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Cellular and Enzymatic Basis for Carnitine-Mediated Attenuation of Ethanol Metabolism

Youn-Soo Cha
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Youn-Soo Cha entitled "Cellular and Enzymatic Basis for Carnitine-Mediated Attenuation of Ethanol Metabolism." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Dileep S. Sachan, Major Professor

We have read this dissertation and recommend its acceptance:

Frances A. Draughon, James W. Bailey, Michael B. Zemel

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Dr. Frances Ann Draughon

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Accepted for the Council:

Associate Chancellor
and Dean of The Graduate School

CELLULAR AND ENZYMATIC BASIS FOR CARNITINE-MEDIATED ATTENUATION OF ETHANOL METABOLISM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Youn-Soo Cha

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ABBREVIATIONS: ADH = alcohol dehydrogenase, MEOS = microsomal ethanol-oxidizing system, NAD = nicotinamide adenine dinucleotide, NADP = nicotinamide adenine dinucleotide phosphate, ALDH = aldehyde dehydrogenase, BEC = blood ethanol concentrations, HEC = Hepatic ethanol concentration, KHB = Krebs and Henseleit buffer, BSA = bovine serum albumin, PMS = Post-microsomal supernatant, SN = supernatant

PART I

INTRODUCTION

Alcohol (ethanol) is the most widely used of psychoactive drug and has been known in almost all civilizations since ancient times (Keller 1979). There are millions of people throughout the world who responsibly enjoy consuming alcoholic beverages served as food, medicines, and euphoriants. However, alcohol abuse, alcohol intolerance, alcohol dependence and other alcohol-related disabilities are some of the most challenging social, economical and public health problems facing our modern-day society. In the United states alone, the annual cost of lost productivitity and health expenses related to alcohol is estimated to be $117 billion (Hoek et al. 1992).

Ethanol is known to cause a wide variety of metabolic abnormalities. Some of these abnormalities are the direct effect of ethanol while others are the result of ethanol induced malnutrition (Lieber 1991). Ethanol administration both in humans and laboratory animals, results in hyperlipidemia, fatty liver, and ultimately the most severe stage of alcoholic liver disease: scarring or cirrhosis of the liver. It has been recognized that the metabolism of ethanol plays a major pathogenic role in the etiology of various diseases associated with alcohol abuse. Approximately 90% of ingested ethanol is metabolized in the liver. The liver enzyme systems able to metabolize ethanol are; alcohol dehydrogenase (ADH), the microsomal ethanol oxidizing system (MEOS), and catalase (Larsen 1959).

Carnitine (3-hydroxy-4N-trimethylammonium butyrate), an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix is synthesized in all mammals, and consequently is present in diets containing meat products (Bieber 1988). Sachan et al. found that carnitine supplementation lowered ethanol-induced increases of various lipid fractions in rat's liver (Sachan and Rhew 1983, Sachan et al. 1984) in a dose related manner (Rhew and Sachan 1986). They hypothesized a state of functional carnitine deficiency in chronic alcoholism and proposed that dietary carnitine supplementation may prevent ethanol-induced hepatic diseases. Further, they demonstrated that dietary carnitine supplementation elevated blood ethanol concentration (BEC) following both acute and chronic doses of ethanol and the increase in BEC was dependent on the level of carnitine supplementation (Berger and Sachan 1986, Sachan and Berger 1987, Berger and Sachan 1991, Sachan 1992). This effect of carnitine is specific to carnitine since it could not be reproduced by choline (Sachan and Berger 1993). It was also shown that at 6 hours post ethanol administration on the third day of carnitine and ethanol treatment, there was significantly higher (96%) hepatic ethanol concentration (HEC) in the non-supplemented rats than in the carnitine supplemented rats without changes in activities of ethanol metabolizing systems (Mynatt and Sachan 1992, Sachan 1992). Those results suggested that

carnitine may modulate enzyme systems via some type of mediator or may be affecting membrane permeability to ethanol.

The main objective of the first study, therefore, was to explore mechanism of carnitine-mediated attenuation of ethanol metabolism by:

1. Determining the effects of carnitine on ethanol metabolism in isolated hepatocytes.
2. Establishing specificity of carnitine inhibition on ethanol metabolism in the hepatocytes.

The results of first study showed that, acetylcarnitine, a metabolite of carnitine, was a far more potent inhibitor of ethanol oxidation in hepatocytes than was carnitine. These results were encouraging in that it explained the lag time in carnitine response in the intact animal (Sachan 1992) and the lack of inhibition of ethanol metabolizing enzymes by carnitine (Mynatt and Sachan 1992).

The main purpose of the second study, therefore, was to reinvestigate mechanism of carnitine-mediated attenuation of ethanol metabolism at the enzymatic level by:

1. Determining the inhibitory effect of acetylcarnitine on ethanol metabolizing enzyme(s), ADH.
2. Investigating the mechanism of acetylcarnitgwe interaction with ADH.

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PART II

LITERATURE REVIEW

ETHANOL METABOLISM

Ethanol is absorbed primarily from the small intestine (80%) by simple diffusion and is distributed throughout the total body water, accumulating in tissues with the highest water content (Goldstein 1983). Studies with experimental animals have shown that the liver is the principal organ responsible for ethanol metabolism (Hawkins and Kalant 1972). The first step in the metabolism of ethanol is its oxidation to acetaldehyde. This conversion is carried out almost entirely in the liver, although other tissues have some oxidative capacity (2-10%). Enzyme systems that can catalyze the oxidation of ethanol to acetaldehyde are alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol-oxidizing system (MEOS) (Li 1977, Branden et al. 1975).

Liver ADH is a soluble enzyme that uses nicotinamide adenine dinucleotide (NAD^+) as a coenzyme in conversion of ethanol to acetaldehyde according to the reaction shown below. The details are discussed in ADH review section (page 19).

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

Microsomal fractions of liver contains microsomal ethanol-oxidizing system (MEOS), which can oxidize ethanol in the presence of molecular oxygen and NADPH. The

mechanism involves cytochrome P-450 mediated hydroxylation of carbon one of ethanol with concomitant oxidation of NADPH (Lieber 1991) according to the following reaction. Lieber

$$CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow{MEOS} CH_3CHO + NADP^+ + 2H_2O$$

and colleagues have characterized this system and believe that it is responsible for a substantial part of the ethanol metabolism in the liver, especially at high and chronic ethanol consumptions (Lieber et al. 1987). In rats, chronic feeding of ethanol increases MEOS activity, hepatic microsomal protein, smooth endoplasmic reticulum, and cytochrome P-450 (Goldstein 1983). One possible explanation is that at low concentrations of ethanol, ADH is already saturated and at higher ethanol concentrations, the MEOS has the ability to oxidize more ethanol. This would be consistent with the known enzyme kinetics of the two systems since the Km for ethanol in ADH system is around 2 $mmol.L^{-1}$ and the Km for ethanol in the MEOS is around 10 $mmol.L^{-1}$. Recently, P-450 IIEI which is a 52,000 dalton protein, has been purified by immunoelectrophoresis and shown to be inducible by ethanol (Shimizu 1990). Since MEOS uses both NADP/NADPH and NAD/NADH coenzymes, ethanol oxidation via MEOS could result not only in accelerated ethanol metabolism, but it may also accelerate the ADH pathway by favoring the re-oxidation of NADH (Domschke et al. 1974).

Catalase is a peroxisomal enzyme that can catalyze the

peroxidative cleavage of ethanol to acetaldehyde in the presence of a H_2O_2 generating system. The contribution of

$$CH_3CH_2OH + H_2O_2 \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

catalase in ethanol oxidation is quite small because the physiological rate of H_2O_2 production is rather low (Lieber et al. 1987, Li 1977). However, there is evidence pointing to significantly larger than accepted rate of ethanol metabolism by catalase (Bradford et al. 1993).

The next step in the completion of ethanol oxidation is the oxidation of acetaldehyde to acetate catalyzed by aldehyde dehydrogenase (ALDH). ALDHs are a group of enzymes

$$CH_3CHO + NAD + H_2O \xrightarrow{\text{ALDH}} CH_3COOH + NADH + H^+$$

catalyzing the conversion of aldehydes to corresponding acids via a NAD^+-dependent irreversible reaction (Ikawa et al. 1983). A more detail review of ALDH is given in the later section (page 19).

Studies using liver perfusion, liver slices and hepatocytes have shown that acetate from ethanol is further metabolized in the liver. The [1-^{14}C]-ethanol was partially recovered as $^{14}CO_2$ and TCA cycle intermediates (Dajani and Orten 1962, Forsander et al. 1965, Smith 1961), indicating further oxidation of acetate in the liver rather than in the extrahepatic tissues.

The incorporation of radioactive carbons of ^{14}C-ethanol

into acetoacetate, β-hydroxybutyrate, triglycerides and cholesterol indicates that acetate is also utilized for synthesis of these compounds in the liver (Smith 1961).

EFFECTS OF ETHANOL ON LIVER

In the early part of this century, dietary deficiencies were considered to be the major cause of ethanol-induced liver diseases. However, even in the absence of dietary deficiencies ethanol can lead to the development of liver diseases in humans (Lieber et al. 1962). More recently, it has been shown that ethanol, as well as malnutrition which has been induced by ethanol, contributes to liver diseases (Lieber 1991). Because ethanol is rich in energy (7.1 $Kcal.g^{-1}$), a large intake of ethanol has profound effects on nutritional status (Scheig 1970). Over consumption of ethanol may cause primary malnutrition by displacing other nutrients in the diet because ethanol lacks essential nutrients other than calories (empty calories). Secondary malnutrition may result from either maldigestion or malabsorption of nutrients caused by gastrointestinal complications. Such primary and secondary malnutrition can induce deficiency of virtually all nutrients (Lieber 1990).

Many disturbances in intermediary metabolism and toxic effects can be linked to (1) acetaldehyde, the product of ethanol oxidation, (2) ADH-mediated generation of NADH and (3) the induction of the microsomal enzymes, especially

P-450 IIE1 (Lieber 1991) as shown in Figure 2.1. First, ethanol oxidation results in production of acetaldehyde which in turn, causes liver injury through a variety of mechanisms due to its nature of high chemical reactivity. Acetaldehyde causes glutathione depletion, lipid peroxidation (Lieber 1991), and stimulates hepatic collagen synthesis (Irving et al. 1991), thereby promoting fibrosis and cirrhosis. Second, ethanol oxidation in the cytoplasm via ADH results in a stoichiometric reduction of NAD^+. Since the mitochondrial membrane is impermeable to NADH, reducing equivalents must be transferred into the mitochondria via the α-glycerophosphate or malate shuttle (Thurman 1977). The oxidation of ethanol in the hepatocyte increases the NADH/NAD ratio (Forsander et al. 1965, Domschke et al 1974). This increased NADH/NAD ratio is responsible for many of the deleterious effects of ethanol, such as ketosis, hyperlipidemia (Mendenhall 1972), hyperuricemia (Lieber 1962), and fatty liver (Lieber 1988). The activity of hepatic MEOS, especially, P-450 IIE1 can be induced even after short-term and relatively light consumption of ethanol (Lieber et al. 1988).

Induced cytochrome P-450 affects drug metabolism and enhances the rate of drug clearance from the blood (Kater et al. 1969). Ethanol pretreatment remarkably stimulates the toxicity and carcinogenicity of xenobiotics such as CCl_4 (Hasumura et al. 1974) and bromobenzene (Hetu et al. 1983).

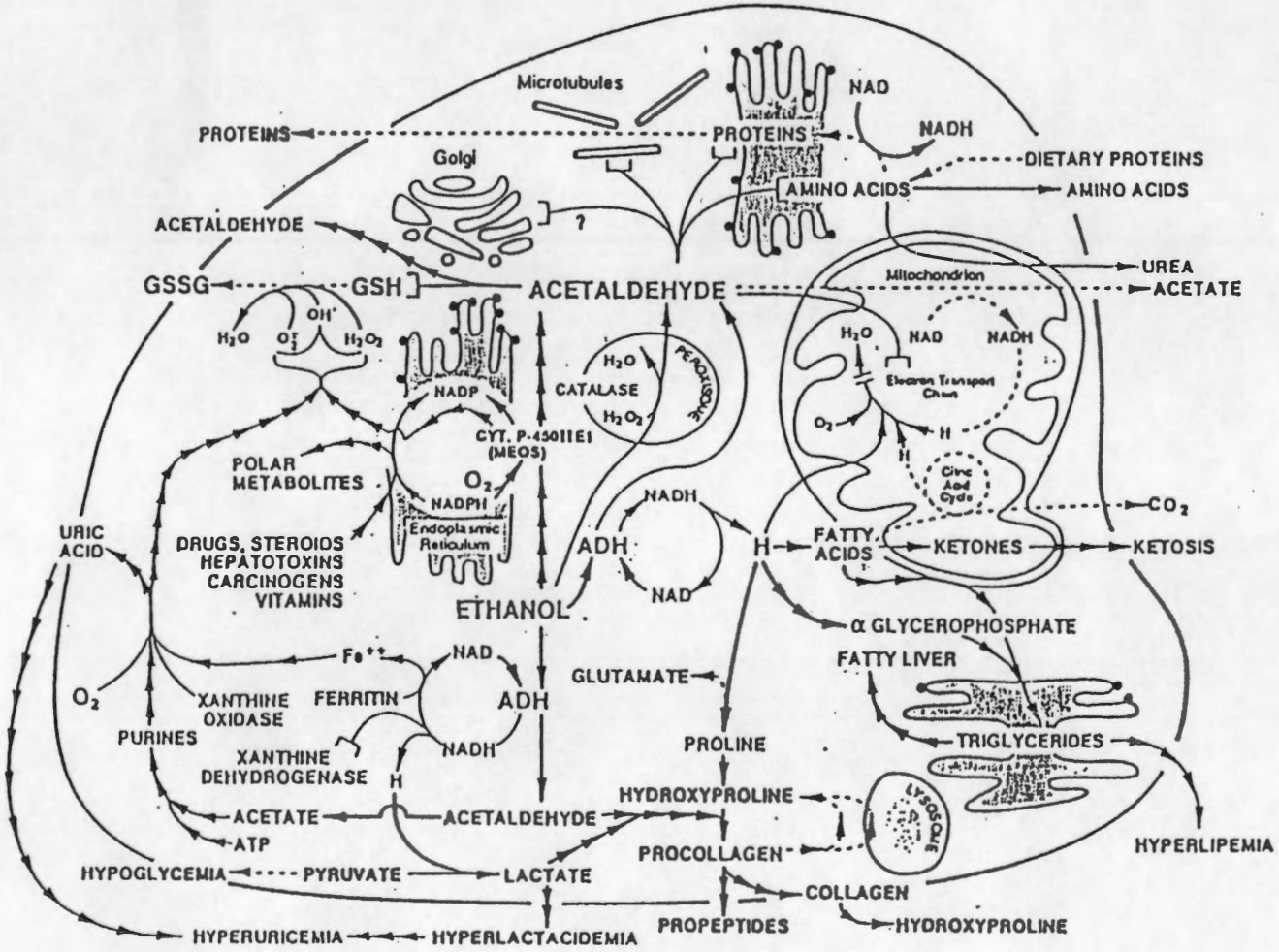

FIGURE 2.1. Oxidation of ethanol in the hepatocyte (Lieber 1991)

ETHANOL METABOLISM IN ISOLATED HEPATOCYTES

Isolated, rat hepatocytes have been used for ethanol oxidation studies under controlled conditions. To relate the results of studies with isolated cells to ethanol metabolism in vivo, a major difference between isolated hepatocytes and the liver in vivo must be considered. A summary of many studies indicates that the rate of ethanol oxidation in rats in vivo is from 2.4 to 4.0 $\mu mol.min^{-1}.g^{-1}$ of wet weight of liver (Cornell et al. 1977). In isolated rat hepatocytes, however, the rates are much lower from 0.7 to 0.9 $\mu mol.min^{-1}.g^{-1}$ of wet weight of cells when these cells are incubated with ethanol and no other substrate is added (Krebs & Stubbs 1975, Crow et al. 1978). Therefore, hepatocytes that are properly prepared and maintained provide an excellent in vitro system for studying liver ethanol metabolism because the isolated cells retain the same metabolic capacities as the intact liver (Cornell et al. 1977). Isolated hepatocytes are also a good system for studying ethanol metabolism in liver because the experimental conditions can be easily controlled.

Effect of ethanol concentration

Many studies have shown that a single large dose of ethanol increases the rate of ethanol oxidation in experimental animals (Yuki and Thurman 1980, Yuki et al. 1980), and in vitro preparations of liver such as liver perfusion and hepatocyte preparation (Yuki et al. 1982). It

has also been shown in vitro that rat-liver parenchymal cells oxidized ethanol at a rate of 2.5 $\mu mol.min^{-1}.mL^{-1}$ packed cells at 65 $mmol.L^{-1}$ ethanol concentration, which was 1.85 times the oxidation rate at 4 $mmol.L^{-1}$ ethanol. At ethanol concentrations above 10 $mmol.L^{-1}$ a significant increase in the ethanol oxidation rate was observed compared to the oxidation rate at 4 $mmol.L^{-1}$ (Grunnet et al. 1973). This phenomenon has been named the "swift increase in alcohol metabolism". The increased oxidation rates attributed to the presence of a high-Km MEOS (Goldstein 1983). An accompanying increase in liver O_2 uptake with large ethanol dose has also been observed (Yuki and Thurman 1980), which may be explained by the following sequence of events: ethanol increases the NADH redox state, which inhibits glycolysis because it is a NAD^+ dependent pathway. Since the glycolytic pathway is an ATP-generating sequence, inhibition of glycolysis makes more ADP available to stimulate mitochondrial electron transport, and increase oxygen uptake (Thurman 1977). In contrast to the above studies, other investigators have shown that ethanol is metabolized at a constant rate over a wide concentration range; however, a significant increase in O_2 uptake is found after acute ethanol pretreatment in hepatocytes isolated from starved, but not from fed, rats (Stowell and Crow 1985). In one study ethanol was oxidized more slowly by rats which were given an ethanol dose of 5.1 $g.Kg^{-1}$ than by

rats which were given an ethanol dose of 1.4 $g.Kg^{-1}$ (Braggins and Crow 1981). This is not consistent with the previous explanation that a high Km MEOS and the rate of NADH reoxidation play an important role in determining ethanol oxidation rates and that the rates of ethanol metabolism are closely linked to the rates of O_2 uptake. The alternate explanation is that ethanol was oxidized more slowly at high ethanol concentrations because ethanol itself was an inhibitor of rat liver ADH. It was also shown that increases in O_2 uptake with ethanol pretreatment may not lead to an increased rate of ethanol metabolism. Therefore, the activity of ADH is the major factor limiting rates of ethanol oxidation in vivo (Braggins and Crow 1981). Some of these observations may be due to the different experimental conditions such as using different strain of animals, duration of starvation of rat, perfusion technique, incubation medium, etc. For example, the effects of ethanol on O^2 uptake by liver depend entirely on the initial state of the liver.

Fructose effect

It has often been reported that fructose causes a marked acceleration of ethanol oxidation in perfused liver and isolated hepatocytes (Berry and Kun 1978, Grunnet et al. 1973, Scholz and Nohl 1976). Scholz and Nohl (1976) have suggested that fructose accelerates ethanol oxidation by increasing the rate of intramitochondrial reoxidation of

NADH due to an increased ATP demand for gluconeogenesis. However, other gluconeogenic precursors such as sorbitol, which also increase respiration, have no stimulatory effect on ethanol oxidation. The stimulation of ethanol oxidation by fructose was completely abolished by 4 mmol.L^{-1} pyrazole (ADH inhibitor), indicating that the "fructose effect" was mediated via ADH (Berry and Kun 1978).

