there is far too little ethanol available. Assuming that rat liver

has the ability to metabolize 2.5 μMoles ethanol min^{-1} g^{-1}, the value we obtained from enzyme assays, one can calculate its potential metabolic capability at the concentration of ethanol found in the Warburg flasks by using a Lineweaver-Burke plot of activity vs. ethanol (Fig. VIII). The tracer ethanol concentration of ethanol is 80 μM, the endogenous concentration is about 70 μM (57), making a total concentration of 150 μM. Extrapolation of the Lineweaver-Burke plot gives an enzymatic oxidative capacity of 4-6 μMoles of ethanol during a Warburg run at an ethanol concentration of 150 μM. There are, however, at most only 0.46 μMoles of ethanol in the flask. Not surprisingly, most of the tracer is utilized during the reaction.

Both Von Wartburg (98) and Bartlett and Barnet (4) made calculations of the metabolic turnover of ethanol based on their tracer work. In both cases the rate of metabolism was not comparable with the results from other sources. This was due to: (1) using the fraction that was incorporated as $^{14}CO_2$ as the measure of oxidation rate, and (2) in both cases the tissue present could maximally metabolize 30 μMoles of ethanol/hr; yet in both cases there was a total of only 30 μMoles of substrate. Thus, as the amount of ethanol declined, the reaction rate declined also. It is our belief that if a tracer study were designed so that the tissues were incubated in a large volume of buffer and substrate, so that during incubation the concentration of substrate would not significantly change, and if the disappearance of alcohol from the medium were used as the

determining rate, rather than the production of $^{14}CO_2$, then the ethanol oxidative capacity measured in this way would approximate the results obtained from enzyme assays and from blood elimination studies. To summarize: *in vitro* measures of the ethanol oxidizing capacity of the liver are not consistent with the results obtained by other measures, at least partly because of the experimental procedures by which they were obtained.

The most striking aspect of the liver's ability to metabolize alcohol is its low capacity to metabolize the acetate formed in the breakdown of ethanol. A number of researchers have reported the accumulation of acetate during ethanol metabolism (5,20,47,48,28) and our work confirms this. After the end of the one hour incubation period, almost one-half of the ethanol carbon is in the form of acetate. Thus there is a major difference between the metabolism of ethanol in the liver and in other tissues. In the liver, at least, a rate-limiting step is not the first step of the breakdown of ethanol but instead the removal of acetate to a site where it can be metabolized. Lundquist has found that acetate is primarily metabolized in the peripheral tissue (49). Therefore, the metabolism of ethanol is an example of a process which requires the integration of organ systems. The liver can oxidize the ethanol to acetate, whereupon it liberates the acetate to the blood for metabolism by the other organ systems. Lundquist finds that the concentration of acetate in the blood is never greater than 5% of that of the

ethanol concentration, indicating that the metabolism of acetate by peripheral tissues is efficient.

To summarize: (1) The liver has the greatest concentration of alcohol dehydrogenase of any tissue tested, and is able to metabolize ethanol at the greatest rate of any of the tissues tested. (2) Measures of maximal ethanol oxidation capacities derived from enzyme assays agree quite well with rates obtained from blood ethanol elimination studies. Therefore, if alcohol dehydrogenase is the enzyme primarily responsible for the breakdown of ethanol, then it must do so at a rate approximating the maximal capacity of the enzyme. (3) In the liver the rate of ethanol metabolism exceeds that of acetate, and consequently acetate accumulates. The acetate is released into the blood and other organs contribute to the subsequent metabolism of acetate.

3. Kidney.

The rat kidney contains about one-sixteenth the amount of alcohol dehydrogenase that is found in rat liver, kidney having an ethanol oxidizing capacity of 10 μMoles hr^{-1} g^{-1}. It must be kept in mind that this is a measure of the maximal rate of ethanol oxidation by the alcohol dehydrogenase of a tissue homogenate. As we have seen, the rate obtained in this fashion is quite consistent with other measures of the ethanol oxidizing capacity in the liver. In the kidney, however, the information obtained from an enzyme assay is not consistent with other calculations of ethanol oxidizing capacity. Both Von Wartburg (98) and Bartlett and