Effects of pyruvate and lactate

Pyruvate increased the oxidation rate of ethanol in isolated rat-liver cells by 130-240 % (Grunnet et al. 1973). An apparent explanation for pyruvate induced acceleration in ethanol metabolism by isolated liver cells is the rapid reoxidation of cytosolic NADH via lactate dehydrogenase reaction which provides conditions more favorable for ethanol oxidation by ADH. By a similar reasoning, it might be expected that lactate added to hepatocyte suspensions would inhibit ethanol metabolism, since lactate oxidation produces NADH and competes with ADH for free cytosolic NAD^+.

It is well documented, however, that lactate increases the rate of ethanol oxidation in hepatocytes (Crow et al. 1978, Krebs and Stubbs 1975). It has been suggested that substrates for glucose synthesis, such as lactate and pyruvate, accelerate ethanol oxidation, as a result of increased ATP utilization for glucose production (gluconeogenesis). Cornell et al. (1977) conducted experiments with quinolinate and tryptophan because these

compounds block gluconeogenesis in the rat by inhibiting phosphoenolpyruvate carboxykinase, thereby preventing 2/3 of the ATP utilization required in the synthesis of glucose from lactate or pyruvate (Ray et al. 1966, Veneziale et al. 1967). They found that quinolinate and tryptophan both effectively decreased glucose synthesis, but neither inhibitor had any effect on the lactate or pyruvate stimulated rate of ethanol metabolism (Cornell et al. 1977). These results indicate that lactate or pyruvate stimulated oxidation is not due to an increased ATP demand for glucose synthesis. Alternatively, it has been proposed that lactate stimulation of ethanol oxidation in hepatocytes is due to an increase in malate and aspartate concentrations (Meijer et al. 1975) which would increase reoxidation of cytosolic NADH via the malate aspartate shuttle (Crow et al. 1983).

In summary, the malate-aspartate shuttle (which controls reoxidation of NADH) intermediates may be rate determining step in ethanol metabolism by hepatocytes under some conditions (particularly in the fasted state) because shuttle intermediates (malate, glutamate, and aspartate) are lost during cell preparation (Cornell 1977, Krebs et al. 1975), which is not the case in vivo. The rate of ethanol oxidation is limited by the level of cytosolic NADH which limits the rate of ethanol oxidation by ADH.

Alcohol dehydrogenase(ADH, EC 1.1.1.1) and Aldehyde dehydrogenase(ALDH, EC 1.2.1.3)

ADH and ALDH are the major enzymes involved in the initial two steps of ethanol metabolism. ADH is universally distributed in living organisms, occurring in microorganisms, plants, and animals (Pietruszko 1983). Yeast and mammalian alcohol dehydrogenase differ in substrate specificity and rate of catalytic activity (Branden et al. 1975). In mammals, this enzyme occurs in highest concentration in the liver, where it is found in the cytoplasmic fraction (Raskin and Sokoloff 1972). Liver ADH exists in multiple molecular forms that arise from the association, in various permutations, of different types of subunits (Strydom and vallee 1982). Extrahepatic tissues contain isozymes of ADH with a much lower affinity for ethanol than the hepatic isozymes: as a consequence, at the concentrations of ethanol reached in the blood, the extrahepatic enzymes are inactive, and therefore, extrahepatic metabolism of ethanol is negligible (Manautou and Carlson 1992), with the exception of the gastrointestinal tract (Algar et al. 1992). Human and rodent ADH exists as a heterogeneous group of isozymes that can be placed into three categories based upon structural and functional distinctions (Strydom and Vallee 1982). Class I ADH is encoded in humans by three closely related genes ADH1, ADH2, and ADH3 showing differential tissue-

specific and temporal expression during human development (Smith 1989). Class I ADH functions as both an ethanol dehydrogenase for detoxification of ingested ethanol (Li 1977) and as a retinol dehydrogenase for retinoic acid synthesis (Mezey and Holt 1971). Ethanol is the classical human class I ADH substrate and acts as a competitive inhibitor of retinol oxidation in human liver extracts. Liver has consistently higher class I ADH activity than other tissues, whereas class III ADH activity is present in practically all tissues (Danielsson et al. 1992).

Human class II ADH reduces an aldehyde intermediate in norepinephrine metabolism (Ooij et al. 1992). The distribution of class II isozymes changes between species. In humans, the class II isozyme has been reported only in the liver, whereas in the rat, the ADH 2 isozyme is characteristic of the digestive tract and external organs such as skin, cornea, tongue, etc., but is not found in the liver (Boleda et al. 1989). Human class III ADH catalyzes an omega-oxidation step in leukotriene B_4 synthesis in neutrophils (Gotoh et al. 1990). Also, human class III ADH has been identified as the glutathione-dependent formaldehyde dehydrogenase that is involved in elimination of formaldehyde produced as a by product of certain metabolic reactions (Jornvall et al. 1991).

Rat tissues contain three different isozymes of ADH. Rat ADH 1 is an anodic isozyme present in high amounts in the

ocular tissues (Pares et al. 1985), stomach and lung. ADH 1 has intermediate properties, with a Km for ethanol of 340 mmol.L^{-1}, a broad substrate specificity and Ki for pyrazol of 0.56 mmol.L^{-1}. Rat ADH 2 is also anodic and has been found in all the rat organs examined (Julia et al 1986). ADH 2 has substrate specificity for long-chain alcohols and aldehydes, cannot be saturated by ethanol and is practically insensitive to pyrazol (Ki=78.4 mmol.L^{-1}). Rat ADH 3 is the group of cathodic ADH's, are mainly present in the liver, and plays a major role in ethanol metabolism (Julia et al. 1987). ADH 3 exhibits a Km for ethanol of 1.4 mmol.L^{-1}, a broad substrate specificity and is strongly inhibited by pyrazole (Ki=0.4 μmol.L^{-1}). There is approximately 80% sequence homology among subunits of human and rat liver ADH (Jornvall and Markovic 1972). Rat ADH 1, ADH 2 and ADH 3 exhibit many analogies with human ADH classes II, III and I respectively (Pares et al. 1985). In the rat, ADH 3 and to some extent ADH 1 are the primary isozymes for external alcohol metabolism whereas ADH 2 may be more important for the metabolism of the endogenous long-chain alcohols and aldehydes (Julia et al. 1987, Julia et al. 1986). Human and rat isozymes are summarized in Table 2.1. In investigations of the interaction in vitro between liver ADH and a large number of heterocyclic compounds, pyrazol was found to be a most potent inhibitor of ADH.

TABLE 2.1

Summary of human and rat ADH isozymes

Species	Isoenzyme	Location	Specificity and function
Human	Class I	Liver	Ethanol oxidation Synthesis of retinoic acid
	Class II	Liver	Norepinephrine metabolism
	Class III	All tissues	Synthesis of leukotriene
Rat	ADH 1	Ocular Stomach Lung	Anodic High Km - ethanol
	ADH 2	All tissues	Anodic Long chain ethanol oxidation Insensitive to pyrazol
	ADH 3	Liver	Cathodic Ethanol oxidation Inhibited by pyrazol

Pyrazole was shown to have a dose-dependent inhibitory effect on ethanol metabolism , ED_{50} in the rat being 0.29-0.43 m mol.kg^{-1} pyrazol (Goldberg and Rydberg 1969). Studies in rats on the effects of various pyrazole analogues and other compounds on ethanol metabolism demonstrated that a dose of less than 0-5 mmol.L^{-1} pyrazol per kg resulted in an inhibition in the rate of ethanol elimination by more than 25% of control value (Golfberg and Rydberg 1969). Therefore, pyrazole and its analogues are widely used to inhibit ADH and to block the metabolism of ethanol (Wendell and Thurman 1979, Grunnet et al. 1973, Yuki et al. 1982, Feierman and Cederbaum 1987). The essential mechanism of action in vivo is most probably the same as the in vitro inhibition of the ADH. Pyrazole is a competitive inhibitor of ADH on ethanol oxidation, which is consistent with the in vivo findings that a dose-response relationship exists between degree of inhibition and dose of pyrazol (Goldberg and Rydberg 1969). Other inhibitors of ADH are summarized in Appendix 8.

Like ADH, NAD-linked ALDH occurs primarily in the liver, with much lower concentrations in other organs. ADH occurs exclusively in the cytoplasm, whereas the ALDH activity is distributed among different subcellular compartment (Marjanen 1972). The majority of the ALDH activity in the rat is localized in the mitochondria, but the enzyme also occurs in the cytoplasm, microsomes, and other membranes.

Two major and several minor isozymes exist in the mammalian liver including man. One of the major isozymes, designated as ALDH1 or E1 is associated with slower anodal electrophoretic mobility, a low Km for NAD, and a high Km for acetaldehyde. Another major isozyme, designated as ALDH2 or E2, is associated with faster anodal electrophoretic mobility, a high Km for NAD, and a low Km for acetaldehyde (Tottmar et al. 1973). ALDH1 isozyme is of cytosolic origin and ALDH2 of mitochondrial origin (Vallari and Pietruszko 1984).

There is some evidence that the interaction of alcohol and hormones may be mediated by ALDH (Maxwell and Topper 1961). Ethanol has also been reported to cause alterations in the steady state levels of biogenic amines through alterations in the rates of ALDH metabolism of biogenic aldehydes (Tank et al. 1976). Thus, ALDH may play a physical role in the metabolism of aldehydes arising from corticosteroid and biogenic amine hormones.

Although it is exceedingly rare in persons of European ancestry, in about 50-70 % of Orientals there is a deficiency of the ALDH enzyme (isozyme ALDH1) that metabolizes acetaldehyde to acetate (Goedde and Agarwal 1983). The deficiency is responsible for high concentrations of acetaldehyde after the ingestion of ethanol. Acetaldehyde has pronounced effects on the body, including shortness of breath, facial warmth (flushing),

decrease in blood pressure, and increase in heart rate (Agarwal and Goedde 1990). These effects of acetaldehyde are generally perceived to be unpleasant, so it is easy to understand why this population, called flusher, become alcoholics far less frequently than nonflushers.

Disulfiram (Antabuse) and Calcium Carbamide (Temposil) are drugs that are sometimes given in the treatment of alcoholism (Giles and Kapur 1991, Pirola 1978). These drugs block the conversion of acetaldehyde by inhibiting ALDH enzyme (Giles and Kapur 1991). Drugs and chemicals that have disulfiram-like activity include cyanamide (Hald et al. 1952), hypoglycemic sulphonylureas (Truitt et al. 1962), anti-parasitic agent, metronidazole, (Lal 1969), and antimicrobial drug, furazolidone, (Perman 1965).

CARNITINE AND ETHANOL METABOLISM

Carnitine facilitates the transport of fatty acids, via carnitine acyltransferase, into the mitochondria for β-oxidation (Fritz et al. 1962). Without carnitine, most of the fatty acids that are eaten by humans could not be metabolized for energy and thus an important energy source would be eliminated and the fatty acids would be stored in adipose tissue (Bieber 1988). Because of the unique role of carnitine in the transport of fatty acids into mitochondria (Fritz et al. 1962) as well as among different organs (Hoppel et al. 1980), investigators studied the possibility

that carnitine might help prevent the ethanol-induced liver diseases. Hosein and Bexton (1975) reported that carnitine had a protective effect on lipid metabolism rat fed ethanol. They found that carnitine (0.1 $mg.g^{-1}$ B.W.) significantly lowered serum triglycerides in rats which received ethanol (6 $g.kg^{-1}$ B.W.). It also significantly reduced hepatic triglycerides in rats injected with ethanol intraperitoneally (4 $g.kg^{-1}$ B.W.). Carnitine depletion in patients hospitalized for advanced cirrhosis was shown by Rudman et al. (1977). Fasting serum carnitine concentration was measured in 16 normal people and 247 cirrhotic patients. They found that the cirrhotic group showed significantly lower total and free carnitine than the normal group.

In recent studies undertaken to study the possible effect of dietary carnitine supplementation on ethanol-induced hepatic steatosis, it was observed that carnitine supplementation lowered ethanol-induced increases of various lipid fractions (Sachan et al. 1984, Sachan and Rhew 1983). They found that supplementation of carnitine in liquid alcohol diet (36 % calories as ethanol) significantly lowered total lipids, free and esterified cholesterol, triglycerides, and phospholipids in the liver. Later, it was found that the effect was dose dependent up to 0.8 % of DL-carnitine supplement (Rhew & Sachan 1986). Intravenous administration of L-carnitine to humans significantly reduced ethanol-induced increase of blood acetate and

promoted urinary excretion of acetylcarnitine following ethanol administration (Adamo et al. 1988). When rats were administered an acute oral dose of ethanol (3g.Kg^{-1} B.W.), the animals supplemented with 1 % DL-carnitine for 3, 5, and 10 days had significantly higher blood ethanol concentrations (BEC) from 2 to 8 hours post ethanol administration than non-supplemented rats (Berger and Sachan 1986, Sachan and Berger 1987). The increase in BEC was dependent on the level of carnitine supplementation and the 0.5 % L-carnitine supplementation for 3 days produced maximum BEC (Berger and Sachan 1991, Sachan and Berger 1993). They found that carnitine did not alter the rates of ethanol appearance in the portal blood, but it significantly elevated the systemic BEC during the same initial 30 min. So, they concluded that carnitine retarded ethanol metabolism without altering the rate of ethanol absorption from the small intestine. Since the simultaneous administration of carnitine with ethanol did not attenuate ethanol metabolism (Sachan 1992), it was proposed that carnitine may modulate enzyme systems or cell structure in some type of inductive manner (Mynatt 1989). Carnitine supplementation significantly reduced [1-^{14}C]-ethanol oxidation to $^{14}CO_2$ and increased plasma concentrations of lactate 2.5 times which resulted in a higher lactate/pyruvate ratio. Carnitine supplementation, however, did not significantly affect the ethanol and acetaldehyde

metabolizing enzyme activities (Mynatt and Sachan 1992). The shift to a more reduced redox state by carnitine supplementation was hypothesized to be the possible mechanism for the attenuation of ethanol oxidation by carnitine. In conclusion, it was suggested that carnitine may modulate enzyme systems via some type of mediator or it may be affecting hepatocyte's permeability to ethanol. As a result ethanol oxidation in the isolated hepatocytes was examined to understand the mechanism of carnitine-mediated attenuation of ethanol metabolism.

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PART III

EFFECT OF L-CARNITINE AND RELATED COMPOUNDS ON ETHANOL METABOLISM IN RAT HEPATOCYTES

ABSTRACT

Previous studies have shown that supplementary carnitine protected liver from fatty infiltration caused by ethanol. One of the reasons was that carnitine retarded ethanol clearance and hence its metabolism in the intact rat. However, in vitro activities of ethanol metabolizing enzymes were not significantly altered by carnitine. Therefore, hepatocytes were targeted to understand the mechanism of action of carnitine on ethanol metabolism. The cells were isolated from male Sprague Dawley rats by a collagenase-perfusion technique. Hepatocytes were incubated in albumin-containing medium with ethanol in presence or absence of added L-carnitine or related compounds. Ethanol oxidation was determined by the loss of ethanol as well as by the products formed against time, cell numbers and ethanol concentrations. The rate of ethanol oxidation in presence of L-carnitine was one half the rate in absence of carnitine (24.69 $nmol.min^{-1}.million^{-1}$ cells). Further it was found that choline mimicked carnitine effect and L-acetylcarnitine was 100 times more active than L-carnitine in inhibiting ethanol oxidation in the isolated hepatocytes. The effect was dose related for both carnitine and acetylcarnitine, however, there was a definite lag time for carnitine effect. The products of ethanol oxidation in presence of acetylcarnitine suggested that site of inhibitory action must be ADH. From these results it is concluded that carnitine is converted

to acetylcarnitine which in turn inhibits ethanol metabolism in the isolated hepatocytes and the intact rat.

INTRODUCTION

Alcoholism is a major social, economical and public health problem throughout much of the world. In the United states alone, the annual cost of lost productivitity and health expenses related to alcoholism is estimated to be $117 billion (Hoek et al. 1992). Alcohol (ethanol) is known to cause a wide variety of metabolic abnormalities (Agarwal and Goedde 1990). Ethanol administration both in humans and laboratory animals results in hyperlipidemia and fatty liver (Lieber et al. 1987).

Carnitine (3-hydroxy-4-N-trimethylammonium butyrate) is widely distributed in living organisms and is biosynthesized in mammals. The foods of animal origin especially, milk and meats are rich dietary sources of carnitine. It is an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix where β-oxidation takes place (Fritz et al. 1962). Without carnitine, most of the ingested fatty acids would not be fully metabolized for energy and thus an important production, and the body would be forced to store the fatty acids as triglycerides in adipose tissue (Bieber, 1988).

In recent studies supplementary carnitine has been shown to minimize ethanol-induced hepatic steatosis in a dose

dependent manner (Sachan et al. 1984, Rhew and Sachan 1986). The underlying mechanism involved retarded clearance of blood-ethanol due to attenuation of ethanol metabolism in the intact rat (Berger and Sachan 1986, Sachan and Berger 1987, Berger and Sachan 1991). This effect of carnitine is specific to carnitine since it could not be reproduced by choline (Sachan and Berger 1993). Further, it is known that hepatic ethanol concentrations are higher in carnitine unsupplemented than supplemented rats (Mynatt 1989). Carnitine reduced oxidation of ^{14}C-ethanol to $^{14}CO_2$ in the intact rat, however, the in vitro activities of ethanol metabolizing enzymes were not significantly affected by carnitine (Mynatt and Sachan 1992). These results suggested that carnitine may modulate enzyme systems via some type of mediator or may be affecting membrane permeability to ethanol.

Therefore, the main objective of this study was to explore mechanism of carnitine-mediated attenuation of ethanol metabolism using rat hepatocytes. The specific objectives were to determine in vitro effects of carnitine on ethanol metabolism in isolated hepatocytes and to establish specificity of the carnitine effect. Ethanol oxidation in rat hepatocytes was determined against time, cell numbers and ethanol concentration in presence or absence of L-carnitine as well as carnitine like compounds.

MATERIALS AND METHODS

Chemicals. L-acetylcarnitine and r-butyrobetaine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Collagenase (Type IV), HEPES (N-2-hydroxy ethylpiperazine), L-carnitine, DL-carnitine, choline, L-acetylcholine, β-OH-butyrate, L-lysine, and palmytylcarnitine were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). Ethanolamine was obtained from Fisher scientific Co. (Fair Lawn, NJ). [1-^{14}C]-ethanol was obtained from New England Nuclear (Boston, MA). All other chemicals were from Baxter Diagnostics Inc.(McGaw Park, IL).

Animals and diets. The experimental protocol was approved by the University of Tennessee animal care committee. Male Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) with an average body weight of about 250 g were individually housed in 7" x 10" x 7" stainless steel wire mesh cages. Room temperature was maintained at 22 °C ± 1 °C and constant relative humidity (52%) with a 12 hour light-dark cycle. All specifications of the animal facility complied with AAALAC regulations. All animals had free access to rat chow (Agway Prolab #3000, Syracuse, NY) and hyperchlorinated city water.

Hepatocyte preparation. About 8:30 A.M. on the day of experiment, a rat was anesthetized by Metofane (Pitman-

Moore, Inc. Mundelein, IL). The abdominal cavity was opened and portal vein was surgically cannulated. The liver was perfused and hepatocytes were isolated according to the method described by Seglen (Seglen 1976) with some modification (Horne et al. 1976). The procedure is described in detail in appendix I. A brief account of the modification is as follow: The concentration of collagenase was 0.05 % (w/v). Ca^{++}-free perfusion buffer for the preparation of the cells was replaced by the physiological saline Krebs-Henseleit buffer, KHB (Krebs 1974) and solution for the final incubation was replaced by KHB saline containing 2.5% (w/v) BSA. The buffer solutions including collagenase buffer were gassed with O_2:CO_2 mixture (95:5) for at least 10 min and Ca^{++} free buffer was gassed throughout the liver perfusion. The cells released by collagenase treatment were filtered though a nylon mesh (size 0.5 mm x 0.3mm). All hepatocyte preparations were washed and cell viability was determined by Trypan Blue exclusion (Crow et al. 1978). The hepatocyte concentration was standardized, kept at room temperature and used for study within hours after preparation.

Ethanol metabolism in hepatocytes. The incubation was carried out in a 25 mL Erlenmeyer flask in total volume of 4 mL KHB (pH 7.4) with 2.5 % BSA. The hepatocytes in the incubation mixture was one million cells.mL^{-1} (24 million cells = 1 g wet cell weight). The standard incubation

mixture contained KHB with 2.5 % BSA, hepatocytes (million. mL^{-1}), ethanol (10 mmol. L^{-1}), and carnitine or related compound (200 μmol. L^{-1}) in 4 mL. Whenever necessary deviation in the concentration of component was made as indicated in that experiment. The flask was flushed with mix of O_2:CO_2 (95:5) before adding ethanol to get the predetermined concentration (usually 10 mmol. L^{-1}) in the incubation mixture. The flask was sealed with rubber stopper and incubated in a Dubnoff water bath with shaking (80 oscillation.min^{-1}) at 37 °C. A control flask was prepared for each flask in which 0.2 mL of 60% $HClO_4$ was added prior to addition of ethanol and rest of the incubation procedure was identical to that of test flask. The reaction was terminated by injecting 0.2 mL of 60% $HClO_4$ with a syringe. The contents were mixed well, and cooled in ice bath for 30 minutes. The mixture was centrifuged in a centrifuge (Backman TJ-6R, 2000 x g) at 4 °C for 10 minutes and supernatant was separated and assayed for ethanol by the enzymatic method (Bernt and Gutmann 1974).

Oxidation of ethanol to CO_2 was determined using [1-^{14}C]-ethanol. The incubation was carried out on a 25 mL Warburg flask. The standard incubation mixture contained KHB with 2.5 % BSA, hepatocytes (million. mL^{-1}), [1-^{14}C]-ethanol (10 mmol. L^{-1}), and carnitine or related compound (200 μmol. L^{-1}) in 3 mL. Whenever necessary, deviation in the concentration of component was made as indicated in that experiment. The

center well contained 0.2 mL of ethanolamine (100 %) and side arm contained 0.2 mL of 60 % $HClO_4$. The flask was gassed with a mix of O_2:CO_2 (95:5) before adding 10 mmol.L^{-1} [1-^{14}C] ethanol. The flask was sealed with rubber stopper. Incubation was carried out at 37 °C in Dubnoff metabolic shaking water bath (80 oscillation.min^{-1}). The reaction was terminated by tipping $HClO_4$ from the side arm into incubation mixture. The flask was allowed to shake an additional 60 min at 37 °C to allow absorption CO_2 in the ethanolamine. The flask was well chilled in ice water for 30 min. Ethanolamine was transferred from center well to scintillation vial. Residual ethanolamine was wash out 3 times with scintillation fluid, 5 mL of scintillation fluid was added to the vial, and radioactivity was counted in a Beckman LS 3801 Liquid Scintillation system ($^{14}CO_2$). The $HClO_4$ treated incubation mixture was centrifuged with in a Beckman TJ-6R tabletop centrifuge (2000 x g) at 4 °C for 10 minutes and SN, pellet were separated, and frozen for the analysis of metabolites.

The pellet was extracted for Lipids with 2:1 of chloroform-methanol mixture and insoluble substances were removed by pipetting out. Soluble mixture was washed with water 2 times to remove non-lipid contaminants, transferred into scintillation vial containing 5 mL of scintillation fluid, and radioactivity was determined in Beckman LS 3801 liquid scintillation system.

Acetyl-CoA and acetylcarnitine were determined spectrophotometrically by the method (Pearson et al. 1974) described in detail in appendix 2. Acetate was determined by the enzymatic method using acetyl-CoA synthetase (Guynn and Veech 1975, Bridges et al. 1992) which is described in detail in appendix 3. Acetaldehyde was determined by the enzymatic method of described in detail in appendix 4 (Lundquist 1974). Acetoacetate was determined by fluorescent spectrophotometric method of described in detail in appendix 5 (Mellanby and Williamson 1974). 3-Hydroxybutyric acid was determined according to the procedure of Williamson and Mellanby (Williamson and Mellanby 1974) adapted for fluorescence which is in detail in appendix 6.

All values shown in the figures and tables are expressed as group mean ± the standard error of the mean (SEM). The Student's t-test (Ott et al. 1987) was used to determine statistical significance of differences between the means of treatment groups at $p \leq 0.05$. For the time course experiment, a split-plot-in-time model was used for analyzing the effects of time and treatment group on ethanol oxidation. A residual analysis indicated that using the natural logarithm transformation of ethanol oxidation data might be more appropriate than using normal ethanol oxidation data. Time, group, and their interaction were treated as fixed effects, rat nested within group was

treated as a random effect. Specific Linear Contrasts were used to test for differences in each group at the different time intervals. For the cell number experiment, a split-plot model was used for analyzing the effects of cell number (1, 2, 3, 4, 8) and group (None and L-Carnitine) on ethanol oxidation. Group was considered the whole-plot, and cell number was considered the split-plot. A residual analysis indicated that no transformation of the data was necessary, so ethanol oxidation was analyzed directly. Cell number, group, and their interaction were treated as fixed effects, rat nested within group was treated as a random effect. Specific Linear Contrasts were used to test for differences in each group at the different cell numbers. For the ethanol concentration experiment, a simple two-way analysis of variance (without interaction) was used to analyze ethanol oxidation. A residual analysis indicated that no transformation of the data was necessary, so ethanol oxidation was analyzed directly. Ethanol concentration was considered as a fixed effect, and rat was considered a random effect. General Linear Mixed Models or GLMM (Blouin et al. 1990) was used to analyze the data (time course, cell number, and ethanol concentration experiment). The GLMM was also used to calculate the Generalized Least Square Means (GLSM) and their SEM for the fixed effects in the model, variance components for the random effects, and the value of the specific contrasts. Data for all rats were

used in all three experiments (time course, cell number, and ethanol concentration) and no data were ignored.

RESULTS

Metabolic integrity of isolated hepatocytes was determined by studying the time course of ethanol oxidation (Figure 3.1) as well as the effect of cell concentration (Figure 3.2) and substrate (ethanol) concentration (Figure 3.3) on ethanol metabolism. Ethanol oxidation was linear with time for 60 minutes. Ethanol oxidation was also linear with cell numbers up to 8 million cells. The ethanol oxidation was increased in proportion to ethanol concentrations up to 4 $mmol.L^{-1}$ at which maximum rate was reached and no increase was observed at higher ethanol concentrations. The Lineweaver-Burk plot of the data (Figure 3.3) provided a K_m of 1.9 $mmol.L^{-1}$ and V_{max} of 11.1 $mmol.L^{-1}$. Ethanol oxidation in rat hepatocyte with and without L-carnitine is shown in Figure 3.4. The rate of ethanol oxidation by rat hepatocytes without carnitine was 24.69 ($nmol.min^{-1}.million^{-1}$ cells) which is 43% higher than that the rate with L-carnitine (14.05 $nmol.min^{-1}.million^{-1}$ cells). The time course of ethanol oxidation in hepatocytes with or without L-carnitine is summarized in Table 3.1. There was a significantly decrease in ethanol oxidation by hepatocytes in the presence of L-carnitine within the first 60 min. However, the inhibitary effect of carnitine was not

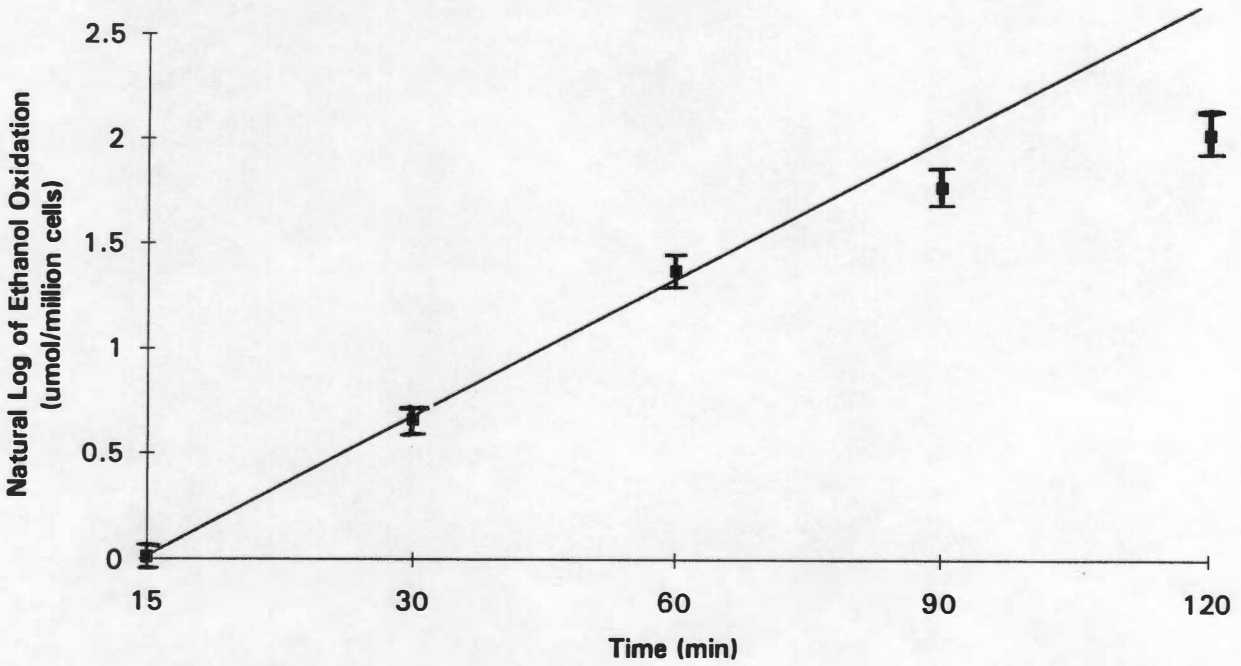

FIGURE 3.1. Time course of ethanol oxidation by hepatocytes Values are mean ± SEM of 3 different hepatocyte preparations (3 replicates from a single hepatocyte preparation and 1 replicate from each of the other 2 different hepatocyte preparations). Incubation mixture consisted of KHB pH 7.4 with 2.5% BSA, hepatocytes (1 million.mL^{-1}), ethanol (10 mmol.L^{-1}), L-Carnitine (200 μmol.L^{-1}) in a total volume of 4 mL.

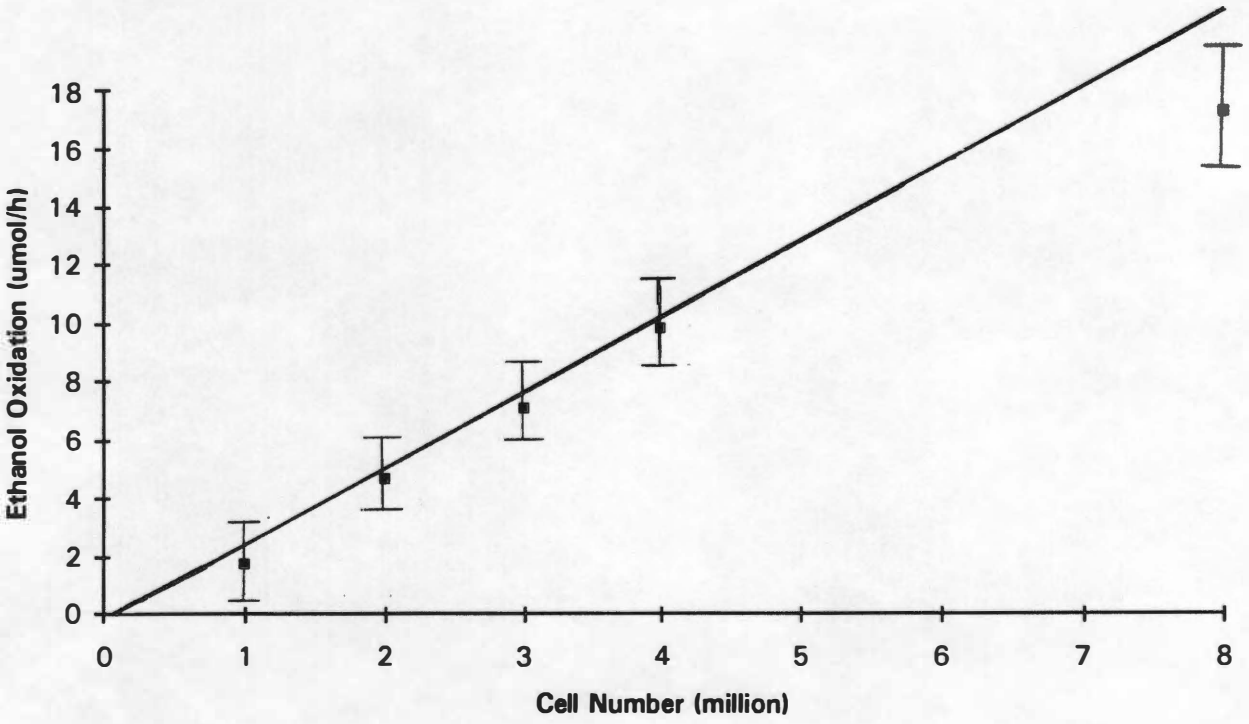

FIGURE 3.2. Effect of hepatocyte concentration on ethanol oxidation

Values are mean ± SEM of 3 different hepatocyte preparations (3 replicates from a single hepatocyte preparation and 1 replicate from each of the other 2 different hepatocyte preparations). Incubation mixture consisted of KHB pH 7.4 with 2.5% BSA, hepatocytes (1 million.mL^{-1}), ethanol (10 mmol.L^{-1}), L-Carnitine (200 μmol.L^{-1}) in a total volume of 4 mL.

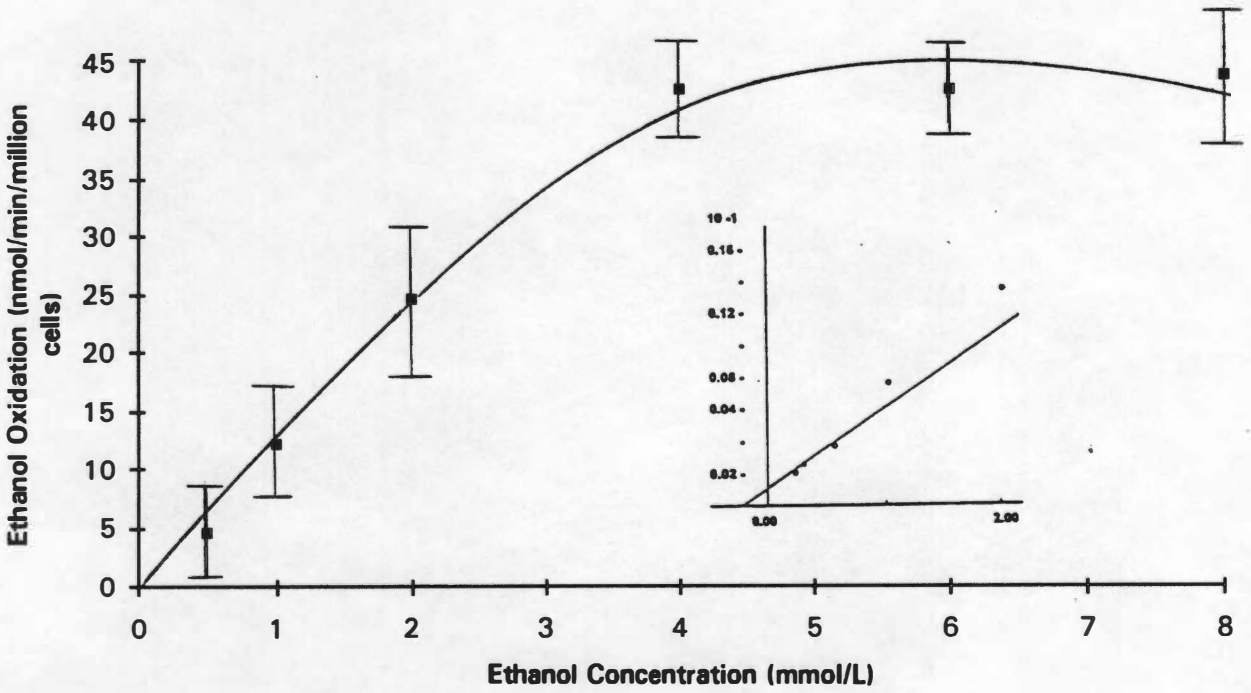

FIGURE 3.3. Effect of ethanol concentration on ethanol oxidation Values are mean ± SEM of 3 different hepatocyte preparations (3 replicates from a single hepatocyte preparation and 1 replicate from each of the other 2 different hepatocyte preparations). Incubation mixture consisted of KHB pH 7.4 with 2.5% BSA, hepatocytes (1 million.mL^{-1}), ethanol (10 mmol.L^{-1}), L-Carnitine (200 μmol.L^{-1}) in a total volume of 4 mL.

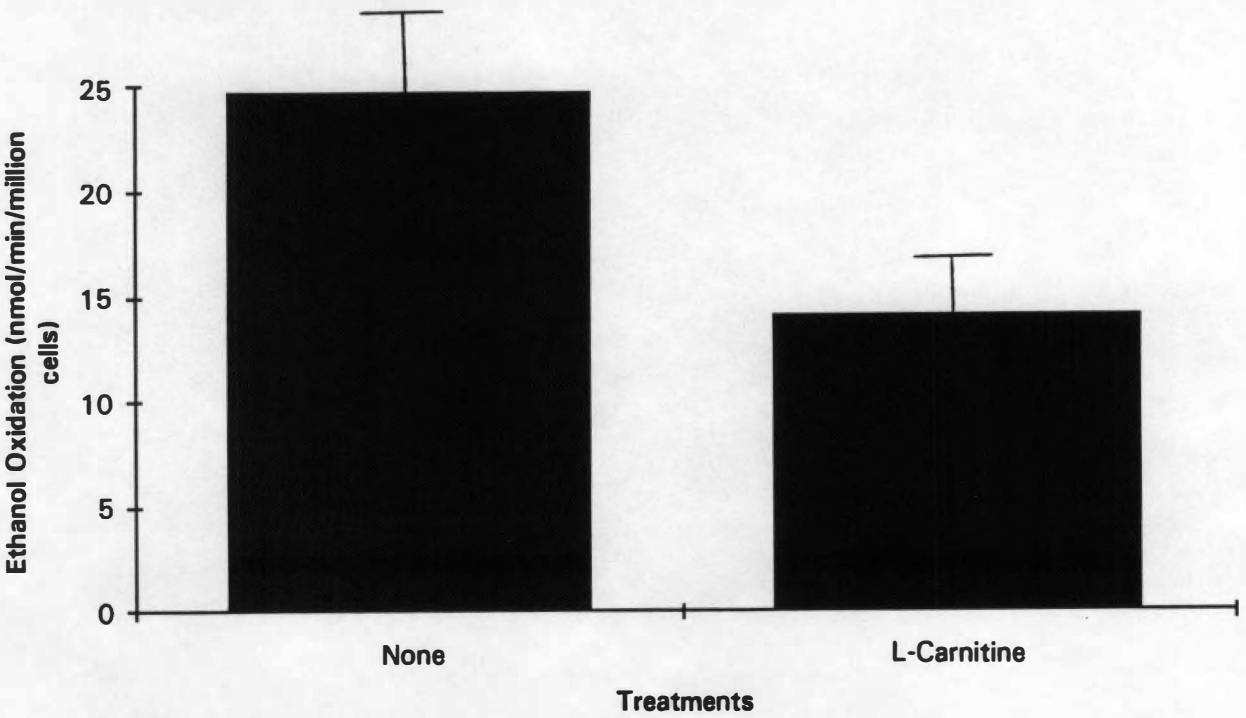

FIGURE 3.4. Effect of L-Carnitine on ethanol oxidation by hepatocytes

Values are mean ± SEM of 4 different hepatocyte preparations (3 replicates from a single hepatocyte preparation and 1 replicate from each of the other 3 different hepatocyte preparations). Incubation mixture consisted of KHB pH 7.4 with 2.5% BSA, hepatocytes (1 million.mL^{-1}), ethanol (10 mmol.L^{-1}), L-Carnitine (200 μmol.L^{-1}) in a total volume of 4 mL.