Barnet (4), using tissue slice preparations, studied the ability of kidney to oxidize 10 mM tracer ethanol into $^{14}CO_2$. This level of ethanol should allow the alcohol dehydrogenase reaction to proceed at a nearly maximal rate. Von Wartburg found that kidney was able to convert approximately 140 μMoles of ethanol into CO_2 hr^{-1} $tissue^{-1}$, whereas Bartlett and Barnet obtained a value of about 60 μMoles hr^{-1} g^{-1}. These values are approximate and required a conversion from dry tissue weight to wet tissue weight, using a ratio of dry weight to wet weight of 0.4. These values are much in excess of the value obtained from enzyme assay. Unfortunately, what is clearly lacking is a carefully designed kidney perfusion study that would accurately measure a simulated *in vivo* rate of kidney ethanol metabolism. In its absence the best measures available are those obtained from the tissue slice experiments. Of course, these values are obtained by measuring the $^{14}CO_2$ produced, rather than the more desirable information obtained by measuring the elimination of ethanol. The metabolic study of kidney ethanol metabolism done by us used a much smaller ethanol concentration than used by Von Wartburg and Bartlett and Barnet, but we did measure the disappearance of ethanol as well as the $^{14}CO_2$ production. We found that about two-thirds of the oxidized ethanol was metabolized fully to $^{14}CO_2$. This is in contrast to the results obtained in liver, where only about 10% of the isotope label is metabolized to $^{14}CO_2$, the greater part being found in acetate. The best estimate of the rate of ethanol

metabolism in the kidney available from kidney slice studies is somewhere around 150 μMoles of ethanol hr^{-1} g^{-1}, about the same rate obtained for liver. Thus, an explanation of the metabolism of ethanol by kidney must be consistent with the following: kidney is able to metabolize ethanol at a rate comparable with that of liver, yet apparently contains only 1/16 the amount of enzyme as does liver.

There are three possible explanations for this result:

1. The amounts of enzyme in the rats used by Bartlett and Barnet and Von Wartburg, from which the rate of ethanol metabolism is based, and those used by us to obtain enzyme assays, might be significantly different. However, this is unlikely in view of the ease with which kidney tissue was able to metabolize ethanol in our Warburg experiments. It is interesting to note that the work Von Wartburg, Bartlett and Barnet, and our work all show that kidney is able to convert tracer ethanol into $^{14}CO_2$ about 2.5 to 3 times as fast as liver. Thus there does not seem to be any evidence of different metabolic capabilities between the rats used by us and other experimenters.

2. The methods used in the assay of alcohol dehydrogenase may not give an accurate estimate of the enzymatic capacity of the tissue. This might be a result of measuring different isozymes of alcohol dehydrogenase. This, as will be shown, is the case in the stomach and intestine. However, an assay of kidney at pH 7.4, in 0.1 M phosphate buffer at 37 C yields about the same activity as does an assay with the standard assay

conditions. This is true of liver as well as kidney, indicating that at least in this respect the enzymes are the same. Indeed, we have found no evidence to indicate that the enzyme from the two sources might be different. Also, Von Wartburg (100) found that rhesus monkey liver and kidney contain the same isozyme of alcohol dehydrogenase.

The possibility that the enzymatic activity of kidney might be located in the microsomal or mitochondrial fractions and thus not released in normal homogenization procedures was investigated by adding the non-ionic surface acting agent Triton X-100 to the homogenizing buffer. A forty percent increase in activity was found. However, this is the same effect that is reported in the literature (75) for rat liver so treated. It was demonstrated that only half the increase in activity was due to liberated enzyme, the other half due to an activating effect on the enzyme itself. Thus one cannot account completely for the enzymatic activity of kidney as being due to unreleased enzyme from particulate fractions. Another possibility is that a strong inhibitor of alcohol dehydrogenase, partitioned from the enzyme in the intact cell but released during homogenization, could mask the enzymatic activity during the assay procedure. Conversely, it is possible that some metabolite is able to activate the enzyme *in vivo* but is unable to do so in the assay situation.

3. It is possible that an enzyme other than alcohol dehydrogenase is responsible for the major part of alcohol metabolism in the kidney.

The possibility has been discussed by Von Wartburg, who believes that catalase is the enzyme responsible for the remarkably high ethanol metabolizing ability of kidney (98). The question of the contribution of catalase to kidney ethanol metabolism is one that deserves further study. Von Wartburg has found that acetaldehyde accumulates in the base in the center well of the Warburg flask during the metabolism of ethanol by kidney but not by liver. He argues that kidney has a very high potential capacity to metabolize ethanol due to the contribution of catalase, and that the limiting factor in the metabolism of ethanol is the rate of acetaldehyde formation. He pretreated rats with 3-amino, 1,2,4 triazole, a known catalase inhibitor, in such a manner that 90% of the body catalase was inhibited. Kidney slices from these animals did not accumulate acetaldehyde with 0.16 M ethanol substrate. However, this concentration of ethanol is sufficiently high to partially inhibit kidney alcohol dehydrogenase, and this could be the cause of the decreased acetaldehyde formation. A much more meaningful experiment would be to measure the ethanol metabolism of kidney slices from 3-amino-1,2,4 triazole treated animals. If catalase plays an important role in kidney ethanol metabolism, the oxidation rate of such tissues should be reduced.