TABLE 3.1

Time course of ethanol metabolism by hepatocytes[1]

Additions	Incubation Time (min)				
	15	30	60	90	120
	Natural Log (μmoles of ethanol metabolized per million^{-1} cells)				
None	0.01 ± 0.13[a]	0.65 ± 0.14[a]	1.37 ± 0.13[a]	1.77 ± 0.13[a]	2.03 ± 0.14[a]
L-Carnitine	-0.6 ± 0.13[b]	0.20 ± 0.13[b]	1.01 ± 0.13[b]	1.68 ± 0.13[a]	1.94 ± 0.13[a]
% Inhibition	59	69	26	5	4

[1]Values are mean ± SEM of 5 determinations from 3 different hepatocyte preparations (3 observations/time for one rat and 1 observation/time for 2 other rats).
Values bearing different superscript letters between rows are significantly differenrt ($p \leq 0.05$). Incubation mixture consisted of KHB pH 7.4 with 2.5 % BSA, hepatocyte (1 million. mL^{-1}), ethanol (10 mmol. L^{-1}), L-Carnitine (200 μmol. L^{-1}) in a total volume of 4 mL. The precincubation period of 10 min preceeded start of reaction by addition of ethanol and incubation at 37°C for time intervals indicated in the table.

significant beyond 60 min. As a result the rates of ethanol oxidation by hepatocytes with L-carnitine was much lower than the rate without carnitine. Similarly, the effect of carnitine on ethanol oxidation by hepatocytes was greater at lower than at higher cell concentration (Table 3.2). The inhibitory effect of carnitine on ethanol metabolism was significant at wide range of cell concentrations (0.25-2 million cells.mL^{-1}).

The effect of carnitine and related compounds on ethanol oxidation is summarized in Table 3.3. Ethanol oxidation was significantly reduced by L-carnitine, DL-carnitine, choline, and L-acetylcarnitine compared to that without any addition. Choline had almost the same effect as carnitine on ethanol oxidation and equimolar DL-carnitine showed half the effect produced by L-carnitine. Most important, however, was the effect of acetylcarnitine which produced a much higher inhibition than did L-carnitine.

The rate of ethanol oxidation in response to different concentrations (0.2-40 μmol. L^{-1}) of L-carnitine and L-acetylcarnitine is shown in Table 3.4. The maximum inhibitory effect of L-carnitine was at the concentration of 20 μmol. L^{-1}. In contrast, there was an almost identical inhibitory response by L-acetylcarnitine at the concentration of 0.2 μmol. L^{-1}. Thus, L-acetylcarnitine was a 100 fold better inhibitor of ethanol metabolism than was carnitine.

TABLE 3.2

Effect of hepatocyte concentration on ethanol metabolism[1]

Additions	Hepatocyte Concentration (Millions.4mL^{-1})				
	1	2	3	4	8
	μmoles of ethanol metabolized per hour^{-1}				
None	1.7 ± 0.35^{a}	4.7 ± 0.35^{a}	7.0 ± 0.34^{a}	9.8 ± 0.35^{a}	17.3 ± 1.13^{a}
L-Carnitine	1.1 ± 0.35^{b}	2.8 ± 0.18^{b}	4.2 ± 0.33^{b}	6.0 ± 0.35^{b}	14.7 ± 0.35^{a}
% Inhibition	35	40	40	39	15

[1]Values are mean ± SEM of 5 determinations from 3 different hepatocyte preparations (3 observations/hepatocytes for one rat and 1 observation/2 the other rats).
Values bearing different superscript letters between rows are significantly different ($p \leq 0.05$). Incubation mixture consisted of KHB pH 7.4 with 2.5 % BSA, hepatocyte (1-8 million.mL^{-4}), ethanol (10 mmol. L^{-1}), L-Carnitine (200 μmol. L^{-1}) in a total volume of 4 mL. The precincubation period of 10 min preceeded start of reaction by addition of ethanol and incubation at 37°C for 60 min.

TABLE 3.3

Effect of carnitine and related compounds on ethanol metabolism in hepatocytes

Additions [200 μmol.L^{-1}]	Ethanol Metabolism (nmol. min^{-1}. $million^{-1}$ cells)	Change (%)
None	37.1 ± 1.17	0
L-Carnitine	27.3 ± 1.44	-26.5
DL-Carnitine	31.4 ± 0.58	-15.4
Choline	27.2 ± 0.23	-26.7
r-Butyrobetaine	42.8 ± 2.31	15.4
β-OH-Butyrate	41.2 ± 1.79	11.0
L-lysine	37.9 ± 0.89	2.2
L-Acetylcarnitine	24.4 ± 0.46	-34.2
Palmytylcarnitine	42.0 ± 1.79	13.2

[1]Values are mean ± SEM of 3 determinations from 2 different hepatocyte preparations. Incubation mixture consisted of KHB pH 7.4 with 2.5 % BSA, hepatocyte (1 million. mL^{-1}), ethanol (10 mmol. L^{-1}), L-Carnitine or related compounds (200 μmol. L^{-1}) in a total volume of 4 mL. The precincubation period of 10 min preceeded start of reaction by addition of ethanol and incubation at 37°C for 60 min.

TABLE 3.4

Effect of carnitine and acetylcarnitine concentration on ethanol metabolism in hepatocytes[1]

	Concentration (μmol. L^{-1})								
Additions	0	0.2	0.5	1	3	6	12	20	40
	Ethanol Oxidation (nmol. min^{-1}. $million^{-1}$ cells)								
None	49.0								
L-Carnitine	-	27.5	26.0	26.0	15.5	14.5	11.5	8.0	8.0
L-Acetylcarnitine	-	8.0	8.0	9.0	11.5	11.0	13.0	15.0	14.2

[1]Values are mean of 2 determinations from a single hepatocyte preparation. Differnent hepatocytes from different rat gave similar results. Incubation mixture consisted of KHB pH 7.4 with 2.5 % BSA, hepatocyte (1 million. mL^{-1}), ethanol (10 mmol. L^{-1}), L-Carnitine or L-Acetylcarnitine (200 μmol. L^{-1}) in a total volume of 4 mL. Incubation period was 20 min at 37°C.

The products of ethanol oxidation by hepatocytes with and without L-carnitine and L-acetylcarnitine is shown in Table 3.5. The metabolism of ethanol was determined by the rate of ethanol disappearance from the medium as well as by the products of ethanol oxidation. There was good balance between ethanol disappearance and the products formed. The major (93-95 %) product of ethanol metabolism was CO_2. The remaining 4 % were other intermediates of ethanol metabolic pathway. Therefore, the major impact of carnitine or acetylcarnitine inhibition was in terms of CO_2 produced. A small but quite relevant effect could be appreciated in concentration of acetaldehyde in presence or absence of these inhibitors.

DISCUSSION

The carnitine-mediated attenuation of ethanol metabolism in the intact rat formed the basis for studying the mechanism at cellular level. The rate of ethanol oxidation by hepatocytes in our study was about 33 $nmol.min^{-1}.million^{-1}$ hepatocytes which is approximately 0.8 $\mu mol.min^{1}.g^{-1}$ of wet cell weight or 24 million cells. These rates are within the range reported (0.7-0.9 $\mu mol.min^{-1}.g^{-1}$) for rat hepatocytes in presence of no other substrate but only ethanol (Krebs 1975, Crow et al. 1978). These rates of ethanol oxidation in isolated hepatocytes are about 70-78% lower than those (2.4 - 4.0 $\mu mol.\ min^{-1}.\ g^{-1}$ of wet weight of liver) observed

TABLE 3.5

Effect of carnitine and acetylcarnitine on ethanol metabolism measured by ethanol disappearance and product formation[1]

	None	L-Carnitine	L-Acetylcarnitine
	Ethanol Metabolism (nmol. min^{-1}. $million^{-1}$ cells)		
Ethanol Disappeared[2]:	34.6 ± 3.0	27.9 ± 2.0	22.4 ± 0.6
Product formed[3]:	30.03	24.27	18.44
$^{14}CO_2$	28.0 ± 2.1	23.2 ± 1.6	17.6 ± 0.1
^{14}C-Fatty acids	0.27 ± 0.05	0.12 ± 0.06	0.11 ± 0.09
Acetaldehyde	1.5 ± 0.06	0.6 ± 0.03	0.3 ± 0.06
Acetyl-CoA	0.13 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Acetylcarnitine	0.13 ± 0.02	0.2 ± 0.01	0.27 ± 0.01
Acetate			
Acetoacetate			
3-hydroxybutyrate			
Recovery (%)	86.8	86.9	82.3

[1]Values are mean ± SEM of 3 determinations from a single hepatocyte preparation.
[2,3]Incubation mixture consisted of KHB pH 7.4 with 2.5 % BSA, hepatocyte (1 million. mL^{-1}), [1-^{14}C]-ethanol (10 mmol. L^{-1}), L-Carnitine or L-Acetylcarnitine (200 μmol. L^{-1}) in a total volume of 3 mL. Incubation period was 20 min at 37°C.

in intact rat (Sachan 1992, Sachan and Berger 1987, Berger and Sachan 1991) and in several in vivo studies summarized by others (Cornell et al. 1977). The decrease in oxidation rate of ethanol in isolated hepatocytes is due to loss of shuttle intermediates during hepatocyte preparation, since addition of malate and/or lactate restored the rates of ethanol oxidation in isolated hepatocytes equal to that found in the intact liver (Cornell 1974, Krebs et al. 1975).

Ethanol oxidation in isolated hepatocytes was linear with time (Figure 3.1), cell numbers (Figure 3.2) and ethanol concentration (Figure 3.3). The data produced a classical Michaelis-Menten kinetic with a Km = 1.9 $mmol.L^{-1}$ and V_{max} = 11.1 $mmol.L^{-1}$. The Km for ethanol is slightly higher than the Km (1.4 $mmol.L^{-1}$) reported for rat liver ADH-isozyme 3 which is the primary isozyme for ethanol metabolism in rat (Julia et al., 1987). In our hepatocyte preparations, ethanol oxidation maximized at ethanol concentration of 4$mmol.L^{-1}$ which is similar to that seen in parenchymal cells of 24 hour starved female Wistar rats (Grunnet et al. 1973). Hepatocytes from fed male Sprague Dawley rats ethanol metabolized at a constant rate at high ethanol concentration (Stowell and Crow 1985) which is in concert with our data.

Carnitine inhibited ethanol oxidation in hepatocytes (Figure 4 and Table 3.1-3.2) which is consistent with the observations in the intact rat (Sachan 1992). The magnitude

of inhibition of ethanol metabolism was about 50% in isolated hepatocytes and about 30% in the intact animal (Sachan and Berger 1986, Mynatt and Sachan 1992). The inhibitory effect was not limited to carnitine alone in hepatcocytes since equimolar concentations of choline was equally effective in decreasing ethanol oxidation (Table 3). In the intact rat, choline did not inhibit ethanol metabolism (Sachan and Berger 1993). The most important finding was that acetylcarnitine at concentrations equal to that of carnitine was far more potent inhibitor of ethanol metabolism in hepatocytes (Table 3.3). A dose response study revealed acetylcarnitine to be 100 times more effective than carnitine alone (Table 3.4). While response to carnitine was preceded by a short lag time, the effect of acetylcarnitine was immediate. This is born out by the data in intact rat where simultaneous administration of carnitine and ethanol did not attenuate ethanol metabolism and a minimum period of about 32 hours was essential before effect of carnitine was seen (Sachan 1992). A 11-15% stimulation of ethanol oxidation by butyrobetaine, β-hydroxybutyrate and palmitylcarnitine is interesting and needs further study.

About 95% of ethanol was oxidized to CO_2 and remaining was found as acetaldehyde, acetyl-CoA, acetylcarnitine and lipids etc. (Table 3.5). Carnitine and acetylcarnitine reduced these products 20% and 40%, respectively. Since, acetaldehyde formation was especially decreased by the two

inhibitors, it suggested inhibition of ADH.

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PART IV

EFFECT OF L-CARNITINE AND L-ACETYLCARNITINE ON ETHANOL METABOLIZING ENZYMES

ABSTRACT

Since acetylcarnitine, a metabolite of carnitine, proved to be a potent inhibitor of ethanol oxidation in hepatocytes (Chapter III), the main objective of this study was to determine effects of acetylcarnitine on ethanol metabolizing enzyme(s). Because 90% of ethanol oxidation is oxidized via ADH pathway, this enzyme was targeted for potential inhibitory effects of acetylcarnitine, carnitine and related compounds. The post-microsomal supernatant (PMS) was prepared from normal male Sprague Dawley rats by differential centrifugation and used for determination of ADH activity in the presence or absence of carnitine derivatives. It was found that the inhibition of ADH activity occurred only when NAD concentration was less than the inhibitor concentration. As NAD concentration was decreased, inhibition by acetylcarnitine was significantly (67%) increased. Carnitine, choline, and acetylcholine were very weak inhibitors of ADH. Inhibition of ADH activity by acetylcarnitine produced a typical Michaelis-Menten kinetics (K_i = 135 $\mu mol.L^{-1}$) and the Lineweaver-Burk plot revealed that inhibition was of competitive nature. Binding studies showed that acetylcarnitine did bind to ADH in a dose related manner provided NAD:acetylcarnitine ratio was low. The MEOS activity was not affected by acetylcarnitine or carnitine. Both crystalline and crude ADH preparations from different vertebrate species were also inhibited by

acetylcarnitine; however, the yeast enzyme was unaffected. It is concluded that attenuation of ethanol metabolism is mediated via the inhibition of ADH by acetylcarnitine under diminishing concentrations of NAD^+.

INTRODUCTION

Ethanol has a wide variety of short- and long-term effects on human physiology (Hawkins and Kalant 1972). Ethanol oxidation occurs primarily in the liver by ADH, MEOS, and catalase (Lieber,1991). ADH catalyzes the oxidation of a wide variety of xenobiotic or endogenous alcohols (Duester 1991, Eklund et al. 1990). In addition to the rate-limiting step in ethanol metabolism (Li 1977), human class I ADH catalyzes vitamin A (retinol) oxidation (Mezey and Holt 1971), which is the rate-limiting step in the conversion of retinol to retinoic acid. Retinoic acid has been called a hormone like substance because it modulates transcription via specific nuclear receptor (Petkovich et al. 1987, Wahli and Martinez 1991).

Mammalian ADHs are polymorphic and the isozymes can be classified based on their function and physicochemical properties (Julia et al. 1987, Strydom and Vallee 1982). It has been found that isozyme ADH 3 is the enzyme responsible for ethanol oxidation in rat liver. It has a Km of 1.4 $mmol.L^{-1}$ for ethanol and Ki of 0.4 $\mu mol.L^{-1}$ for pyrazole (Julia et al. 1987).

The role of MEOS in ethanol metabolism has been controversial. However, following chronic abuse and high doses of ethanol, contribution of MEOS to total ethanol oxidation increases significantly (Lieber, 1987). The peroxidation of ethanol by catalase is limited by the rate of H_2O_2 formation which, under physiological conditions, would be too low to contribute significantly to total ethanol oxidation (Li 1977, Lieber et al. 1987, Hawkins and Kalant 1972).

Carnitine (3-hydroxy-4N-trimethyl ammonium butyrate) is an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix where β-oxidation takes place (Murthy and Pande 1984). It was this role of carnitine that first prompted investigators to study the possibility that carnitine might help prevent the ethanol-induced hepatic steatosis. In a chronic alcohol feeding trial (36% calories as ethanol), carnitine prevented ethanol-induced accumulation of total lipids, free and esterified cholesterol, triglycerides and phospholipids in the liver and the effect was dose related (Sachan and Rhew, 1983, Sachan et al. 1984, Rhew and Sachan 1986).

Carnitine offered this protection by retarding ethanol clearance due to attenuation of ethanol metabolism under both acute and chronic conditions of ethanol administration (Berger and Sachan 1986, Sachan and Berger 1987, Berger and Sachan 1991, Sachan and Berger 1993). Further, oxidation of

orally administered [1-^{14}C]-ethanol to $^{14}CO_2$ was significantly reduced 4-12 hours following ethanol administration in the carnitine supplemented intact rats. However, in vitro activities of ADH, MEOS, catalase and acetaldehyde dehydrogenase were not significantly altered by carnitine (Mynatt and Sachan 1992, Sachan 1992). Since plasma concentrations of lactate were increased 2.5 fold in the carnitine supplemented rats after ethanol administration, the explanation was that carnitine attenuated ethanol oxidation by causing a large shift in the redox state of the system (Mynatt and Sachan 1992).

In the part III, we have shown that acetylcarnitine is a more potent inhibitor of ethanol oxidation in hepatocytes than was carnitine. Therefore, the purpose of this study was to determine potential inhibitory effect of acetylcarnitine on ADH and MEOS. The purified (commercial) and semipurified (PMS, microsomes) enzyme preparations were used for these studies.

MATERIALS AND METHODS

Chemicals. ^{14}C-acetyl-CoA was purchased from Moravek Biochemicals, Inc. (Brea, CA). L-acetylcarnitine and r-butyrobetaine were obtained from Aldrich Chemical Co. (Milwaukee, WI). L-carnitine, DL-carnitine, choline, L-acetylcholine, β-OH-butyrate, L-lysine, gamma-aminobutyrate, Folin & Ciocalteu's phenol reagent, NAD

(Grade III), ADH (Bakers Yeast), NADPH (Tetrasodium Salt), Glutathione (reduced form), and palmitylcarnitine were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin was obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). All other chemicals were from Baxter Diagnostics Inc. (McGaw Park, IL).

Animals and diets. The experimental protocol was approved by committee on Humen Care and Use of Laboratory Animals. Male sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) with an average body weight of 200 g were individually housed in 7" x 10′ x 7′ stainless steel wire mesh cages. Room temperature was maintained at 22 °C ± 1 °C and constant relative humidity (52%) with a 12 hour light-dark cycle. All specifications of the animal facility complied with AAALAC regulations. All animals had free access to rat chow (Agway Prolab #3000, Syracuse, NY) and chlorinated city water.

Enzyme preparation. On the day of experiment, at 8:30 A.M., liver of a rat was surgically cannulated and perfused with cold 0.15 M KCl in 0.01 mol.L^{-1} phosphate buffer, pH 7.4. The liver was homogenized in ten volumes of KCl-phosphate buffer, pH 7.4, in a teflon/glass homogenizer. The homogenate was centrifuged at 600 x g for 10 minutes at 2 °C in a Beckman J2-21M induction drive centrifuge (Beckman Instruments, Inc.,Palo Alto, CA). The 600 x g supernatant

(SN) was carefully transferred to another centrifuge tube and centrifuged at 10,000 x g for 10 min. The 10,000 x g SN was centrifuged at 100,000 x g for 1 hr. at 4 °C in a L5-50 Ultracentrifuge. An aliquot of the PMS was then saved and used for ADH analysis and 100,000 x g pellet (microsomes) was suspended in KHB (pH 7.4) and used for determination of MEOS. Protein concentration in the PMS and microsomes was determined using Folin & Ciocalteu's phenol reagent (Lowry et al. 1951).

Determination of ADH activity. ADH activity was estimated by using a modified procedure of Bergmeyer (Bergmeyer et al. 1974). Each reaction was performed in a total volume of 3 mL at 37 °C. The components and final concentration in the reaction mixture were sodium pyrophosphate buffer (55 $mmol.L^{-1}$), pH 7.4, ethanol (20 $mmol.L^{-1}$), NAD^+ (0.2-2 $mmol.L^{-1}$), reduced glutathione (1.1 $mmol.L^{-1}$), semicarbazide (55 $mmol.L^{-1}$), glycine (1.6 $mmol.L^{-1}$), and enzyme preparation (2-3 mg SN protein). Whenever necessary deviation in the concentration of a component was made as it is indicated in that experiment. The reaction mixture was preincubated for 3 minutes at 37 °C in a temperature controlled Beckman Model 34 dual-wavelength spectrophotometer. The reaction was initiated with the addition of ethanol and absorbance at 340 nm was recored for at least 3 minutes.

Ethanol oxidation by MEOS. It was determined using a modified procedure of Lieber (Lieber and Decarli 1970).

Flasks were divided into three groups; (a) no inhibitor, (b) with 200 μmol.L^{-1} L-carnitine or (c) 200 μmol.L^{-1} L-acetylcarnitine. Each reaction was performed in a total volume of 3 mL at 37 °C in a 25 mL Erlenmeyer flask. The components and final concentration in the reaction mixture were Na_2HPO_4/KH_2PO_4 buffer (100 mmol.L^{-1}), pH 7.4, $MgCl_2$ (5.0 mmol.L^{-1}), disodium-EDTA (1.0 mmol.L^{-1}), NADPH + H^+ (1 mmol.L^{-1}), ethanol (10 mmol.L^{-1}), and enzyme preparation (8-10 mg of microsomal protein). The NADPH + H^+ and ethanol were added last. Following NADPH + H^+ addition the flask was flushed with O_2+CO_2 (95:5) for 20 seconds and the reaction was started by adding ethanol. The flask was stoppered with a serum cap and incubated in a shaking (80 oscillations/ min) water bath at 37 °C for 120 min. A control flask was prepared for each test flask in which 0.2 mL 60% $HClO_4$ was added and contents mixed prior to oxygenation and addition of ethanol and incubation. The reaction in test flasks was terminated by injecting 0.2 mL of 60% of $HClO_4$ with a syringe and mixing the contents which were then cooled for 30 min in ice bath. The reaction mixture was centrifuged at 2000 x g for 10 min and SN were assayed for ethanol concentration by an enzymatic method (Bernt and Gutmann, 1974).

Acetylcarnitine binding assay. [1-^{14}C]-acetylcarnitine was synthesized according to the method described in appendix 7 (Murthy and Pande 1984). In a 1.5 mL micro

centrifuge tubes (conical bottom with attached closure) the total volumes of 0.5 mL contained sodium pyrophospate buffer, pH 7.4 (55 mmol.L^{-1}), NAD^+ (0, 1, 2, and 4 μmol.L^{-1}), reduced glutathione (1.1 mmol.L^{-1}), [1-^{14}C]-acetylcarnitine (2 μmol.L^{-1}, specific radioactivity 123,659 dpm.$nmole^{-1}$ or 2061 Bq.nmole), and horse ADH (0.17 IU or 100 μg protein). The ratio of [NAD]:[^{14}C-acetylcarnitine] in each tube was controlled by adding different NAD concentration stated above resulting in NAD: [1-^{14}C]-acetylcarnitine ratio of 0, 0.5, 1, and 2 (see Appendix 7). The reaction mixture was incubated for 5 minutes at 37 °C in a Dubnoff metabolic shaking water bath (80 oscillation. min^{-1}). The reaction mixture was then transferred to an 30,000 NMWL Ultrafree-MC filter unit (Millipore, Bedford, MA) and centrifuged at maximum speed (15,000 x g) for 5 min in an Eppendorf centrifuge, model 5412 (Sybron/Brinkmann Instruments, Westbury, NY). The residue on membrane sample cup was transferred to a scintillation vial containing 5 mL scintillation fluid and the radioactivity was counted in a Beckman LS 3801 Liquid Scintillation counter.

All values are expressed as group mean ± SEM. The student's t-test (Ott et al. 1987) was used to determine significant differences between the means of two groups at the level of $p < 0.05$. When 2 or more treatments were compared, an ANOVA was employed and followed with a Duncan's Multiple Range Test (Steel and Torrie 1960).

RESULTS

Under the standard conditions of ADH assay, carnitine or acetylcarnitine did not affect ADH activity (Table 4.1). However, when NAD^+ concentration was made limiting, ADH activity was significantly inhibited by acetylcarnitine which was inversely related to the ratio of NAD^+:acetylcarnitine (Table 4.2). Inhibition by carnitine and choline was less than 5% compared to 45% by acetylcarnitine. The inhibition by acetylcholine ranged from 2-17% depending on the ratio of NAD:acetylcholine. The inhibitory effect of acetylcarnitine (22%) was specific as other compounds inhibited ADH activity little (3-10%) or none under identical conditions (Table 4.3). Effect of various acetyl derivatives on ADH activity is summarized in Table 4.4. At equimolar concentration and at NAD:acetyl derivative ratio of 1:2, inhibition of ADH was 28% by acetylcarnitine, 10% by acetylcholine or acetylCoA, 8% by ammonium acetate and 5% by sodium acetate. Carnitine and choline inhibited ADH activity of 4% and 5%, respectively. As shown in Table 4.5, the oxidation of ethanol by MEOS was not affected by carnitine or acetylcarnitine under the standard conditions.

The effects of two concentrations of acetylcarnitine at various concentrations of NAD^+ on the activity of ADH is shown in Table 4.6 and Figure 4.1. Either concentration of acetylcarnitine produced greater inhibition of ADH at lower

TABLE 4.1

Effect of carnitine and acetylcarnitine on ADH activity[1]

Inhibitors [200 μmol.L^{-1}]	ADH Activity[2] (nmoles NADH.min^{-1}.mg^{-1})
None	5.11 ± 0.05
L-Carnitine	5.21 ± 0.03
L-Acetylcarnitine	5.40 ± 0.03

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55 mmol.L^{-1}), pH 7.4, NAD (2 mmol.L^{-1}), reduced glutathione (1.1 mmol.L^{-1}), enzyme (mg SN protein) and inhibitor (200 μmol.L^{-1}). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol(20 mmol.L^{-1}). Absorbance was monitored at 340 nm for at least 3 min.
[2]Values are the means ± SEM of 3 determinations.

TABLE 4.2

Effect of carnitine, choline, acetylcarnitine, and acetylcholine on ADH activity different NAD concentration[1]

Inhibitors	NAD concentration (μmol.L^{-1})			
	25 [1:8][2]	50 [1:4]	100 [1:2]	200 [1:1]
	ADH Activity (nmoles NADH. min^{-1}. mg^{-1})[3]			
None	0.96±0.005^{aw}	1.84±0.012^{ax}	2.68±0.005^{ay}	4.78±0.005^{az}
L-Carnitine	0.92±0.017^{aw} (4.06)[4]	1.80±0.17^{ax} (2.38)	2.41±0.029^{by} (9.83)	4.69±0.035^{az} (1.84)
Choline	0.92±0.017^{aw} (4.06)	1.75±0.012^{ax} (4.78)	2.58±0.005^{ay} (3.29)	4.69±0.017^{az} (1.84)
L-Acetylcarnitine	0.53±0.005^{bw} (45.20)	1.20±0.012^{bx} (40.49)	1.93±0.005^{by} (27.89)	4.43±0.017^{bz} (7.38)
L-Acetylcholine	0.83±0.012^{cw} (13.23)	1.62±0.023^{bx} (11.89)	2.23±0.005^{by} (16.71)	4.69±0.012^{az} (1.84)

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55mmol.L^{-1}), pH 7.4, NAD (25-200 μmol.L^{-1}), reduced glutathione (1.1 mol.L^{-1}), enzyme (2-3 mg SN protein), and inhibitor (200 μmol.L^{-1}). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol (20 mmol.L^{-1}). Absorbance was monitored at 340 nm for at least 3 min.

[2]The NAD:inhibitor ratio.

[3]Values are the means ± SEM of 3 determinations. Values bearing different superscript letters (a-c) between rows are significantly different ($P \leq 0.05$). Values bearing different superscript letter (w-z) between columns are significantly different ($P \leq 0.05$).

[4]Values in parenthesis indicate percent inhibition with reference to the control.

TABLE 4.3

Effect of carnitine and related molecules on ADH activity[1]

Additions [200 μmol.L^{-1}]	ADH Activity[2] (nmoles NADH.min^{-1}.mg^{-1})	Inhibition (%)
None	2.47 ± 0.02^{a}	0
L-Carnitine	2.24 ± 0.01^{b}	9.3
DL-Carnitine	3.39 ± 0.02^{a}	0
Choline	2.31 ± 0.01^{a}	6.5
Choline(pH 7.0)	2.31 ± 0.03^{a}	6.5
Acetyl-L-Carnitine	1.92 ± 0.01^{b}	22.3
r-Butyrobetaine	2.39 ± 0.02^{a}	3.2
β-OH-Butyrate	2.54 ± 0.02^{a}	0
L-Lysine	2.54 ± 0.03^{a}	0
r-Aminobutyrate	2.47 ± 0.02^{a}	0

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55 mmol.L^{-1}), pH 7.4, NAD (100 μmol.L^{-1}), reduced glutathione (1.1 mol.L^{-1}), enzyme (2-3 mg SN protein) and inhibitor (200 μmol.L^{-1}). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol (20 mmol.L^{-1}). Absorbance was monitored at 340 nm for at least 3 min.

[2]Values are the means ± SEM of 3 determinations. Values bearing different superscript letters between rows are significantly different ($P \leq 0.05$)

TABLE 4.4

Effects of acetyl derivatives on ADH activity[1]

Additions [200 μmol.L^{-1}]	ADH Activity[2] (nmoles NADH.min^{-1}.mg^{-1})	Inhibition (%)
NS	3.33 ± 0.02^{a}	0
L-Carnitine	3.21 ± 0.03^{a}	3.96
Choline	3.16 ± 0.01^{a}	5.25
Acetylcarnitine	2.37 ± 0.02^{b}	28.95
Acetylcholine	2.98 ± 0.02^{b}	10.53
Amonium Acetate	3.07 ± 0.01^{a}	7.92
Na-Acetate	3.15 ± 0.02^{a}	5.28
Acetyl-Co A	2.98 ± 0.03^{b}	10.53

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55mmol.L^{-1}), pH 7.4, NAD (100 μmol.L^{-1}), reduced glutathion (1.1 mol.L^{-1}), enzyme (2-3 mg SN protein), and inhibitor (200 μmol.L^{-1}). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol (20 mmol.L^{-1}). Absorbance was monitored at 340 nm for at least 3 min.

[2]Values are the means ± SEM of 3 determinations. Values bearing different superscript letters between rows are significantly different ($P \leq 0.05$)

TABLE 4.5

Effect of carnitine and acetylcarnitine on ethanol oxidation by rat liver microsomes (MEOS)[1]

Additions [200 μmol.L^{-1}]	Ethanol Oxidation[2] ($mmol.L^{-1}.h^{-1}.g^{-1}$ microsomal protein)
None	30.55 ± 0.98
L-Carnitine	31.68 ± 1.14
L-Acetylcarnitine	31.84 ± 1.50

[1]Condisions: 3 mL of reaction mixture contained sodium potassium phosphate buffer (100 $mmol.L^{-1}$), pH 7.4, $MgCl_2$ (5.0 $mmol.L^{-1}$), disodium-EDTA (1 $mmol.L^{-1}$), NADPH + H^+ (1.0 $mmol.L^{-1}$), enzyme (8-10 mg microsomal protein), and ethanol (10 $mmol.L^{-1}$). Reaction mixture was incubated for 120 min. at 37 °C in methods.
[2]Values are the means ± SEM of triplicate from 3 different rats.

TABLE 4.6

Inhibition of ADH activity at different concentrations of NAD and L-acetylcarnitine[1]

Inhibitor	NAD Concentration (μmol.L^{-1})						
	25	50	100	200	400	1000	2000
	ADH Activity (nmoles NADH.min^{-1}.mg^{-1})[2]						
None	1.39	1.93	2.93	4.32	4.77	5.10	5.49
L-Acetyl-carnitine [100 μmol.L^{-1}]	0.67 (51.8)[3]	1.61 (16.5)	2.45 (16.3)	4.23 (2.1)	4.77 (0)	5.10 (0)	5.49 (0)
L-Acetyl-carnitine [200 μmol.L^{-1}]	0.46 (64.7)	1.12 (41.9)	1.93 (34.1)	2.94 (31.9)	4.00 (16.1)	5.10 (0)	5.49 (0)

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55mmol.L^{-1}), pH 7.4, NAD (25-2000 μmol.L^{-1}), reduced glutathione (1.1 mol.L^{-1}), enzyme (2-3 mg SN protein), and inhibitor (100 and 200 μmol.L^{-1}). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol (20 mmol.L^{-1}). Absorbance was monitored at 340 nm for at least 3 min.

[2]Values are the means of 2 determinations.

[3]Values in parenthesis indicate percent inhibition with reference to the control.

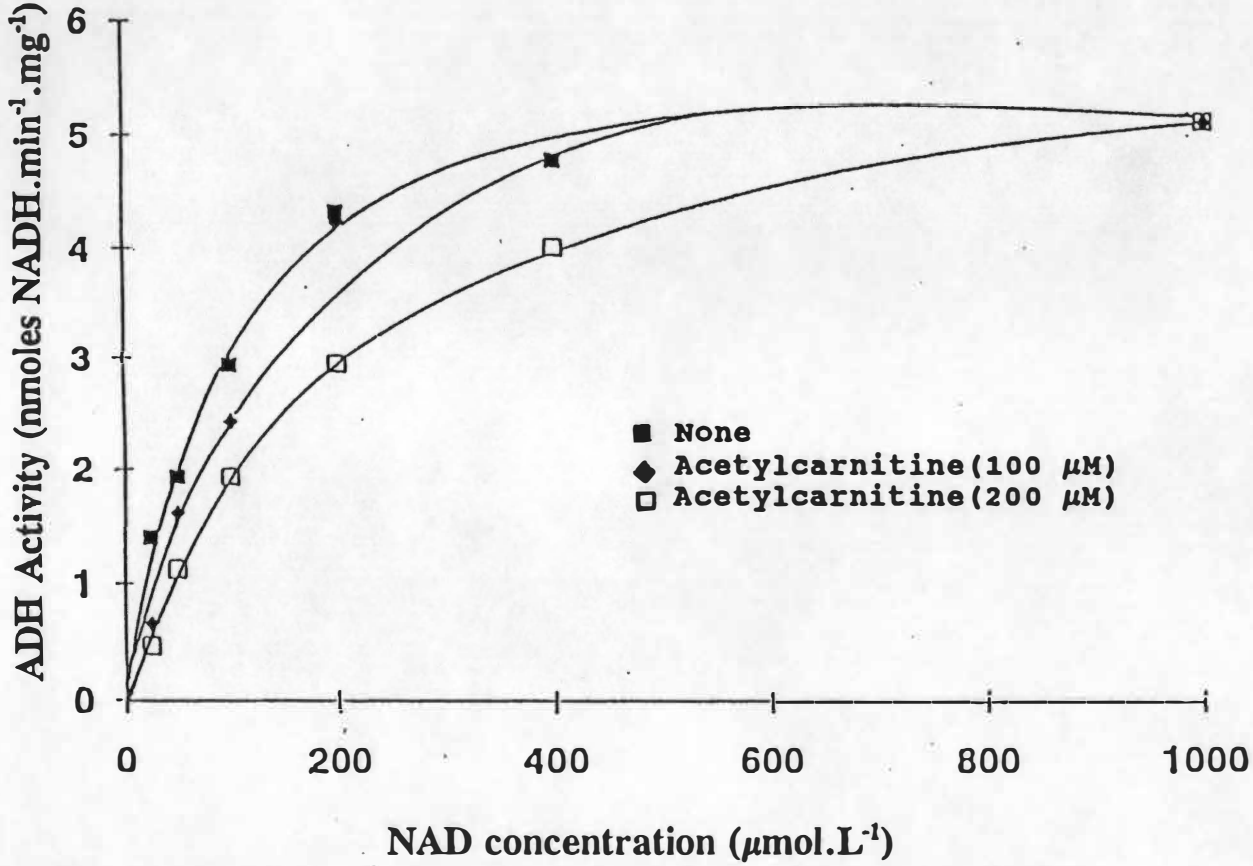

FIGURE 4.1. Alcohol dehydrogenase activity on different NAD concentration

than at higher concentrations of NAD^+. At a given concentration of NAD^+, higher concentration (200 $\mu mol.L^{-1}$) of acetylcarnitine was proportionally more inhibitory than lower concentration (100 $\mu mol.L^{-1}$). The plot of reaction velocity and the concentration of NAD (co-substrate) resulted in 3 separate hyperbolic curves which obeyed classical Michaelis-Menten kinetics (Figure 4.1). A Lineweaver-Burk plot of this data (Figure 4.2) produced a definite shift in the Km without affecting Vmax which is the characteristic of competitive inhibition (Table 4.7). The Ki at 100 $\mu mol.L^{-1}$ and 200 $\mu mol.L^{-1}$ of acetylcarnitine were 184.5 and 135.1 $\mu mol.L^{-1}$, respectively.

In order to access the site of acetylcarnitine interaction, NAD^+ and acetylcarnitine were preincubated with ADH and reaction velocity was measured (Table 4.8). When enzyme was preincubated with acetylcarnitine alone or with acetylcarnitine plus NAD^+, the inhibition was essentially identical, however, when ADH was preincubated with NAD^+ first the inhibition by acetylcarnitine was decreased. This suggested that interactions were at the level of enzyme and not NAD. A more direct assessment was made by studying the binding [1-^{14}C]-acetylcarnitine to ADH at different ratio of NAD:[1-^{14}C]-acetylcarnitine (Table 4.9). The binding of [1-^{14}C]-acetylcarnitine to ADH was decreased as the ratio of NAD:acetylcarnitine was increased.