4. The Stomach and Alcohol Metabolism.

Prior to this study no metabolic study had been done to confirm or deny the possible role of stomach tissue in alcohol metabolism, but Spencer (87) reported alcohol dehydrogenase levels in stomach eight times

greater than those of liver. We were able to reproduce the results that Spencer obtained, but were not able to show an extremely high enzyme assay with our assay procedure. We then discovered the reason for this incongruity. Our assay used 1 µl of ethanol; Spencer used .100 µl, as recommended by Bonnicshen and Brink (8). A comparison of ethanol activity revealed that stomach activity increased with increasing ethanol concentration up to 1M, and therefore exhibited no substrate inhibition. This was surprising since all previous alcohol dehydrogenases from animal tissues had exhibited substrate inhibition by ethanol at 2 to 20 mM. The yeast enzyme, a molecule over twice as large as the liver enzyme, with twice as many active sites, does not exhibit substrate inhibition. The possibility occurred to us that the enzyme might come from stomach bacteria that were not washed free from the tissue. However, thorough washing does not remove the enzyme, and separation of the stomach mucosa from the stomach muscle wall revealed that the activity was located in the muscle wall.

Another surprising feature of the stomach enzyme was discovered when stomach homogenate was assayed at pH=7.4, in sodium phosphate buffer using 100 µl of ethanol. The change in pH and buffer resulted in a decline in activity to one-seventeenth its previous value. When the same change in pH and buffer was employed with liver homogenate, only a decline of about one-half the activity was found. Since pH=7.4 is more physiological than pH=9.6, we made an attempt to estimate the true physiological

activity of the enzyme. By the time one has changed to a pH=7.4, 0.1 M phosphate buffer and decreased the ethanol concentration to 10 mM, it is clear that the remaining activity is very low. We plotted the activity of a stomach homogenate vs. the concentration of ethanol on a Lineweaver-Burke plot and found a straight line. It was necessary to extrapolate the activity of the homogenate to a concentration of 10 mM, a state of light intoxication when this concentration is present in the blood, because the activity is so low it cannot be measured directly. A rough correction for Q_{10} was made, since the data was collected at 26°C instead of 37°C. The resultant activity was 1.0-1.5 μMoles ethanol hr^{-1} g $tissue^{-1}$. When one compares this to 150 μMoles hr^{-1} g $tissue^{-1}$ for liver, it is apparent that the activity of rat stomach alcohol dehydrogenase is quite low. Two important conclusions can be drawn from these results.

1. The results obtained by Spencer (87) indicating a high ethanol oxidizing capacity for rat stomach are an artifact of the assay conditions employed.

2. The alcohol dehydrogenase present in rat stomach is kinetically quite different from that found in other tissues. Consequently, an assay of the enzyme from this source should not be compared directly with other isozymes of alcohol dehydrogenase.

In order to confirm that the rat stomach enzyme was actually different from the liver-kidney enzyme, and that the results obtained were not due to modification of the activity of the enzyme by some constituent of the

homogenate, stomach tissue extracts were fractionated in $(NH_4)_2\ SO_4$ and dialyzed against 0.005M, pH=6.6 sodium phosphate buffer. The kinetic properties did not change. It was interesting to note that the enzyme precipitated in the 30%-55% $(NH_4)_2\ SO_4$ fraction rather than in the 55%-80% fraction, as is the case with the liver enzyme.

Another aspect of stomach ethanol metabolism deserves mention. When discussing ethanol metabolism it is convenient to consider the situation where the blood ethanol concentration is about 10-20 mM, since blood alcohol concentrations generally don't get too much higher than this and since the enzyme activity decreases when the ethanol concentration exceeds this value due to substrate inhibition. However, in the stomach and the rest of the gastrointestinal tract, alcohol concentrations may greatly exceed blood alcohol concentrations. Consider the case of the individual who drinks a great deal on an empty stomach. It would appear on the basis of Lineweaver-Burke plots that if a sufficiently high ethanol concentration were obtained that the stomach could make a significant contribution to ethanol metabolism while the ethanol was still in the stomach. Of course, the question of the permeability of the ethanol into the stomach wall must be considered. Still, it is interesting that the one part of the body that could have an extremely high ethanol concentration also has an enzyme that would not be "shut down" by the high concentration. Of course, this is just speculation and conformation would have to come from further experimental work.

Studies of stomach tissue slices indicate that this tissue is capable of metabolizing ethanol. 9.4% of the ethanol label was metabolized hr^{-1} 100 mg $tissue^{-1}$. It therefore metabolizes ethanol quite a bit less readily than either liver or kidney. Unlike liver, most of the label is not accumulated as acetate.