The effect of carnitine and acetylcarnitine on the

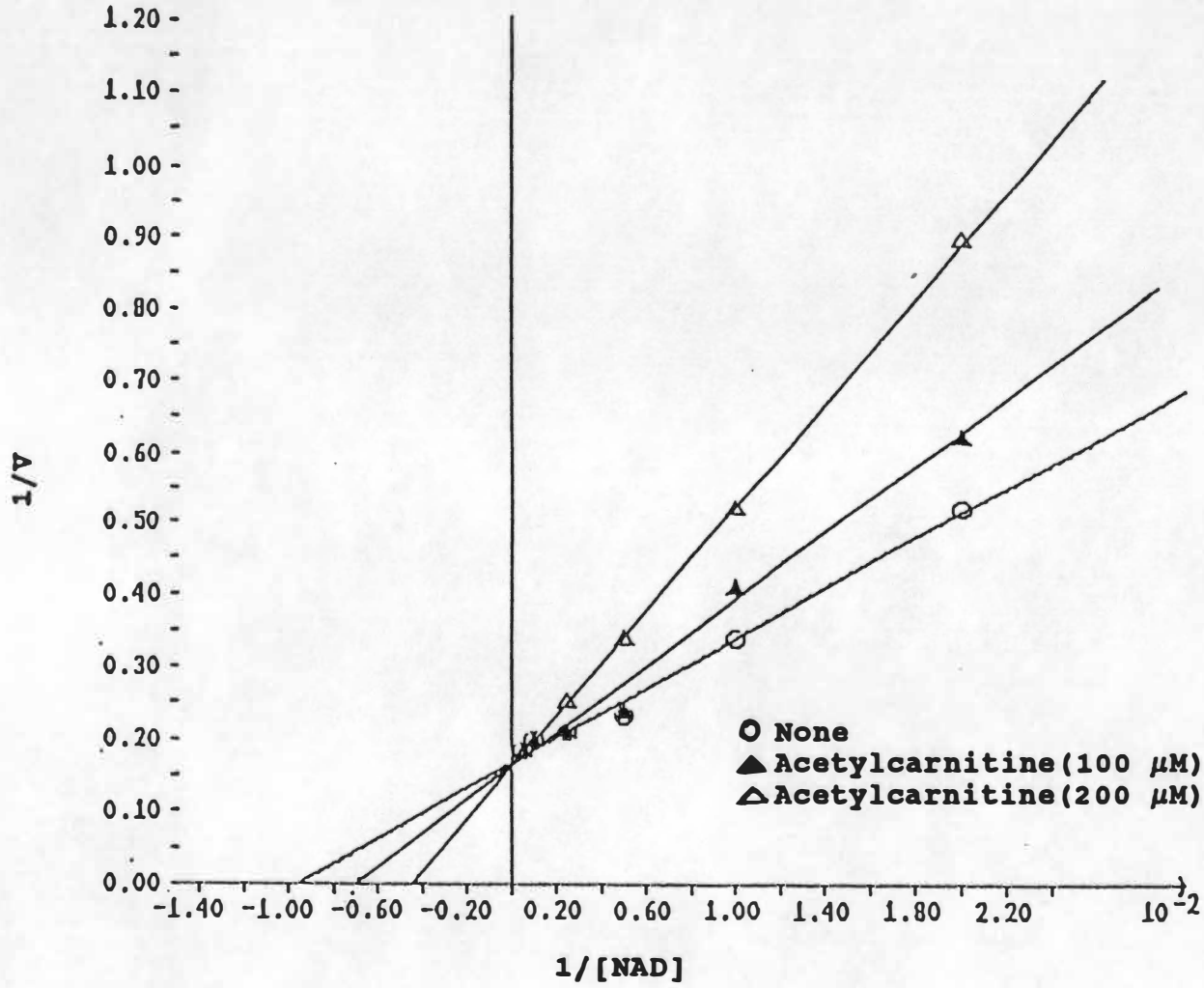

FIGURE 4.2. Linweaver-Burk plot

TABLE 4.7

Kinetic parameters for ADH activity at varying concentrations of acetylcarnitine[1]

Additions	Km (μmol.L^{-1})	Vmax (μmol.L^{-1})	Ki (μmol.L^{-1})
None	87.64	5.71	
Acetyl-L-Carnitine [100 μmol.L^{-1}]	113.02	5.71	184.52
Acetyl-L-Carnitine [200 μmol.L^{-1}]	217.00	5.71	135.09

[1]Kinetic parameters were calculated from the values in Table 4.5 using computer program (Enzfitter).

TABLE 4.8

Inhibition of ADH activity by carnitine, choline,acetylcarnitine, and acetylcholine under different experimental condition

Inhibitor:NAD Conc. [200:100 μmol.L^{-1}]	Experimental Condition[1]		
	A	B	C
	ADH Activity (nmoles NADH.$min.^{-1}.mg^{-1}$)[2]		
None	2.67	3.33	2.67
L-Carnitine	2.41 (9.83)[3]	3.20 (3.96)	2.63 (1.64)
L-Acetylcarnitine	1.93 (27.87)	2.37 (28.95)	2.19 (18.05)
Choline	2.59 (3.29)	3.16 (5.25)	2.63 (1.64)
L-Acetylcholine	2.23 (16.71)	2.98 (10.53)	2.41 (9.83)

[1]Experimental Conditions were as described in table 4 except the following
A: NAD + inhibitor preincubated for 3 min. prior to ethanol addition
B: Inhibitor preincubated for 3 min. prior to NAD + ethanol addition
C: NAD preincubated for 3 min. prior to inhibitor + ethanol addition
[2]Values are the means of 2 determinations.
[3]Values in parenthesis indicate percent inhibition with reference to the control.

TABLE 4.9

Binding of [1-^{14}C]-acetylcarnitine to horse alcohol dehydrogenase at different ratio of [NAD]:[1-^{14}C]-acetylcarnitine[1,2]

NAD:[1-^{14}C] Acetylcarnitine^{-1}	[^{14}C] DPM/mg Enzyme protein	% Binding
0	5051 ± 252^{a}	5.1 ± 0.23^{a}
0.5	3845 ± 240^{b}	3.9 ± 0.23^{b}
1	3499 ± 214^{c}	3.5 ± 0.17^{c}
2	1772 ± 102^{d}	1.7 ± 0.05^{d}

[1]Conditions: 0.5 mL of reaction mixture contained sodium pyrophosphate buffer (55 mmol.L^{-1}), pH 7.4, NAD (0-2 nmol.L^{-1}), reduced glutathione (1.1 mmol.L^{-1}), [1-^{14}C]-Acetylcarnitine (1 nmol.L^{-1}), and horse ADH (0.17 μmol.L^{-1} or mg protein). Reaction mixture was incubated for 5 min. at 37 °C, filtered through millipore filter by centrifugation, and counted ^{14}C in the filter.

[2]The values are means ± SEM of 3 determinations.

Values bearing different superscript letters between rows are significantly different ($P \leq 0.05$)

activity of ADH derived from different species is shown in Table 4.10. Neither carnitine nor acetylcarnitine inhibited the yeast ADH activity. However, the activities of ADH from horse, rat, guinea pig, mouse and broilers were significantly inhibited by acetylcarnitine.

DISCUSSION

The analysis of the products of ethanol oxidation by hepatocytes (Table 3.5) revealed 80% inhibition of acetaldehyde formation in presence of acetylcarnitine suggesting that the site of inhibition must be at the enzymatic level. Since ADH is the main enzyme of ethanol oxidation, it was considered to be the target enzyme for studying the effect of carnitine, acetylcarnitine, and related compounds. The results showed that acetylcarnitine inhibited ADH only when the ratio of NAD:acetylcarnitine was low. The inhibition was minimum (7%) at the ratio of 1:1 and maximum (45%) at ratio of 1:8 (Table 4.2). When this ratio was higher than 1:1, there was no effect of acetylcarnitine on ADH (Table 4.1). On the other hand, at any ratio of NAD:carnitine, thheinhibition of ADH was no more than 10%. In previous studies (Mynatt and Sachan 1992) also, carnitine produced little or no inhibition of ADH.

The specificity of ADH inhibition by acetylcarnitine was determined using structurally related compounds e.g. choline and acetylcholine or carnitine precursors namely, r-

TABLE 4.10

Inhibition of ADH from different species by carnitine and acetylcarnitine[1]

Species	Inhibitor		
	None	L-Carnitine	L-Acetylcarnitine
	ADH Activity (nmoles $NADH.min^{-1}.mg^{-1}$)[2]		
Yeast[3]	36.18 ± 0.1[a]	36.18 ± 0.2[a] (0)[5]	36.18 ± 0.1[a] (0)
Horse[3]	0.53 ± 0.01[a]	0.48 ± 0.07[a] (9.2)	0.38 ± 0.01[b] (27.3)
Rat[4]	3.33 ± 0.2[a]	3.20 ± 0.2[a] (3.3)	2.36 ± 0.1[b] (27.9)
Guinea Pig[4]	7.33 ± 0.5[a]	6.58 ± 0.4[a] (8.0)	5.37 ± 0.4[b] (26.8)
Mouse[4]	6.49 ± 0.05[a]	6.02 ± 0.1[a] (7.3)	5.03 ± 0.2[b] (22.5)
Broilers[4]	4.06 ± 0.2[a]	3.85 ± 0.2[a] (5.2)	3.01 ± 0.3[b] (15.3)

[1]Conditions: 3 mL of reaction mixture contained sodium pyrophosphate buffer (55 $mmol.L^{-1}$), pH 7.4, NAD (100 $\mu mol.L^{-1}$), reduced glutathione (1.1 $mol.L^{-1}$), enzyme (2-3 mg SN protein), and inhibitor (200 $\mu mol.L^{-1}$). The reaction mixture was preincubated for 3 min at 37 °C before starting reaction by addition of ethanol. Absorbance was monitored at 340 nm for at least 3 min.

[2]Values are the means ± SEM of triplicate determinations. Values bearing different superscript letters between columns are significantly different ($P \leq 0.05$)

[3]The ADH was purchased from the Sigma Chemical Co.

[4]The ADH was prepared by differential centrifugation and 100,000 x g supernatant was used.

[5]Values in parenthesis indicate percent inhibition with reference to the control.

butyrobetaine and lysine. Among these compounds, only choline, acetylcholine, and butyrobetaine inhibited ADH approximately 5%, 14%, and 3%, respectively (Table 4.2-4.3). Because of relatively high degree of inhibition by acetylcarnitine and acetylcholine compare to carnitine and choline, it was necessary to examine effect of acetyl moiety. Acetyl group by itself was not able to inhibit ADH any more than choline or carnitine, however, acetyl-CoA was as effective as acetylcholine, both of which at equimolar concentration produced only about 30% of the inhibition caused by acetylcarnitine (Table 4.4). Therefore, it is concluded that the inhibition of ADH by acetylcarnitine is specific and can not be matched by the related compounds we studied. These result provide explanation for the lack of significant (10%) inhibition by choline (Sachan and Berger 1993) and the requirement of a pretreatment period with carnitine for its inhibition of ethanol metabolism in the intact rat (Sachan 1992). In the intact system, it would be difficult to maintain a low ratio of NAD:acetylcarnitine because NAD^+ is being constantly regenerated and acetylcarnitine is being excreted in urine. However, the picture may be quite different in the chronic alcoholic condition.

There are a large number of molecules which have some degree of inhibitory effect on ADH (Table A8.1). Among these Pyrazol and its 4-substituted derivatives are most

potent and widely studied compound with regard to ADH inhibition (Goldberg and Rydberg 1969, Danielesson et al. 1992). The inhibition varies with the isozyme involved e.g. rat ADH 3 has a Ki = 0.4 μM, ADH 1 ki = 0.56 μM but ADH 2 is insensitive to pyrazol (Julia et al. 1986). Pyrazol and its analogues are competitive inhibitors of ADH (Goldberg and Rydberg 1969) which is brought about by formation of an extremely tight ADH-NAD^+ and pyrazol ternary complex (Khalifah and Sutherland 1979). The precise mode of binding of pyrazoles has not been elucidated. However, it is proposed that heterocyclic ring of pyrazole forms a bridge through its two nitrogen atoms and the catalytic zinc atom and C-4 of the nicotinamide moiety of NAD (Branden et al. 1975). The mechanism of acetylcarnitine inhibition may be similar to that of pyrazole since it does have quaternary nitrogen.

Inhibitor molecules bind to enzyme protein either at the coenzyme binding site or at the substrate binding site. Three main coenzyme binding sites where inhibitors are known to bind are 1) the adenosine binding cleft for aromatic molecules, 2) the anion binding site where the pyrophosphate group of the coenzyme binds, and 3) the nicotinamide binding region (Branden et al. 1975). Acetylcarnitine appears to bind to ADH at the site of NAD binding since NAD inhibited acetylcarnitine binding to ADH in a dose dependent manner. When the ratio of NAD:acetylcarnitine was increased from 0

to 2, binding of [1-^{14}C-acetylcarnitine to ADH decreased 67% (Table 4.10). The affinity of ADH for binding is greater for NAD than acetylcarnitine as indicated by preincubation experiments shown in Table 4.9. However, these experiments do not inhibitory rule out the possibility of alteration of NAD configuration by acetylcarnitine.

Acetylcarnitine-NAD^+ interactions inhibited ADH preparations from broiler chicken, guinea pig, horse, mouse, rat but not the enzyme from yeast (Table 4.11). The specificity may be related to different isozymes of the species (Julia et al. 1987). The ADH is not a single enzyme but a system consisting of many isozymes which are species specific to a certain degree. Yeast and mammalian ADH differ in substrate specificity and rate of catalytic activity (Branden et al. 1975). Human and rodent ADH exists as a heterogeneous group of isoenzymes that can be placed into three categories based upon structural and functional distinctions (Strydom and Vallee 1982). There are clear analogies between human ADH classes and rat ADH isoenzymes. There is approximately 82% sequence homology among subunits of human and rat liver ADH (Julia et al. 1987). The kinetic and molecular properties of rat ADH 3 are similar to those of human class I isoenzymes. It is well known that ethanol is the substrate for classical human class I ADH (Li 1977) and rat ADH 3 (Julia et al. 1987) isoenzyme. Both human class I and rat ADH 3 are mainly localized in liver and rat

enzyme exhibits a Km of 1.4 mmol.L^{-1} for ethanol and it is strongly inhibited by pyrazole, ki = 0.4 μmol.L^{-1}.

This data provides molecular explanation for carnitine-mediated attenuation of ethanol metabolism in the intact rat (Sachan and Berger 1987, Mynatt and Sachan 1992), mouse (Sachan 1992) and broiler chicken (Smith et al. 1993). These similarities and differences among species must be born in mind in extrapolation of rat data to humans.

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APPENDICES

APPENDIX 1

HEPATOCYTE PREPARATION

A. Preparation of solutions for perfusion

Table A 1.1.

Composition of buffers used for collagenase perfusion

Chemicals (g)	Ca_{++}-free perfusion buffer (L)	Collagenase buffer (L)	Suspension buffer (L)	Krebs-Henseleit buffer (L)
NaCl	8.3	0.39	0.4	9
KCl	0.5	0.05	0.04	0.46
$CaCl_2$		0.529	0.0136	0.37
$MgCl_2.6H_2O$			0.013	
KH_2PO_4			0.05	0.21
$MgSO_4$				0.38
Na_2SO_4			0.01	
$NaHCO_3$				2.73
HEPES	2.4	2.4	0.72	
TES			0.69	
Tricine			0.65	
1 M NaOH	5.5	6.6	5.25	
Collagenase		0.05		
pH	7.4	7.6	7.6	7.4

B. Preparation of apparatus

We used the perfusion apparatus similar to that described by Seglen (Seglen 1976) and shown in Figure A 1.1.

1. It's most important unit is the water-jacketed coiled tube (1), which maintains the perfusate temperature at 37 °C (2 x 4 mm glass tube in 22 to 24 coil; coil diameter 30 mm; coil length 100 mm).
2. A combined filter unit and bubble trap (2) is made of a short piece of silicone rubber tubing with a rubber stopper in each end; stainless-steel cannulas are used to pierce the stopper. A cotton plug

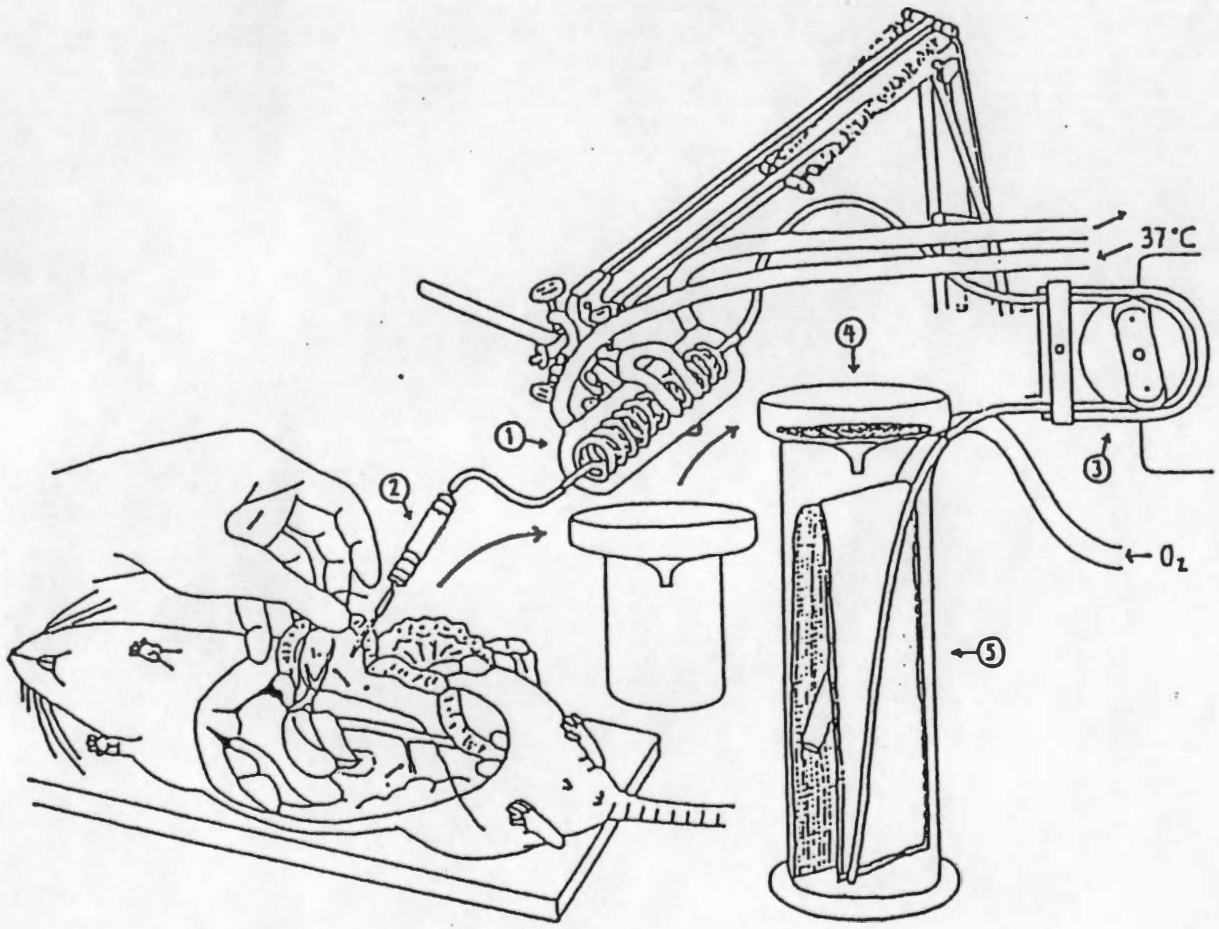

FIGURE A 1.1 Experimental apparatus for liver perfusion
(Seglen 1976)

is used as a filter; the cotton is carefully packed at the portal, end of the fluid-filled filter unit, taking care to avoid the trapping of bubbles within the cotton.

3. From the other end of the coiled tube a long stretch (90 Cm) of 2 x 4 mm silicone rubber tubing leads through the pump (3) to the buffer reservoir.
4. The coiled tube is fixed with a clamp onto a flexible lamp holder. The whole coiled tube-filter-cannula assembly can thus be moved freely in all directions, which facilitates exact positioning of the portal cannula and easy transfer of the cannulated liver from the body to the liver dish.
5. The latter is a flat glass bottle (diameter 80 mm) with a conical outlet (4); the isolated liver is placed on a nylon net in the bottom of the dish.
6. When Ca^{++}-buffer is recirculated before perfusion, the oxygenation (5) is provided to sufficient surface area for complete oxygenation of the perfusate.

C. Surgical procedure and perfusion

1. The rat was anesthetized by Metofane (Pitman-Moore, Inc. Mundelein, IL) in jar placed a hood , it was carefully transferred to the operating tray.
2. The rat was placed on its back on the operating tray with its neck stright. The abdomen was opened by a V-

shaped incision, begining at lower abdomen and extending to the back near last left ribs. The intestines were displaced to the left side of the abdominal cavity (right of operator) so that portal vein and posterior vena carva were in right.

3. A loose ligature was placed around the portal vein at about 1 Cm poster to entry point in liver (Fig. A 1.1). The portal cannula was then placed adjacent to the portal vein.
4. The perfusate flow (Ca^{++}-free perfusion buffer) is started at a rate of 30 mL per min. The oxygenator cylinder is used as a reservoir for the perfusion buffer (about 500 mL), which was maintained at 37 °C and saturated with oxygen.
5. A deep cut was made in the posterior vena cava and using a fine scissors a small (hafeway through wall) cut was rapidly made in the potal vein a few mm inferior to ligature (Fig. A 1.1: upper arrow).
6. The portal cannula was rapidly inserted into the vein through the cut and pushed up towards lower so that it lies about portal 2-4 mm inside the ligature. The ligature was securely tied and secured in place with an extra knot.
7. The perfusate flow was increased to 50 mL per minute. The first 2-3 minutes of preperfusion was allowed while the liver remained intact in situ.

8. While preperfusion is continued, the liver was removed from the carcass by cutting all attachments including the vascular and biliary supply distal to the ligature (one cut), both ends of the vena cava as close to the liver as possible, and the thin, clear ligaments to the intestines and the abdominal wall. Care was exercised to avoid twisting of the hepatic lobes, cutting into the liver tissue, and rupture of the liver capsule due to stretching of the ligaments.
9. The liver is transferred to the liver perfusion dish (temporarily placed on top of a 250 mL beaker) and laid flat in a normal position similar to them in situ position. A good perfusion of liver should allow rapid perfusate afflux with no swelling, and the whole liver should have a uniform, light-tan color.
10. When 500 mL of the Ca^{++}-free perfusion buffer in the oxygenator-reservoir has been used about 10 min, the flow pump was momentarily stopped, and reservoir was changed from Ca^{++}-free buffer to collagenase buffer.
11. The perfusate flow was immediately reswitched at its maximal rate (50 mL.min), and the liver dish was placed on top of the collagenase buffer beaker.
12. The liver is perfused with recirculating collagenase buffer 50 mL. min for 7-10 minutes; during this time it swells to more than twice its original size.

13. The liver is then transferred to a watch glass containing 5 mL of KHB with 2.5% BSA. The liver is held up with forceps (portal region) and gently shaken, the cells are liberated from the liver connective and vascular stroma by careful raking with a stainless-steel fork.

D. Cell collection and viability test

1. The cell suspension was distributed in a 10 mL KHB with 2.5% BSA and centrifuged in a Beckman TJ-6R tabletop centrifuge at 50 x g for 3 min at 25 °C.
2. The supernatant and lose pellet was discared and heavy cells fluid was resuspened in 10 mL of KHB with 2.5% BSA and centrifuged as above.
3. The final packaged cells were suspened in KHB with 2.5% BSA and kept at room temperature until the experiment.
4. Cell suspension was diluted 1:10 and 100 μL of it was treated with 0.6% trypan blue (1:1). The cells were counted in a hemacytometer, 4 outer squares, using an Olympus TO41 microscope. Cell numbers was calculated as follow: Cells/mL = # of counted cell x DF / 4 and % of viability was determined (should be upper 90 %).

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APPENDIX 2

ACETYLCARNITINE AND ACETYL-CoA DETERMINATION

Acetylcarnitine was determined spectrophotometrically by the method of Pearson et al. (Pearson et al.1974).

Principle

$$\text{(1) Acetylcarnitine + CoASH} \xleftrightarrow{\text{CAT}} \text{carnitine + Ac-CoA}$$

$$\text{(2) Ac-CoA + Oxaloacetate} \xrightarrow{\text{citrate synthase}} \text{Citrate + CoASH}$$

$$\text{(3) Malate + } NAD^+ \xleftrightarrow{\text{MDH}} \text{Oxaloacetate + NADH + } H^+$$

The acetyl-CoA formed in reaction (1) reacts with oxaloacetate in the presence of citrate synthase to give citrate [eqn.(2)]; CoASH is regenerated. The consumption of oxaloacetate leads to disturbance of the equilibrium of the malate dehydrogenase (MDH) reaction (3) with consequent reduction of NAD. The increase in the NADH concentration, as meassured by the extinction at 340 nm, is the unit of measurement.

Equipment

Spectrophotometer for measurements at 340 nm.

Reagents

1. 1 M Potassium malate: Dissolve 13.4 g L-malic acid in water, adjust to pH 7.5 with 5 N KOH, and make vol. to 100 mL with distilled water.
2. 10 mM β-Nicotinamide-adenine dinucleotide: Dissolve 23.1

mg NAD in approx. 1 mL distilled water, add 0.045 mL 1N KOH, and dilute to 3 mL with distilled water.

3. 10 mM Coenzyme A: Dissolve 10 mg CoA in 1 mL distilled water.
4. Malate dehydrogenase, MDH (1 mg protein.mL^{-1}): Dilute stock suspension to get desired concnetration with 3.2 M ammoniumsulphate solution (pH=6.0)
5. Citrate synthase,CS (1 mg protein.mL^{-1}): Dilute stock suspension to ge desired concentration with 3.2 M ammonium sulphate solution (pH=6.0).
6. 1 M Tris buffer (pH 7.8): Dissolve 12.1 g tris in 65 mL 1 N HCl, and make up to 100 mL with distilled water.
7. 50 mM Ethylenediaminetetra-acetate, EDTA: Dissolve 0.46 g EDTA-$Na_2H_2.2H_2O$ in 20 mL distilled water,adjust pH to 8 with 1 N KOH, and make up to 25 mL with water.
8. Carnitine acetyltransferase, CAT(1 mg protein. mL^{-1}): Dissolve 1 mL suspention in 4 mL potassium phosphate buffer.
9. 0.5 M Phosphate buffer(pH 7.5): Dissolve 6.8 g KH_2PO_4 in approx. 70 ml of water, adjust to pH 7.5 with approx. 2.2 mL 5 N KOH, and make up to 100 mL with distilled water.
10. 50 mM Perchloric acid:Dilute 4.2 mL 70% $HClO_4$ to 100 mL with distilled water.
11. 30 mM Acetylcarnitine:
12. 30 mM Acetyl-CoA:

Procedure

A standard curve was prepared by adding 0, 10, 20, 40, 60, 80 and 100 µl (Table A 2.1) of acetylcarnitine standard (30 mM) to tubes containing 100 µl 10% BSA, 300 µl of 3.0 M $HClO_4$ and double distilled water to a total volume of 1.65 mL. The tubes were vortexed vigorously for 30 sec and allowed to sit in an ice water bath for 5 min, vortexed again and centrifuged for 10 min at 1500 x g at 2 °C.

1.0 mL of the supernatant was removed and added to another conical tube on ice. 200 µl of 3 M $KHCO_3$ was added, vortexed and allowed to sit for 15 min, then centrifuged for 10 min at 1500 x g at 2 °C. The supernatant was then poured off into another tube on ice.

A 100 µl aliquot of the neutralized standard or samples (cell supernatant) were added to 1.86 mL of reaction mixture (1) in a 3 mL polystyrene disposable cuvette (Baxter Scientific Products, Atlanta, GA) and mixed by inversion and read extinction (E_1). MDH suspention (2) was pipetted into the cuvette, mixed, waited until equilibrium was reached, and read extinction (E_2). CS suspension (3) was added,mixed, waited until reaction stoped, and read extinction (E_3). Acetyl-CoA in the sample was reacted. CAT solution (4) was mixed, waited until reaction stoped, and read extinction (E_3). Acetylcarnitine was reacted in this reaction (5-15 min).

$@E_1 = E_2 - E_1 \quad @E_2 = E_3 - E_2 \quad @E_3 = E_4 - E_3$

Table A 2.1

Reaction mixture and summary of procedure

Reagent	volume(ml)	Final Con. in assay mixture(mM)
(1) 1 M Tris buffer	0.20	100
L-Malate solution	0.02	10
EDTA solution	0.05	1.25
NAD solution	0.10	0.5
CoA solution	0.025	0.125
Water	1.465	Vt =1.96
(2) MDH suspention	0.01	5 $\mu g.mL^{-1}$ = 5.5 $U.mL^{-1}$
(3) CS suspension	0.02	10 $\mu g.mL^{-1}$ = 0.7 $U.mL^{-1}$
(4) CAT solution	0.01	5 $\mu g.mL^{-1}$ = 0.6 $U.mL^{-1}$

Table A 2.2

Example of an assay

No.	30 mM Ac-CNE			30mM Ac-CoA			D.W. (μL)	10%BSA (μL)	3 M $HClO_4$ (μL)	3 M $KHCO_3$ (μL)
	μL	μmol	[μM]	μL	μmol	[μM]				
1B	0	0	0	0	0	0	1250	100	300	200
2RS1	10	0.3	9.1	10	0.3	9.1	1230	100	300	200
3RS2	20	0.6	18.2	20	0.6	18.2	1210	100	300	200
4RS3	40	1.2	36.4	40	1.2	36.4	1170	100	300	200
5RS4	60	1.8	54.5	60	1.8	54.5	1130	100	300	200
6RS5	80	2.4	72.7	80	2.4	72.7	1090	100	300	200
7RS6	100	3.0	90.9	100	3.0	90.9	1050	100	300	200
8S1	100 -1000 μL							100	300	200

V_t = 1.65

RS = Refereced Standared, S = Sample

Calculations

Extinction coefficient of NADH:

$c = 6.22\ cm^2.\ \mu mole_{-1}$ at 340 nm

#1. Acetyl-CoA:

$$c = \frac{\alpha \times @E_1}{6.22} \times 2\ [\mu mole.cuvette^{-1}\ contents]$$

Where $\alpha = \frac{\beta^2 + 2\beta}{\beta + 1}$ and $\beta = \frac{@E_2}{@E_1}$

#2. Acetylcarnitine + Acetyl-CoA:

$$c = \frac{\alpha' \times @E_1}{6.22} \times 2[\mu mole.cuvette\ contents^{-1}]$$

Where $\alpha' = \frac{(\beta')^2 + 2\beta')}{\beta' + 1}$ and $\beta' = \frac{@E_2 + @E_3}{@E_1}$

The value for aceylcarnitine is found from the difference between equations #2 and #1. If the sample contains no acetyl-CoA, $@E_2 = 0$, and the calculation is simplified.

Normal Values: Tissues of rats 377 ± 62 n mole per g of frozen heart muscle and 41 ± 9 n mole per g of frozen liver.

Reference:

1. Pearson, D. J., Tubbs, P. K. & Chase, J. F. A. (1759) Canitine and acylcarnitines. In: Methods of enzymatic analysis, Vol. 3 (Bergmers, H. V., ed. pp.1758-1818, Verlay chemie Intern, Deerfield Beach, Fla.

APPENDIX 3

ACETATE DETERMINATION

Acetate was determined by a enzymetic determination by using acetyl-CoA synthetase as described by Guynn and Veech (Mellanby & williamson 1974), with some modifications. The acetate concentration was determinated as the difference in the initial and final readings by using a potassium acetate standard curve.

Principle

The method is based upon the enzyme sequence:

$$Acetate^{-} + ATP^{4-} + CoA = AMP^{2-} + PP^{4-} + acetyl\text{-}CoA + H^{+}$$

$$Acetyl\text{-}CoA + oxaloacetate^{2-} = citrate^{3-}\ CoA + H^{+}$$

$$Malate^{2-} + NAD^{+} = oxaloacetate^{2-} + NADH + H^{+}$$

$$Net:\ Acetate^{-} + ATP^{4-} + malate^{2-} + NAD^{+} = AMP^{2-} + PP^{4-} + citrate^{3-} + 3H^{+} + NADH$$

The assay depends upon the conversion of acetate to acetyl-CoA and the measurement of the acetyl-CoA by a coupled system of malate dehydrogenase and citrate synthase.

Equipment

Spectrophotometer for measurements at 340 nm.

Reagents

a) Stored at 0-4 °C

1. 0.2 M Tri-HCl, pH 8.0 containing KCl, 0.1 M:
2. 0.1 M Magnesium chloride:
3. 5.0 mM NADH, in 0.3% (w/v) $KHCO_3$, Stable for 5-7 days :
4. 1.2 M Perchloric acid:

b) Stored below -15 °C

5. 27 mM Adenosine 5'-triphosphate, Tris, Potassium or sodium salt:
6. 24 mM NAD^+, free acid:
7. 0.1 M Malate, Potassium salt:
8. 1.2 mM Coenzyme A, free acid, containing 3.0 mM of mercaptoethanol, prepare fresh good for 1 week
9. Bovine serum albumin, 100 $mg.mL^{-1}$:
10. Acetyl-CoA synthtase:

Procedure

Preparation of reagent cocktail:

All reagents were prepared in deionized water and neutralized when necessary with either HCl or KOH.

A cocktail was prepared containing 90 mmol Tris-HCl.L^{-1} (pH8), 2.7 mmol ATP.L^{-1}, 0.075 mmol NADH.L^{-1}, 0.6 NAD mmol.L^{-1}, 12 mmol coenzyme A.L^{-1}, 0.6 mmol malate.L^{-1}, 4.5 mmol magnesium chloride.L^{-1}, 0.3 mmol mercaptoethanol.L^{-1}, 45 mmol potassium chloride.L^{-1}, 12 $U.mL^{-1}$ malate dehydrogenase, 0.5 $U.mL^{-1}$ citrate synthase, and 50 mg ddialized BSA.mL^{-1}(Table A 3.1).

A standard curve was prepared by adding 0, 25, 50, 100, 150, and 200 μl of 1 mM of potassium acetate standard to tubes and made total volume 1 mL.

1 mL of standard and incubated hepatocyte supernatant (SN) was centrifuged at 2000x g for 10 min at 25 °C.

0.5 mL of SN filtrate was transferred to a cuvette and 1 ml of the cocktail was added. Each cuvette was mixed by

inverting and the optical density (OD) was read in a spectrophotometer at 340 nm(A_1).

2μL of stock enzyme (36 U. mL^{-1}) acetyl-CoA synthetase was added to get 0.075 U. mL^{-1}, mixed, and allowed to stand for 40 min. The OD of the sample was again read at 340 nm (A_2)

The acetate concentration was determined as the difference in the initial and final readings (A_2- A_1) by using a potassium acetate standard curve.

Reference:

1. Guynn, R. W. & Veech, R. L. (1975) Enzymatic determination of acetate. In: Methods of enzymology, vol. 35 (Lowenstein, J. M., ed). pp. 302-7. Academic press, New York, NY.

2. Bridges, S. R., Anderson, J. W., Deakins, D. A., Dillon, D. W. & Wood, C. L. (1992) Oat bran increases serum acetate of hypercholesterolemic men. Am. J. Clin. Nutr. 56: 455-9.

Table A 3.1

Preparation of A cocktail

Required con.	uL/1 mL	Stok Conc.
90 mmol Tris-HCl.L^{-1} (pH8):	450	200 mM
2.7 mmol ATP.L^{-1}:	100	27 mM
0.075 mmol NADH.L^{-1}:	15	5 mM
0.6 mmol NAD.L-1:	25	24 mM
0.12 mmol coenzyme A.L^{-1}:	12	10 mM
0.6 mmol malate.L^{-1}:	60	0.1 M
4.5 mmol magnesium chloride.L^{-1}:	50	0.1 M
0.3 mmol mercaptoethanol.L^{-1}:	100	0.3 mM
45 mmol potassium chloride.L^{-1}:	45	1 M
50mg.mL^{-1} dialized BSA	20	10% BSA
12 U.mL^{-1} malate dehydrogenase.	10	1000 U/mL
0.5 U.mL^{-1} citrate synthase,	3	1.67 U/mL
0.075 U.mL-1 Acetyl coA synthase	2	36 U/mL

Table A 3.2

Example of an assay

No.	W-std 1 mM K-Ac nmole	D.W (ml)	Cock tail (ml)	AcCoAs (µl)	V_t (ml)	Final Con. [µM]	A_2 A_1
1B	0	1000	1000	20	1.50	0	
2RS1	25	975	1000	20	1.50	12.26	
3RS2	50	950	1000	20	1.50	24.51	
4RS3	100	900	1000	20	1.50	49.02	
5RS4	150	850	1000	20	1.50	73.53	
6RS5	200	800	1000	20	1.50	98.0	
7S1	500 µL		1000	20	1.50	-	

APPENDIX 4

ACETALDEHYDE DETERMINATION

Principle

$CH_3CHO + NAD^+ \rightleftharpoons CH_3COOH + NADH + H^+$

Although the equilibrium of the reaction lies in favour of acetate and NADH, quantitative oxidation of acetaldehyde is possible with the yeast enzyme. Therefore the rate of reaction which is proportional to the aldehyde concentration over a limited range, is used.

Equipment

Spectrophotometer for measurements at 340 nm.

Reagents

1. 0.1 M Potassium chloride: Dissolve 74.5 g KCL in distilled water and make up to 1 L.
2. 1 M Tris buffer pH 8.0: Dissolve 30 g tris in 150 mL distilled water, adjust to pH 8.0 with 4 N HCL and dilute to 250 mL with distilled water.
3. 0.1 M EDTA: Dissolve 3.7 g EDTA-$Na_2H_2.2H_2O$ in distilled water and make up to 100 mL.
4. 0.1 M Mercaptoethanol: dissolve 0.78 g mercaptoethanol in distilled water and make up to 100 mL.
5. 15 mM β-NAD: Dissolve 100 mg NAD in distilled water and make up to 10 mL.
6. NAD-buffer mixture: Immediately before use mix 3 volumes of solution 1, 6 volumes solution 2,0.5 volume solution 3, 0.5 volume solution 4, and 0.5 volume solution 6.

7. 2 M Acetaldehyde stock solution: Prepare a 10 % solution of freshly distilled acetaldehyde in distilled water.
8. 5 U.mL^{-1} Aldehyde dehydrogenase: Thaw the frozen enzyme solution and dilute accordingly with distilled water.

Procedure

1. 2 mL of incubation SN was rapidly pipette into a cuvette.
2. 1 mL of NAD-buffer mixture was added,mixed rapidly, put on cuvette lid, and read extinction E_1.
3. 0.025 mL Adehyde dehydrogenase suspension(150 U.3 mL^{-1}) was added,mixed.
4. After exactly 10 min, read extinction E_2.
5. Calculation: @E = E_1 - E_2.

Table A 4.1

Reaction mixture & summary of procedure

Pipette into cuvettes mix.		Vol.(mL)	Con.in assay
Deproteinized, neutralized Sample or Std.		2	
NAD-Buffer Mixture			
1 M KCl	3 p		94mM
1 M Tris buffer (pH 8.0)	6 p	1	0.189M
0.1 M EDTA	0.5 p		1.58mM
0.1 M Mercaptoethanol	0.5 p		
15 mM β-NAD	0.5 p		0.24mM
Read E_1 at 340 nm		Vt = 3mL	
Add aldehyde dehydrogenase			> 50 $mU.mL^{-1}$ 150 $mU.3mL^{-1}$ 0.15 $U.3mL^{-1}$
Mix, immediately start a stop watch and read E_2 exactly 10 min. later @E = $E_2 - E_1$ is used for the calculations.			

Table A 4.2

Example of an assay

No.	W - Std		D.W. (mL)	NAD-Buffer Mixture (mL)	(μL)
	2 mM (μL)	Acetaldehyde (μmole)			
1B	0	0	2	1	30
2RS1	50	0.1	1.95	1	30
3RS2	100	0.2	1.90	1	30
4RS3	200	0.4	1.80	1	30
5RS4	300	0.6	1.70	1	30
6RS5	400	0.8	1.60	1	30
7RS6	500	1	1.5	1	30
S1	up to 2 mL			1	30

Reference:

1. Lundquist, F. (1974) Determination with dehydregenase. In: Methods of enzymology, vol 3 (Lowenstein, J. M., ed). pp. 1509-1513. Academic press, New York, NY.

APPENDIX 5

ACETOACETATE DETERMINATION

Acetoacetate was determinated by the fluorescent spectrophotometry method (Mellanby and Williamson 1974). The decrease in NADH concentration was measured by the decrease in fluroescence as acetoacetate was reduced to 3-Hydroxybutyrate.