Thus stomach tissue contains a small amount of alcohol dehydrogenase and apparently the stomach is capable of metabolizing ethanol. Its contribution to the elimination of ethanol from the blood is probably small. However, the rat stomach appears to contain an isozyme of alcohol dehydrogenase characterized by a lack of substrate inhibition, and it may be that the stomach is able to metabolize ethanol while it is still in the stomach, where its concentration may be quite high. If this is the case, its contribution to ethanol metabolism might be quite significant.

5. The Intestine.

The intestine, like the stomach, was reported by Spencer (87) to contain alcohol dehydrogenase, although its concentration was less than one-tenth that of stomach. These findings are confirmed by our data. The enzyme from the intestine, like that of the stomach, does not demonstrate substrate inhibition, and it is reasonable to assume that both tissues contain the same isozyme of alcohol dehydrogenase.

It would be expected from the results of the alcohol dehydrogenase assays that the intestine would be less capable than stomach of oxidizing tracer ethanol. Such was not found to be the case. Tissue slice studies

indicate the intestine is even more capable than stomach of metabolizing ethanol. One intestine (not included in the experimental data) had the amazingly high rate of ethanol utilization of 24% label incorporation hr^{-1} 100 mg $tissue^{-1}$. Eleven percent of this was present as acetate, representing an accumulation of acetate such as found in liver. Just why intestine is more capable of metabolizing ethanol than stomach while having so much less enzyme is not clear. It is possible that it possesses an enzyme other than alcohol dehydrogenase that contributes to the ethanol metabolism. It may also be a question of the availability of coenzyme or an ability of stomach to oxidize acetaldehyde.

Like stomach, rat intestine may play a role in the metabolism of injested ethanol. Since substrate inhibition does not occur when the ethanol concentration becomes high, the intestine, like the stomach, increases its ethanol oxidizing capacity as the concentration of ethanol increases. It is possible that the intestine is able to metabolize part of injested ethanol during the process of absorption.

6. Ethanol Metabolism by Muscle.

Muscle tissue from rat abdominal wall was tested for the presence of alcohol dehydrogenase. No activity was found. This is consistent with a study in which the hind limbs of a rat were perfused with blood containing tracer ethanol and very little $^{14}CO_2$ was produced (29). However, both Bartlett and Barnet (4) and Von Wartburg (98) noticed a slight conversion of C^{14} ethanol into $^{14}CO_2$ by muscle. These isotope studies were consistent

with our own results in which .06% of the label hr^{-1} 100 mg $tissue^{-1}$ was fully oxidized to $^{14}CO_2$. Whether this slight metabolism is due to a very small amount of alcohol dehydrogenase or a result of some other enzyme is not known. However, despite its very high mass in comparison to other tissues in the body, the contribution of the musculature to total body ethanol metabolism is probably quite small.

SUMMARY AND CONCLUSIONS

I. A procedure for the assay of alcohol dehydrogenase from a tissue homogenate was developed. The procedure is sufficiently sensitive to measure alcohol dehydrogenase levels in those tissues that make a significant contribution to ethanol metabolism *in vivo*. Endogenous inhibitors in the homogenate may effect the assay results.

II. An isozyme of alcohol dehydrogenase was discovered in rat stomach and intestine that, unlike the enzyme isolated from other animal sources, does not exhibit substrate inhibition.

III. Alcohol dehydrogenase levels were determined in a number of rat tissue homogenates and in the liver and kidney tissue homogenates of several other species.

IV. Metabolic studies were made of the ability of tissue slices from several sources to oxidize ethanol-1-^{14}C. The results were interpreted in the light of tissue alcohol dehydrogenase levels, and the following conclusions drawn:

A. The liver was able to oxidize the tracer ethanol the most readily of any tissue. Acetate, a metabolic product of ethanol metabolism was accumulated. Enzyme levels were consistent with the interpretation that alcohol is metabolized in the liver primarily by the action of alcohol dehydrogenase.

B. The kidney is able to metabolize ethanol rapidly. There

does not appear to be sufficient alcohol dehydrogenase present to account for this rapid rate of metabolism.

C. The stomach is not able to metabolize tracer ethanol very rapidly. The high values obtained in the assay of alcohol dehydrogenase do not reflect a high enzymatic oxidative capacity for the tissue. There is a possibility that the organ may be able to oxidize an appreciable amount of ethanol when a very high concentration of ethanol is presented to the stomach *in vivo*.

D. The intestine possesses considerable variability in its ability to metabolize ethanol, in some cases being quite high. Alcohol dehydrogenase levels predict a low enzymatic oxidative capacity for the tissue.

E. Muscle tissue is able to slowly oxidize ethanol. No alcohol dehydrogenase was found in this tissue.

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