Principle

$$\text{D-(-)-3-Hydroxybutyrate} + NAD^+ \xleftrightarrow{\text{3-HBDH}} \text{Acetoacetate} + NADH + H^+$$

Reagents

1. 1 M K_2HPO_4.NaH_2PO_4 (pH 7.0): Dissolve 21.8 g K_2HPO_4 and 19.0 g NaH_2PO_4 $2H_2O$ in 200 mL of double distilled water adjust pH to 7.0 as necessary with 4 M KOH and bring up to 250 mL with double distilled water. Store at 4 °C.
2. 5 mM NADH: Dissolve 3.5 mg β-NADH-Na_2, Grade III from yeast (Sigma, St. Louis, MO) per mL of carbonate buffer. Heat to 60 °C for 15 min and store at -20 °C. Make fresh weekly.
3. 0.1 M Na_2CO_3.$NaHCO_3$ (pH 10.6): Dissolve 0.85 g Na_2CO_3 and 0.17 g in 75 mL double distilled water adjust pH as necessary with 4 M KOH and bring up to 100 mL double distilled water. Store at -20 °C.
4. 20 mM Tris Buffer (pH 8.0): dissolve 0.24 g Tris (Sigma, St. Louis, MO) in 75 mL of double distilled water adjust pH to 8.0 with 1 M HCl add 200 μL 10% BSA and bring to 100 mL. Store at -20

°C.

5. 3-Hydroxybutyrate Dehydrogenase. type II from Rhodopseudomonas spheroides in 3.2 M $(NH_4)_2SO_4$ (Sigma, St, Louis, MO).
6. 1 mM Acetoacetate: Dissolve 11.5 mg Acetoacetic acid- Lithium salt (Sigma, St. Louis, MO) per mL double distilled water. This yields a 110 mM solution. Dilute to 1 mM by adding 100 µL to 9.9 mL double distilled water.
7. 3 M $HClO_4$: Dilute 25 mL 70% $HClO_4$ to 100 mL with double distilled water. Store at 4 °C.
8. 10% BSA: Dissolve 10 g BSA in 75 mL double distilled water bring to 100 mL with double distille water.
9. 3 M $KHCO_3$: Dissolve 30 g of $KHCO_3$ in double distilled water and make up to 100 mL. Store at -20 °C.
10. Reagent Mixture: Add 10 mL 1 M phosphate buffer and 100 µL of 5 mM NADH to 90 mL double distilled water. Prepare fresh daily.

Procedure

1. A standard curve was prepared by adding 0, 10, 20, 40, 60, 80 and 100 µL(Fig. A 5.1) of acetoactate standard to tubes containing 100 µL 10% BSA, 300 NL 3 M $HClO_4$ and double distilled water to a total volume of 1.65 mL.
2. The tubes were vortexed vigorously for 30 sec and

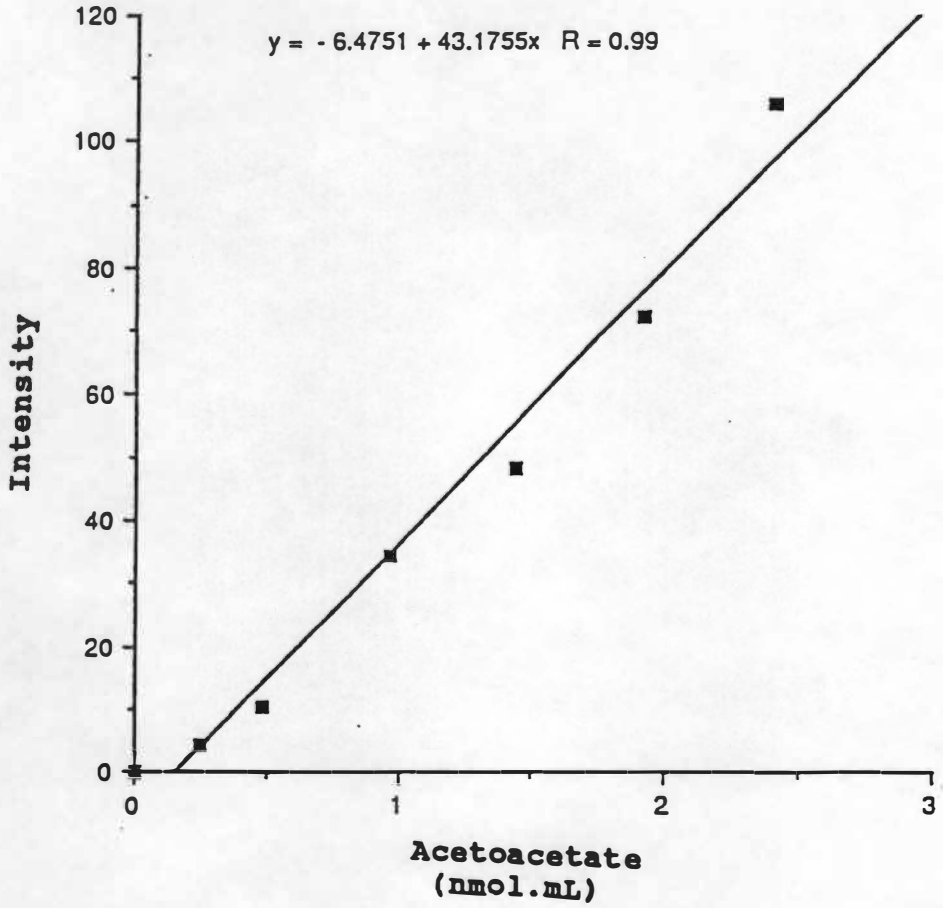


FIGURE A 5.1 Acetoacetate standard curve

allowed to sit in an ice water bath for 5 min vortexed again and centrifuged for 10 min at 1500 x g at 2 °C.

3. The supernatant was then poured off into another tube on ice.
4. A 100 μL aliquot of the neutralized standard or tissue supernatant was added to 1.9 mL of reaction mixture in a 4.5 mL polystyrene Ultra-Vu disposable cuvette(Baxter Scientific Products, Atlanta, GA) and mixed by inversion.
5. Baseline fluorescence was determined in a Hitachi F-2000 Fluorescence Spectrophotometer(Exitation 360 and Emission 400 nm) 10 μL of BHBDH was then added.
6. The cuvettes were mixed by inversion and let sit at room temperature for 60 min in the dark.
7. After 60 min fluorescence was determined again. The decrease in fluorescence was proportional to the concentration of acetoacetate.

Calculations

Fluorescence x 1.slope x 5 = nmoles.mL^{-1} SN

Fluorescence x 1.slope .mg liver = nmoles.mg^{-1} liver

Fluorescence x 1.slope x 10 = nmoles.mL^{-1} urine

Reference:

1. Mellanby, J. & Williamson, D. (1974) Acetoacetate In: Methods of enzymatic analysis (Bergmeyer, H. U. ed) vol.4. pp. 1840-1843. Verlag Chemie. Intl. Deerfild Beach, FL.

APPENDIX 6

3-HYDROXYBUTYRATE DETERMINATION

3-Hydroxybutyric acid was determined according to the procedure of Williamson and Mellanby (1974) adapted for spectrophto fluorometer. The concentration of NADH is measured by an increase in fluorescence as 3-Hydroxybutyrate is oxidized to acetoacetate.

Reagents

1. 1 M 2-Amino-2-methyl-1-propanol buffer(pH 10): Dissolve 8.9 g 2-amino-2-methyl-1-propanol(9.5 mL if liquid) in double distilled water, adjust pH to 10 with 12 M HCl and dilute to 100 mL with double distilled water. Store at -20 °C.
2. 20 mM Tris buffer(pH 8): Dissolve 0.24 g Tris (Sigma, St. Louis, MO) in double distilled water adjust pH to 8 with 1 M HCl add 200 μL 10% BSA and dilute with distilled water to 100 mL. Store at -20 °C.
3. 20 M Hydrazine hydrate: (Sigma,St.Louis, MO)
4. 0.1 M β-Nicotinamide dinucleotide (Grade III) from yeast (Sigma, St. Louis, MO): Dissolve 70 mg NAD in 1 mL double distilled water. Store at -20 °C.
5. Lactate dehydrogenase, LDH: Dilute stock LDH, Bovine heart Type III in 2.1 M $(NH_4)_2SO_4$ (Sigma, St. Louis, MO) with 20 mM Tris buffer.
6. 1 mM 3-hydroxybutyrate: Dissolve 15.4 mg DL-3-hydroxybutyric acid-sodium salt (Sigma, St. Louis, MO) per mL double distilled water this yields a 0.1 M solution. Dilute to 1 mM by adding 200 μL to 9.8

mL double distilled water. Make fresh daily.

7. 3 M Perchloric acid: Dilute 25 mL of 70% $HClO_4$ with double distilled water to 100 mL.
8. 3 M Potassium hydrogen carbonate: Dissolve 30 g $KHCO_3$ with double distilled water and make up to 100 mL.
9. Reagent mixture: Mix 10 mL buffer(1), 250 µL Hydrazine hydrate and 50 µL NAD and bring to 100 mL with double distilled water. Make fresh daily.

Procedure

1. A standard curve was prepared by adding 0, 10, 20, 40, 60, 80 and 100 µL(Fig. A 6.1) of 3-Hyfroxybutyrate standared to tubes containing 100 µL 10% BSA, 300 µL 3 M $HClO_4$ and double distilled water to a total volume of 1.65 mL.
2. The tubes were vortexed vigorously for 30 sec and allowed to sit in an ice water bath for 5 min, vortexed again and centrifuged for 10 min at 1500 x g at 2 °C.
3. 1 mL of the supernatant was removed and added to another to another conical tube on ice. 200 µL of 3 M $KHCO_3$ were added and allowed to sit for 15 min, then centrifuged for 10 min at 1500 x g at 2 °C. The supernatant was then poured off into another tube on ice.
4. A 100 µL aliquot of the neutralized standard or tissue supernatant was added to 1.9 mL of reaction mixture in 4.5 mL polystyrene Ultra-Vu disposable

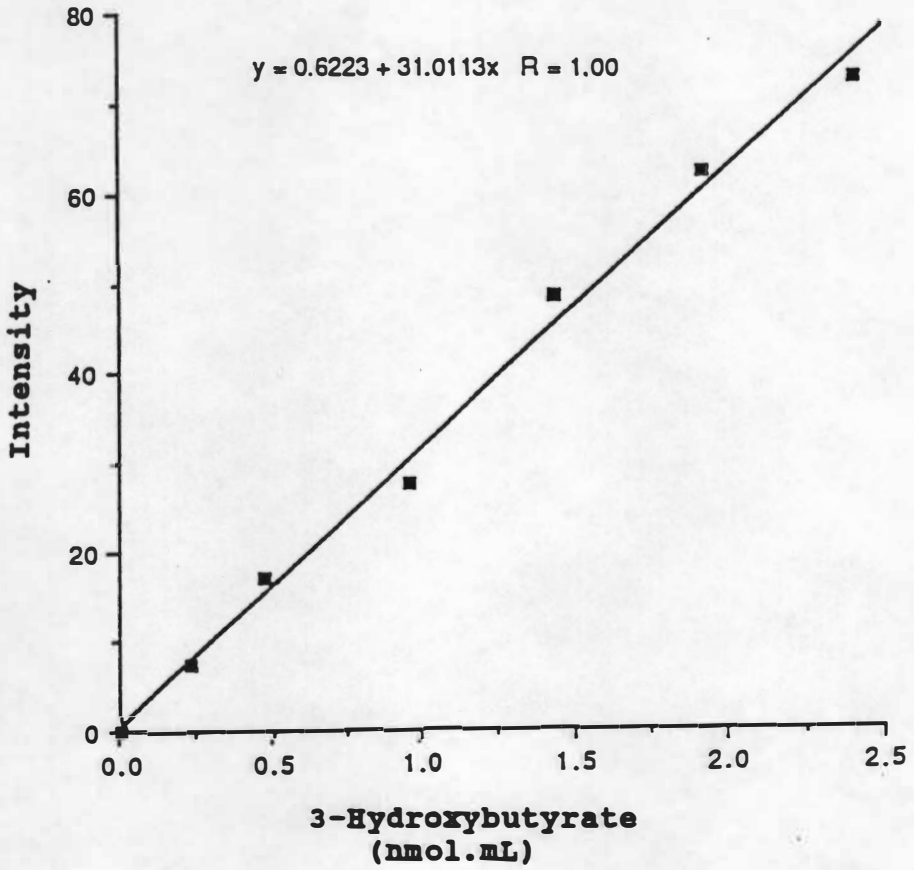


FIGURE A 6.1 3-Hydroxybutyrate standard curve

cuvette (Baxter Scientific Products, Atlanta, GA) and mixed by inversion.

5. Baseline fluorescence was determined in a Hitachi F-2000 Fluorescence Spectrophotometer(Exitation 360 and Emission 460 nm). 10 μL of BHBDH were then added, the cuvettes were mixed by inversion and let sit at room temperature for 60 min in the dark.
6. After 60 min fluorescence was determined again. The decrease in fluorescence was proportional to the concentration of 3-Hydroxybutyric acid.

Calculations

Fluorescence x 1.slope x 5 = nmoles.mL^{-1} SN

Fluorescence x 1.slope.mg liver = nmoles.mg^{-1} liver

Fluorescence x 1.slope x 10 = nmoles.mg^{-1} liver

Reference:

1. Williamson, D. H. & Meanby, J. (1974) D-(-)-3-Hydroxybutyrate. In: Methods of enzymatic analysis (Bergmeyer, H.U. ed.), Vol. 4 pp. 1836-1839. Verlag Chemie Intl. Deerfild, FL.

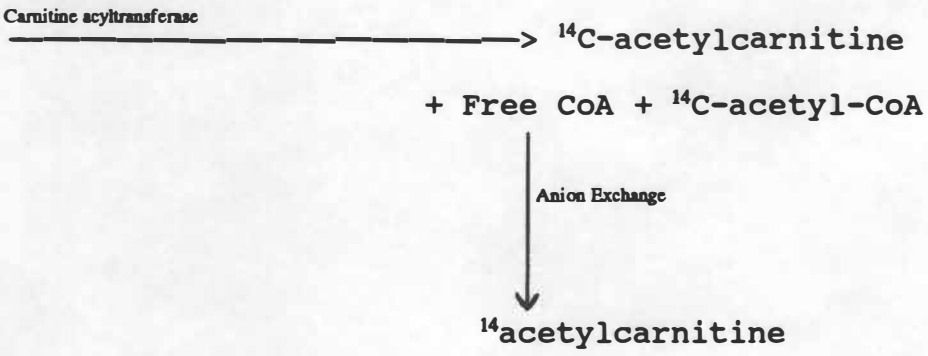
APPENDIX 7

ACETYLCARNITINE BINDING STUDY

1.SYNTHESIS OF [1-^{14}C] ACETYLCARNITINE

Principle

Carnitine + ^{14}C-acetyl-CoA



Procedures

1. Incubate the total volume 3.5 mL of incubation mixture (Table AVII.1) at 30 °C for 60 min.
2. Terminate reaction with 200 μL of 36% of perchloric acid.
3. Cool it up and neutralize acidic soup with 300 μL of 4 N KOH followed by centrifugation.
4. Take supernatant and pass through anion exchange colum(0.5 x 6 cm) of AG 2- x 8, Cl^- form, 200-400 mash in water.
5. Elutriate [1-^{14}C] Acetylcarnitine with 3 times with 0.5 mL of water.(yield 85%)

Table A 7.1.

Compositions of incubation mixture

Ingredient	$\mu mol.L^{-1}$	$\mu mol.mL^{-1}$	$\mu mol.3.5\ mL^{-1}$
[1-^{14}C] acetyl-CoA (50 μCi. μmole)	171	0.171	0.595
L-Carnitine	157	0.157	0.549
Oxidized Glutathione	5000	5	17.5
EDTA(EthyleneDiamine -TetraAcetate)	1000	1	3.5
Potassium phosphate (pH = 7.4)	100,000	100	350
CAT(Carnitine Acyl-Transferase)		4 Units	

Reference

1. Murthy, M. S. R. & Pande, S. V. (1984) Mechanism of carnitine acylcarnitine translocate-catalyzed import of acylcarnitines into mitochondria. J. Biol. Chem. 259: 9082-9089.

2. Binding study

1. In a 1.5 mL micro centrifuge tubes (conical bottom with attached closure) the total volumes of 0.5 mL contained sodium pyrophospate buffer, pH 7.4 (55 $mmol.L^{-1}$), NAD (0, 1, 2, 4 $umol.L^{-1}$), reduced glutathione (1.1 $mmol.L^{-1}$), [1-^{14}C]-acetylcarnitine (2 $umol.L^{-1}$, specific radioactivity 123,659 dpm.nmole or 2061 Bq.nmole), and horse ADH (0.17 unit or 100 ug protein).
2. The reaction mixture (Table AVII.2) was incubated for 5 min at 37 °C in a Dubnoff metabolic shaking water bath (80 oscillation. min^{-1}).
3. The reaction mixture was transferred to an 30,000 NMWL Ultrafree-MC filter unit (Millipore, Bedford, MA) and centrifuged at maximum speed (15,000 x g) for 5 min in an Eppendorf centrifuge, model 5412 (Sybron/Brinkmann Instruments, Westbury, NY).
4. The residue on membrane sample cup was transferred to a scintillation vial containing 5 mL scintillation fluid and the radioactivity was counted in a Beckman LS 3801 Liquid Scintillation counter.

Table A 7.2

Incubation mixture for binding study

Tube No.	1	2	3	4
Mixture		(mL)		
Buffer(pH7.4) 75 mmol.L^{-1}	0.37	0.37	0.37	0.37
NAD (100 μmol.L^{-1})	0	0.005	0.01	0.02
Reduced Glutathione (100 mmol.L^{-1})	0.0055	0.0055	0.0055	0.0055
Horse ADH (100 μg protein or 0.17 unit)	0.01	0.01	0.01	0.01
[1-^{14}C]-acetylcarnitine (123,659 dpm.nmole or 2061 Bq.nmole)	0.1	0.1	0.1	0.1
G.D.W.	0.02	0.015	0.01	0

APPENDIX 8

ADH INHIBITORS

TABLE A8.1

Inhibitors of ADH

Inhibitor	Ki	Competition	Mode of action	Ref#
Pyrazol	0.22	Sub.	Form terary complexes with ADH and NAD	2
4-Metyl pyrazol	0.013	"	Heterocyclic ring of pyrazol forms a bridge	6
4-Propyl pyrazol	0.004	"	through its two nitrogen atoms and the catalitic	7
4-Pentyl pyrazol	0.000	"	zinc atom and C-4 of the nicotinamide moiety of NAD	3
Benzamide	530	Sub.	Ternary complexes with ADH and NADH	2
Isobutyramide	140	"		8
Auramine	9	"	Form strong and specific complexes with ADH in the absence and presence of coenzyme	2
2,2,-Bipyridine		Cosub.	Bind of ADP-ribose and coenzyme fragments	2
Phenanthroline		"	Bind to the active zinc atoms and ternary complexes (ADH, ADP.ribose, and phenanthroline)	1 4
Imidazole		Sub.& Cosub.	Form the binary complexes of ADH and imidazole also form ternart complexes(ADH, imidazole, NAD) in the presence of coenzyme.	7
Primaquine Quinoline derivatives Amphetamines Propranolol Folic acid Flavensomycin metronidazole			Without characterization of the mode of inhibition	2

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VITA

Youn-Soo Cha was born in Chonju, ChonBuk in Korea on October 17, 1959. She graduated from Chonju Women's High School in spring of 1978. After attending the ChonBuk National University in Korea from the spring of 1978 until spring of 1982, she graduated with a Bachelor of Science degree in Food Science. From 1982 to 1984 she held the position of Research assistant and earned a Master of Science degree in Food Science at the Sookmyung Women's University. Until she came in United State, she taught Nutrition and Food Science classes in several Universities in Korea as a part time lecturer. Also during that time she got married and one son was born.

In the fall of 1989, she began studies toward a Ph.D. degree in the Nutrition department at the University of Tennessee and accepted the positions of graduate teaching assistant and Research Assistant.